A P P E N D I X

Makrozoobenthos-Einfluß und mikrobielle Kaltadaptation -

Schlüsselfaktoren für die Effizienz biochemischer Umsetzungen des Kohlenstoffs in marinen Sedimenten

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Temperature Characteristics of Psychrotrophic and Psychrophilic Bacteria

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When the Arrhenius equation is applied to microbial growth rates, a temperature characteristic (μ_A) represents the activation energy. While these μ_A values describe the energetics favouring growth, there is still no convincing evidence from empirical data to support the idea of a widely applicable relationship between μ_A values and optimal growth temperatures, which may serve as a basis for the classification of psychrophiles, psychrotrophs, mesophiles and thermophiles. The μ_A values for the exponential growth rates of 16 psychrophilic and psychrotrophic bacterial strains, including 9 from the literature, varied from 3 to 33 (kcal mol^{-1}). A plot of μ_A versus growth temperature optimum deviated considerably from empirical equations reported recently (Mohr & Krawiec, 1980) for a different selection of strains. Moreover, Arrhenius profiles with two distinct slopes at suboptimal temperatures were not restricted to bacteria with growth temperature optima higher than 37 °C, as had been suggested in that report. A psychrotrophic strain of *Cytophaga johnsonae* with a growth temperature optimum of 23–25 °C was characterized by two different temperature characteristics. Furthermore, in the upper range of suboptimal temperatures (11–24 °C), temperature characteristics (μ_{A1}) were also affected by temperature acclimation of the inoculum.

INTRODUCTION

Growth–temperature relationships of bacteria are adequately described by temperature characteristics representing Arrhenius constants (μ_A) of the exponential growth rates (Arrhenius, 1908; Dean & Hinshelwood, 1966). However, some attempts to establish a relationship between μ_A and the temperature ranges used for the classification of psychrophiles, psychrotrophs, mesophiles and thermophiles (Ingraham, 1958) could not be confirmed (Shaw, 1967; Hanus & Morita, 1968; Tai & Jackson, 1969). Nevertheless, a relationship between μ_A and growth temperature optima has recently been described on the basis of two empirical equations (Mohr & Krawiec, 1980), suggesting a distinctive pattern for bacteria with growth temperature optima below 15 °C and 30 °C, i.e. psychrophiles and psychrotrophs, respectively (Morita, 1975). Our data, obtained from a set of psychrophilic and psychrotrophic freshwater isolates, do not confirm the general validity of those equations nor is there substantial published evidence for their assumption.

Mohr & Krawiec (1980) described the occurrence of complex Arrhenius profiles consisting of more than one negative slope. They suggested that only organisms with optimum growth temperatures above approximately 37 °C can have two distinct temperature characteristics at suboptimum temperatures. In contrast to this suggestion, we show here that disparate Arrhenius profiles can also occur in psychrotrophic bacteria with growth temperature optima far below 37 °C.

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METHODS

Seven psychrophilic and psychrotrophic isolates from cold (<5 °C) freshwater environments were grown at 6, 10, 14, 18, 22, 26 and 30 °C (shaking water bath, operated at 150 rev. min^{-1}) in 50 ml Erlenmeyer flasks containing 10 ml half-strength Difco nutrient broth. Growth rates were calculated from readings, taken at 12 h intervals, of apparent absorbance at 578 nm (0·2 ml microcuvette, 1 cm path-length).

A psychrotrophic strain of *Cytophaga johnsonae* (strain C21) had been isolated from lake water (Reichardt, 1974). Its exponential growth rates were determined in a shaking (40 strokes min^{-1}) Temperature Gradient Incubator (Scientific Industries Inc., Mineola, N.J., U.S.A.) constructed for duplicate incubation in side-arm tubes. These contained 10 ml of autoclaved basal mineral medium [containing (g l^{-1}): $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0·2; $CaCl_2$, 0·003; $FeCl_3$, 0·0003; together with 10 mM-KH_2PO_4/Na_2HPO_4 buffer, pH 7·0] supplemented with 10 mM filter-sterilized *N*-acetylglucosamine as sole organic carbon and nitrogen source. Tubes were inoculated with 0·1 ml portions of late-exponential phase cultures grown at 10 or 23 °C (apparent absorbance at 500 nm of 0·5; Bausch & Lomb Spectronic 20). Growth of cultures was monitored at 3–8 h intervals for up to 72 h using the same instrument. From semi-logarithmic plots of apparent absorbance, the exponential growth rates (k) were determined using the equation: $k = \ln (A_n/A_0)/\Delta t$, in which A_0 and A_n are the apparent absorbances at the beginning and end, respectively, of the time interval Δt.

To calculate temperature characteristics, a specific version of the Arrhenius equation applicable to microbial growth rates (Dean & Hinshelwood, 1966) was employed, i.e. $k = Ae^{-\mu_A/RT}$, in which μ_A, the temperature characteristic, is here substituted for the activation energy, R is the gas constant (1·987 cal K^{-1} mol^{-1}), T is the absolute temperature (K), and A is a constant. In Arrhenius plots showing ln k (h^{-1}) as a function of $1/T$ (K^{-1}), the negative slopes of the linear portions of the curves obtained at suboptimal growth temperatures equalled $-\mu_A/R$, with r^2 values describing their goodness of fit.

RESULTS AND DISCUSSION

Temperature characteristics have generally been given the units kcal mol^{-1}. However, SI guidelines require the use of the joule (1 cal = 4·184 J), and the meaning of the molar dimension in connection with bacterial growth is obscure. Hence, we suggest calculating Arrhenius constants in kcal mol^{-1} but without expressing the units, until a meaningful alternative is found. This is in agreement with the original description of μ_A (Arrhenius, 1908) and a few later publications (Hanus & Morita, 1968; Baker, 1974).

General validity of the Mohr–Krawiec equations

In order to check the general applicability of the empirical relationship between temperature characteristics (μ_A) and growth temperature optima of Mohr & Krawiec (1980), μ_A values of seven of our isolates and nine from the literature were plotted against growth temperature optima up to 30 °C (Fig. 1). The μ_A values of these psychrophilic and psychrotrophic bacteria varied over a relatively wide range, from 3 to 33. The empirical curve obtained by Mohr & Krawiec (1980) for a corresponding range of growth temperature optima, i.e. ≤30 °C, was described by the equation: $\mu_1 = (2{\cdot}73 \times 10^5)\, t^{-0.738}$, with μ_1 corresponding to our μ_{A1}. This equation was based on μ_1 values ranging from 12·7 to 51·9, and suggested a very steep increase of the temperature characteristic in the lower range of the growth temperature optima, i.e. below 25 °C. The steepness was caused exclusively by two psychrophiles with extremely high μ_1 values (51·9 and 39·0). However, when considering the μ_A versus temperature optimum plot obtained for a larger number of psychrophilic and psychrotrophic bacteria (Fig. 1), it seems unlikely that the μ_A values on which the empirical equation of Mohr & Krawiec (1980) was based, were characteristic of the majority of bacteria with low temperature optima. The temperature characteristics of most of our psychrophilic and psychrotrophic isolates and those based on literature values for other bacteria of these groups were considerably lower. Moreover, the Arrhenius constants of the two *Vibrio marinus* strains used in the empirical curve of Mohr & Krawiec (1980) (39·0 and 23·2) were much higher than those reported in an earlier paper (16·2 and 16·4, respectively; Hanus & Morita, 1968). Thus, the empirical equations presented by Mohr & Krawiec (1980) are not applicable to other psychrophilic and psychrotrophic bacteria nor are they

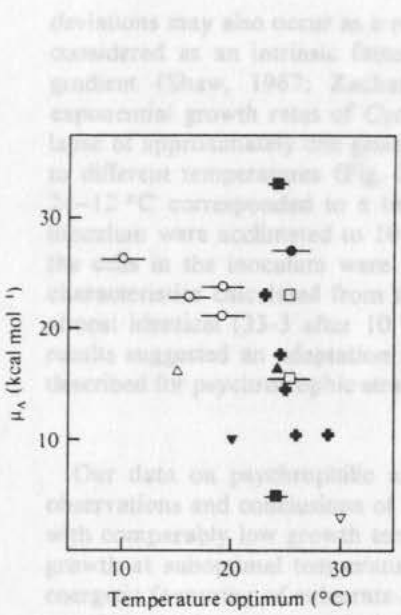

Fig. 1

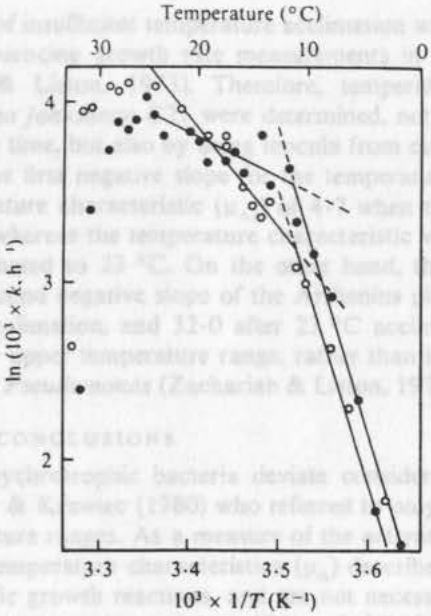

Fig. 2

Fig. 1. The μ_A values of psychrophilic and psychrotrophic bacteria and ranges of optimal growth temperatures. ○, Gram-negative rods with respiratory metabolism, isolated from Rhône Glacier, Switzerland; ●, *Bacillus* sp., from a subalpine stream, S.W. Germany; □, pseudomonads, from profundal lake sediment (1000 m, Lake Baikal, Siberia); ■, *Cytophaga johnsonae* C21, from a eutrophic lake, West Germany ($\mu_{A1} = 4{\cdot}7$ and $\mu_{A2} = 33{\cdot}3$); △, *Vibrio marinus* MP1 (Hanus & Morita, 1968); ▲, *V. marinus* PS-207 (Hanus & Morita, 1968); ▽, *Pseudomonas fluorescens* (estimated value of $\mu_{A1} = 3{\cdot}2$) (from Lynch *et al.*, 1975); ▼, *Micrococcus cryophilus* (Tai & Jackson, 1969); +, psychrotrophic bacteria, from Antarctic peat (Baker, 1974). Data points from the present study are marked by horizontal bars indicating the length of the temperature intervals employed during measurement of the optimal growth temperatures.

Fig. 2. Arrhenius plots for a psychrotrophic strain of *Cytophaga johnsonae* (C21) after growth of the inoculum at 10 or 23 °C. Data points contributing to the negative slopes in each experiment were grouped by linear regression analysis to produce two straight lines with r^2 values maximized over the entire range of suboptimal growth temperatures tested. Temperature characteristics calculated for the upper (μ_{A1}) and lower (μ_{A2}) ranges of suboptimal temperatures, together with r^2 values (in parentheses) were: inoculum grown at 10 °C (●), $\mu_{A1} = 4{\cdot}7$ (0·64), $\mu_{A2} = 33{\cdot}3$ (0·98); inoculum grown at 23 °C (○), $\mu_{A1} = 12{\cdot}3$ (0·93), $\mu_{A2} = 32{\cdot}0$ (0·85).

independent of the different techniques used to measure exponential growth rates (as shown for *Vibrio marinus*).

One of our psychrotrophic isolates (*Cytophaga johnsonae* C21) showed two distinct temperature characteristics (see below), although its temperature optimum was only 23–25 °C. According to Mohr & Krawiec (1980), such disparate Arrhenius profiles can only be expected for bacteria with growth temperature optima higher than 37 °C. Furthermore, calculations of μ_A values based on published data of another psychrotrophic bacterium (*Pseudomonas fluorescens* strain E20) indicated that this was not a singular finding (Fig. 1; Lynch *et al.*, 1975).

Effect of temperature acclimation of the inoculum on temperature characteristics

Deviations within the negative slope of Arrhenius plots have been interpreted as due to uncoupling of energy production from energy utilization (Senez, 1962; Ng, 1969). Such

deviations may also occur as a result of insufficient temperature acclimation which should be considered as an intrinsic factor influencing growth rate measurements in a temperature gradient (Shaw, 1967; Zachariah & Liston, 1973). Therefore, temperature-dependent exponential growth rates of *Cytophaga johnsonae* C21 were determined, not only after the lapse of approximately one generation time, but also by using inocula from cultures adapted to different temperatures (Fig. 2). The first negative slope for the temperature range from 24–12 °C corresponded to a temperature characteristic (μ_{A1}) of 4·7 when the cells in the inoculum were acclimated to 10 °C, whereas the temperature characteristic was 12·3 when the cells in the inoculum were acclimated to 23 °C. On the other hand, the temperature characteristics calculated from the second negative slope of the Arrhenius plots (μ_{A2}) were almost identical (33·3 after 10 °C acclimation, and 32·0 after 23 °C acclimation). These results suggested an adaptation in the upper temperature range, rather than in the lower as described for psychrotrophic strains of *Pseudomonas* (Zachariah & Liston, 1973).

CONCLUSIONS

Our data on psychrophilic and psychrotrophic bacteria deviate considerably from the observations and conclusions of Mohr & Krawiec (1980) who referred to only three isolates with comparably low growth temperature ranges. As a measure of the activation energy for growth at suboptimal temperatures, temperature characteristics (μ_A) describe primarily the energetic favouring of substrate-specific growth reactions, and are not necessarily linked to certain growth temperature ranges or temperature optima (Dean & Hinshelwood, 1966; Kovacs *et al.*, 1968; Hanus & Morita, 1968; Reichardt, 1971; Baker, 1974; Morita, 1975). Our results show that neither the empirical relationship between optimal growth temperatures and temperature characteristics (μ_A) nor the reported restriction of disparate Arrhenius profiles to mesophiles and thermophiles, as suggested by Mohr & Krawiec (1980), are generally applicable phenomena.

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Survival stages of a psychrotrophic *Cytophaga johnsonae* strain[1]

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Cells of a psychrotrophic strain of *Cytophaga johnsonae*, when exposed to starvation in a mineral salts medium, produced short viable rods, viable coccoids, and moribund elongated cells as a function of the incubation temperature. Starvation for 14 days or longer resulted in an increase of the cellular DNA to protein ratio. While rod-shaped cells possessed an intermediate layer identified as murein sacculus, coccoid cells displayed the common properties of spheroplasts, and their formation was frequently preceded by a pestlelike intermediate stage. During survival, substrate affinities ($1/K_m$) for transport, respiration, and incorporation of glucose increased in both rod and coccoid cells. The rods appeared to possess dual, concentration-dependent metabolic pathways for glucose. Glucose incorporation by starved cells into macromolecular pools had lower K_m values (10 to 203 times lower) than the values found for exponentially growing and nonviable rods. Coccoid cells demonstrated a slightly increased resistance to mild heat stress compared with log phase cells but were equally susceptible to ultrasonic vibration and ultraviolet irradiation. The data obtained indicated that unbalanced cellular divisions of multinucleate cells at low temperature and starvation result in coccoid cells. The coccoid cells are probably the survival forms in nature when low temperatures and nutrient deprivation exist.

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Mises en condition de famine dans un milieu minéral salin, les cellules d'une souche de *Cytophaga johnsonae*, psychrotrophes, produisent des bâtonnets courts et viables, des coccoïdes viables et des cellules allongées moribondes, dépendant de la température d'incubation. Une disette de 14 jours ou plus conduit à une augmentation du rapport entre le DNA et la protéine cellulaire. Tandis que les cellules en bâtonnet possèdent une couche intermédiaire identifiée comme un saccule de muréine, les cellules coccoïdes présentent des caractéristiques communes aux sphéroblastes et leur formation est fréquement précédée d'un stade intermédiaire en forme de pilon. Au cours de leur survivance, les affinités du substrat ($1/K_m$) pour le transport, la respiration et l'incorporation du glucose augmentent tant chez les cellules en bâtonnet que chez les coccoïdes. Les bâtonnets semblent posséder deux sentiers métaboliques pour le glucose, dépendant de la concentration. L'incorporation du glucose par les cellules affamées en des regroupements macromoléculaires a des valeurs K_m inférieures (de 10 à 203 fois inférieures) à celles trouvées pour les bâtonnets en croissance exponenntielle et les non viables. Les cellules coccoïdes présentent une résistance légèrement accrue au stress d'une chaleur douce, comparativement aux cellules en phase log, mais elles sont également sensibles aux vibrations ultrasoniques et à l'irradiation en ultraviolette. Les données obtenues indiquent qu'en condition de famine et de basse température les divisions des cellules plurinucléées sont irrégulières et résultent en des cellules coccoïdes. Ces cellules coccoïdes sont probablement la forme de survie en nature lorsque les températures sont basses et que les nutriments sont déficients.

[Traduit par les journal]

Introduction

Knowledge concerning unbalanced growth, as defined by Shockman (1965), and starvation comes primarily from the study of Enterobacteriaceae, pseudomonads, and vibrios (Shockman 1965; Harrison and Lawrence 1963; Felter et al. 1969; Clifton 1967; Fan and Rodwell 1975; Baker and Park 1975). In older cultures of some gliding bacteria such as certain *Cytophaga* spp., coccoid forms are produced which are considered to be spheroplasts (Leadbetter 1974), but sufficient data for this conclusion has never been published.

The occurrence of coccoid forms in older cultures of *Cytophaga johnsonae* was first described by Stainer (1947). Attempts to classify coccoid forms of strains related to *Cytophaga* as microcysts (Graf 1962; Graf and Sturzenhofecker 1964) have not been adopted (Leadbetter 1974; Christensen 1977). Only few of the criteria compiled by Sudo and Dworkin (1973) to define bacterial resting cells have been applied to cellular forms of *Cytophaga* spp. Though viability has been excluded for coccoid forms of certain *Cytophaga* spp. (e.g., *C. krzemeniewskae*, *C. fermentans*, *C. salmonicolor*) (Stainer 1942; Bachmann 1955; Veldkamp 1961), the status of the coccoids formed by other species including *C. johnsonae* has remained uncertain. As far as morphogenetic changes of Cytophagaeceae other than rod–coccoid transformations are concerned, observations made on a *Flexibacter* strain (Poos et al. 1972) indicate an intrinsic impact of temperature.

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While resting cells (microcysts) and fruiting bodies are typically formed by gliding bacteria (Myxobacterales) from terrestrial habitats, no such survival forms appear to exist for aquatic *Cytophaga*-like bacteria whose population dynamics appear often to follow an opportunistic pattern (Reichardt 1981). This paper addresses the question of a survival state of an aquatic *Cytophaga* resulting from the deprivation of organic nutrients as a function of temperature. Survival is important in nature in order to perpetuate the species.

Materials and methods

Organisms

Cytophaga johnsonae Stainer, strain C-21, isolated from lake water (Reichardt 1974) was employed in this study. W. Reichardt, R. Y. Morita, and R. R. Colwell (1980. Abstr. Annu. Meet. Am. Soc. Microbiol. N63) evaluated its psychrotrophic nature.

Cultivation and growth rates

Autoclaved mineral base (MB) contained, in 1 L of distilled water, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 3 mg $CaCl_2$, and 0.3 mg $FeCl_3$ in 10 m*M* KH_2PO_4 buffer, pH 7.0 (autoclaved separately). MB supplemented with 10 m*M* filter-sterilized glucose was used for growing the organism. Cells were usually grown in 200 or 500 mL of medium using 500- and 1000-mL Erlenmeyer flasks, respectively, at 5, 15, and 25°C on a rotary shaker at 100 rpm. Temperature-adapted late log phase cells (OD_{500} = 0.500) were used as inocula to give an initial OD_{550} of 0.01 (Bausch & Lomb Spectronic 20). To determine the influence of temperature on growth and viability, a temperature gradient incubator (Scientific Industries, Inc., Mineola, NY) with the standard L-shaped tubes was employed. Each tube in the temperature gradient incubator contained 10 mL of medium. The shaking rate of the instrument was set at 40 strokes per minute. Exponential growth rates were calculated from OD_{500} readings taken at 3- to 8-h time intervals.

Light microscopy

Cell morphology was examined under phase-contrast microscopy. The relative portion of the predominant forms was estimated from proportional counts. Coccoid to rod conversions were studied by a sealed microculture slide system employing a thin film of Difco plate count agar.

Transmission electron microscopy

Cell suspensions were fixed for 1 h in 3% glutaraldehyde (buffered with 0.1 *M* KH_2PO_4–Na_2HPO_4 buffer, pH 7.0) and then washed in Kellenberger buffer and postfixed in 1% OsO_4 (Ryter and Kellenberger 1958; Kellenberger et al. 1958). The cells were stabilized with uranyl acetate (saturated in 70% acetone) for 20 min followed by dehydration in an acetone series and embedded in Spurr's epoxy resin (Humphrey et al. 1979). The sections were then cut employing a Sorvall ultramicrotome unit. Lead acetate was used for poststaining (Reynolds 1963).

Shadowed preparations were obtained from freeze-dried cell suspensions on Formvar grids, using Pt–Pd coating at an angle of 30°. The extent of slime production on the cell surfaces was made visible by negative staining with phosphotungstic acid (Follet and Webley 1965).

Viable counts

Colony-forming units (CFU) were determined in triplicate on dried Difco plate count agar plates using the spread plate technique. Serial dilutions were made in 5 m*M* KH_2PO_4–Na_2HPO_4 buffer, pH 7.0, containing 10 mg Tween 80·L^{-1}. Plates were incubated at 22°C for 3 days. The moderately spreading colonies developed separated colony centers which could also be counted, but statistically, according to chi-square tests, agreement with the Poisson distribution at $P = 0.05$ was usually restricted to fewer than 40 nonoverlapping colonies per plate.

Cellular protein and DNA

Cell suspensions (25 mL, OD_{550} = 0.10, employing a Bausch & Lomb Spectronic 20) were centrifuged for 20 min at 12 000 to 16 000 × *g*, and the resulting cellular pellet was resuspended in MB and then treated twice with 5% cold trichloroacetic acid (TCA) for at least 30 min. The centrifuged precipitate resulting from the TCA treatment was extracted with 0.5 *N* perchloric acid (2.5 mL for 25 min at 70°C). One-millilitre portions of the extract were used for DNA assays (Burton 1956) employing calf thymus DNA (Sigma Chemical Co.) as the standard. The precipitate containing the protein was dissolved in 5.0 mL of 0.5 *N* NaOH, heated to 100°C for 5 min, chilled, and used for the determination of the protein content by the Herbert et al. (1971) modification of the Lowry et al. (1951) method. Bovine serum albumin (Sigma Chemical Co.) was used as the protein standard.

Assays with [$U^{-14}C$] glucose

[$U^{-14}C$]Glucose with a specific activity of 329 mCi·$mmol^{-1}$ (1 mCi = 37 MBq) (New England Nuclear) was employed as the sole organic carbon source to determine transport kinetics, glucose incorporation into macromolecular components, and respiration.

Transport kinetics were determined by the following procedure. To 0.5 mL of MB cell suspensions (OD_{550} = 0.05) 0.1 mL of [$U^{-14}C$]glucose (0.2 μCi) and 0.2 mL of unlabelled glucose (various concentrations) were added. The eight different glucose concentrations employed were such that the total glucose content varied in the cell suspensions from 12 to 4000 μ*M*. The cell suspensions were incubated at 15°C for 1, 2, and 3 min. The transport process by the cells was stopped by Millipore membrane (HA, 0.22 μm) filtration followed by immediate rinsing of the filters four times with 5.0-mL portions of MB and air drying at 50°C for 1 h. The dried membranes were then placed into respective scintillation vials to which 10 mL of toluene-based Omnifluor (New England Nuclear) was added. Radioactivity was estimated employing a Beckman LS 1000 scintillation counter. Blanks were obtained by treating the cell suspension with 2 m*M* dinitrophenol 1 h before the glucose was added.

The kinetics of glucose respiration (Hobbie and Crawford 1969) and of glucose incorporation into macromolecular compounds (Baross et al. 1975) were determined by incubating 10 mL of cell suspension (OD_{550} = 0.01 to 0.04) with 0.2 μCi of [$U^{-14}C$]glucose and unlabelled glucose to make an eight-step glucose concentration gradient (12 to 40 000 μ*M*). The incubation mixtures were dispensed into 50-mL

serum bottles fitted with rubber stoppers according to the procedure of Harrison et al. (1971) and incubated at 15°C on a rotary shaker at 100 rpm for 100 min. The assay was terminated by injecting 0.4 mL of 1*N* H_2SO_4 resulting in a decrease of the pH to 2.0. After 1 h of shaking (100 rpm) at 5°C the radioactivity incorporated by the cells was determined following 0.22-μm membrane filtration and further treatment as described above. Respiration was determined as transformation of ^{14}C-labelled glucose to $^{14}CO_2$. The $^{14}CO_2$ was absorbed on filter paper wicks soaked with 0.15 mL of phenylethylamine. Radioactivity was estimated as above.

K_m and V_{max} values were calculated for those concentration ranges which matched the linear transformation of the Michaelis–Menten equation by Eadie (1942). These parameters were obtained from the linear regressions and considered only if correlation coefficients were significant on a 95% probability level ($p = 0.05$).

Resistance to physical stress

The heat resistance of the organism was determined in a temperature gradient incubator set at approximately 1° temperature intervals. Cell suspensions (10 mL) in MB containing 5 × 10^5 CFU·mL^{-1}were incubated for 30 min. CFU were then determined on the suspensions. Rates of heat death were calculated from CFU counts obtained at 3-min intervals for cell suspensions exposed to 38.5 and 40.5°C.

Cell suspensions of 10^7 CFU·mL^{-1} in MB were used to determine the death rates due to ultraviolet irradiation and ultrasonic vibration. Two-millilitre portions of the cell suspensions were exposed to a germicidal lamp (producing a fluence of 17.5 J·m^{-2}) and gently agitated in a 9-cm glass petri dish at a distance of 21 in. (1 in. = 25.4 mm) from the lamp and then plated out immediately in semidarkness and incubated in the dark. Ultrasonic treatment of cells (25-mL suspensions) was carried out in a 50-mL Pyrex beaker (cooled in an ice bath) using a Bronwill Biosonic III ultrasonic power unit with an output of 200 W at 20 kHz.

Results and discussion

Exponential growth rates of *C. johnsonae* indicated a broad, near optimal growth temperature range extending from 22 to 30°C (Fig. 1, lower curve). When cell suspensions were washed and reincubated in MB without any organic nutrients, the cells kept dividing. Continued cell division in menstruum without nutrients is not unusual among Gram-negative bacteria (Novitsky and Morita 1977). After 5 days of starvation considerable increase in CFU was noted, especially in the lower temperature range (12 to 15°C) (Fig. 1, upper curve). Starved cells lost their gliding motility concomitantly with the loss of slime and convolutions from the outer membrane. As a result of temperature-dependent starvation, three different morphological types were noted microscopically which were short viable rods (1–2 × 0.3–0.6 μm) formed at suboptimal temperatures, viable coccoids (0.8–1.2 μm in diameter) formed at the optimal growth temperature, and elongated thin moribund rods (0.1–0.2 × 10–14 μm) formed at temperatures above 28°C. Neither cell divisions nor transformation to coccoids occurred when cells were starved at near maximal temperatures.

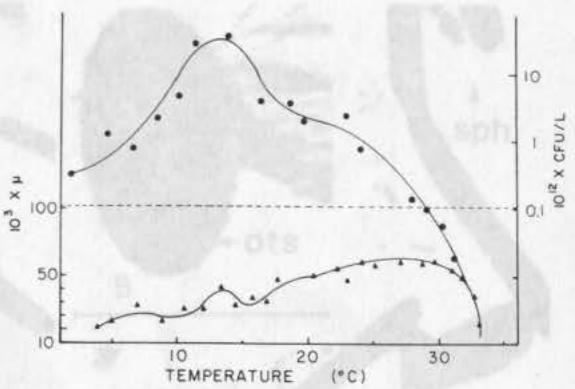

FIG. 1. Influence of temperature on exponential growth rates and viability of *C. johnsonae* C-21. Lower curve: growth rates μ (per hour) versus growth temperature. The inocula were acclimatized to 10 and 23°C for growth studies from 0 to 22°C and 23 to 34°C, respectively. Upper curve: CFU per litre counted after 5 days of starvation in MB versus incubation temperature. The starved cells were precultivated at 15°C until midlog phase. The broken line indicates the level of CFU per litre before transfer to a temperature gradient incubator. The minor peak around 13°C is only observed after acclimation to low temperatures (< 20°C). It is significantly different from both its neighboring growth rates on a 95% probability level (Student's *t*-test).

When stationary phase cultures (OD_{550} = 0.050) were incubated at approximately 25°C, approximately 70% of the rods were converted to coccoids within 6 days. This transformation, as determined by microscopy, was initiated by the thickening of one end of the rod resulting in a pestlelike transient form. Up to 90% of the cells when exposed to organic nutrients in a microscope slide culture retained the ability to form rods as well as to divide. This conversion from coccoid cells to rods was accompanied by a gradual formation of refractile cells in less than 24 h at 22°C. The formation of rods was triggered by adding glucose to a suspension of coccoids in MB at 22°C. The first evidence of transformation was observed microscopically 4 to 5 h after the addition of glucose. Approximately 1 m*M* glucose was the lower threshold level required for complete transformation to normal-size rods. Concentrations of glucose lower than the threshold level (0.1 to 100 μ*M*) produced only a few very short rods. All cellular conversion sequences were observed by light microscopy, and electron microscopy provided additional structural details.

Negatively stained preparations of motile rods from exponentially growing cultures were surrounded by profuse slime strands (Figs. 2A, 2D). An envelope consisting of trilaminar outer and inner membranes as well as an intermediate layer representing the murein

FIG. 2. Transmission electron micrographs of *C. johnsonae* C-21. *c*, coccoid cell form; *s*, slime extrusion; *sph*, spheroplasts (nonviable and only weakly refractile); *ots*, oval transient stage during coccoid to rod transformation; *ms*, murein sacculus. (A) Rods and coccoid cell, negatively stained. 14 560 ×. (B) Oval transient stage during coccoid to rod conversion (refractile) adjacent to nonviable spheroplast. (C) Thin section of log phase rod. 220 000 ×. (D and E) Late stages of coccoid to rod conversion, shadowed. 23 660 ×. (F) Starved short rod, thin section. 146 000 ×. Bars represent 1 μm in Figs. 2A, 2B, 2D, 2E, and 2F and 0.1 μm in Fig. 2C.

sacculus was always present in thin sections of rods (Figs. 2C, 2F, 3B). Growing rods were also characterized by undulations of their outer membranes (Fig. 3E). These undulations were not found in short rods from starved cell suspensions (Fig. 2F) nor were they associated with the coccoid stages (Fig. 3D). Thin sections revealed that slime layers and undulations of the outer membrane were typical for exponentially growing rods but they were lacking or insignificant in starved rods. The utilization of slime material as a source of endogenous substrate appeared probable but not confirmed.

Coccoid cells were generally characterized by a lower electron density compared with rods (Figs. 2A, 3D) without a murein sacculus (Fig. 3D). In the pestlelike transient stages only the nonconverted rod portion showed an intermediate layer between the inner and outer membranes (Fig. 3A). In the more advanced state of transformation, most of the cytoplasm appeared concentrated in the mesosomal structures that were surrounded by a unit membrane (Fig. 3C).

Thin sections of the coccoid stages (Fig. 3D) did not appear much different from the spheroplasts of other Gram-negative bacteria (Thorsson and Weibull 1958; Weibull et al. 1967; Brown et al. 1962; Martin 1963). Neither structurally nor morphogenetically did these coccoid forms resemble the microcysts of related bacteria (Holt and Leadbetter 1967; Sudo and Dworkin

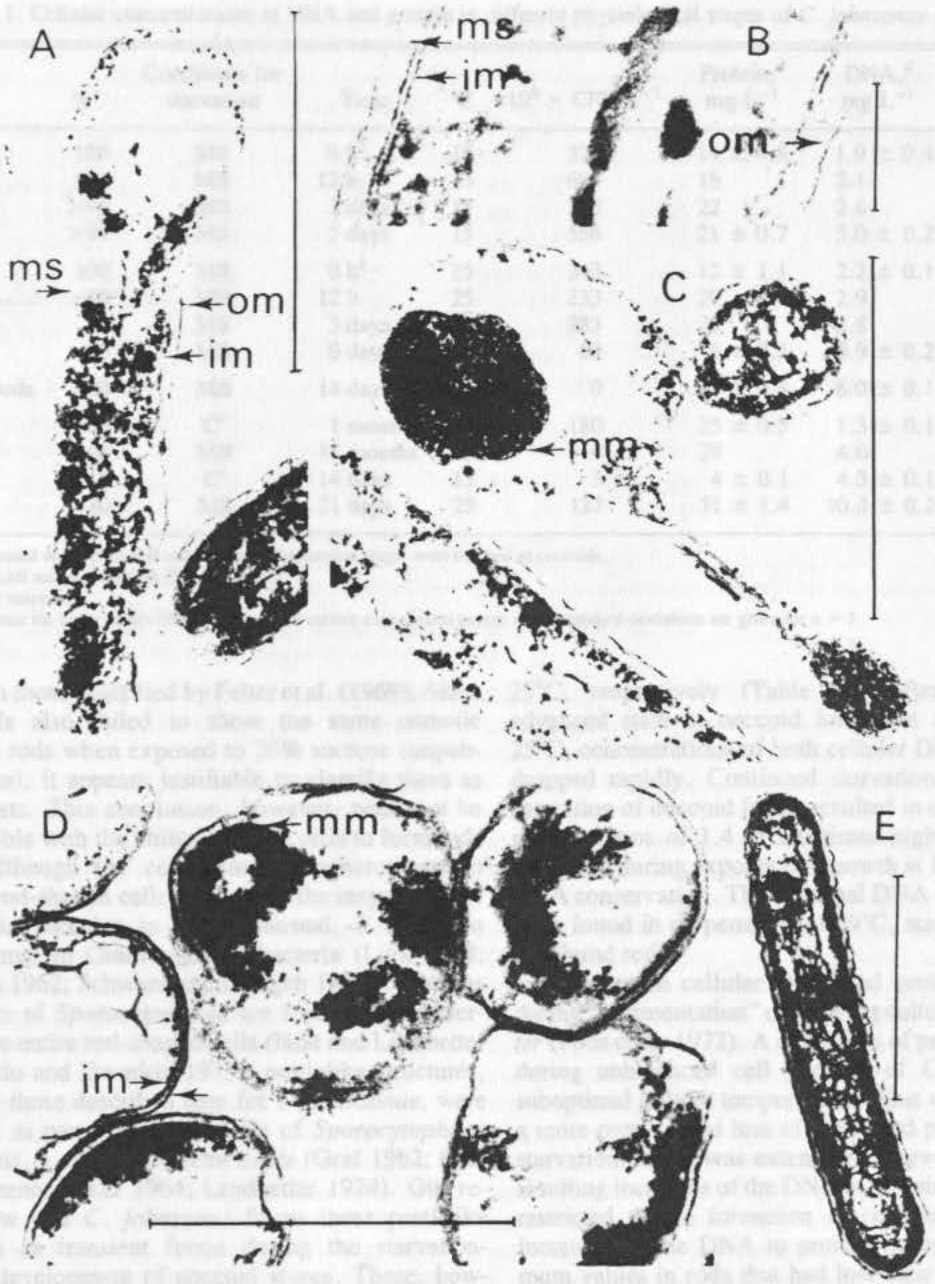

FIG. 3. Transmission electron micrographs of *C. johnsonae* C21. *om*, outer membrane; *im*, inner membrane; *mm*, mesosomal membrane; *ms*, murein sacculus. (A) Starved long rod during conversion to coccoid stage, thin section through pestlelike transient stage. 77 400 ×. (B) Cell walls of starved short rod. 280 000 × (= part of Fig. 2F at higher magnification). (C) Later stage of rod to coccoid conversion during starvation, section. 78 260 ×. (D) Completed coccoid stages, thin section. 78 260 ×. (E) Regular rod showing undulation of the outer membrane, thin section. 49 140 ×. Bars represent 1 μm in Figs. 3A, 3C, 3D, and 3E and 0.1 μm in Fig. 3B.

1973). In addition, they lacked the capacity to divide like truely dimorphic cell forms such as *Arthrobacter* (Ensign and Wolfe 1964; Krulwich et al. 1967; Boylen and Pate 1973). Not every coccoid cell could be induced by nutrient additions to show viability, as evidenced by acquiring a higher refractility and eventually being converted to a rod (Figs. 2B, 2D, 2E). Some of the coccoid forms had lost a considerable portion of their cytoplasma (Fig. 3D) and resembled the nonviable spheres of *Vibrio* reported by Baker and Park (1975)

TABLE 1. Cellular concentrations of DNA and protein in different physiological stages of *C. johnsonae* strain C–21

Cell type	%[a]	Conditions for starvation	Time	°C	$10^9 \times$ CFU·L^{-1}	Protein,[d] mg·L^{-1}	DNA,[d] mg·L^{-1}	DNA/protein ratio
Viable rods	100	MB	0 h[b]	15	324	19 ± 4.8	1.9 ± 0.4	10
	100	MB	12 h	15	605	16	2.1	13
	>95	MB	2 days	15	553	22	2.6	12
	>95	MB	5 days	15	556	21 ± 0.7	3.0 ± 0.2	14
	100	MB	0 h[b]	25	243	12 ± 1.1	2.2 ± 0.1	19
	>95	MB	12 h	25	233	29	2.9	10
	90	MB	3 days	25	283	29	2.8	10
	60	MB	6 days	25	69	8 ± 0.5	0.9 ± 0.2	11
Moribund rods	100	MB	14 days	29	0	20 ± 0.4	6.0 ± 0.1	31
Coccoids	30	C[c]	1 month	5	180	25 ± 0.5	1.3 ± 0.1	20
	98	MB	18 months	5	4	28	4.0	14
	15	C[c]	14 days	15	3	4 ± 0.1	4.3 ± 0.1	18
	80	MB	21 days	25	127	51 ± 1.4	10.2 ± 0.2	20

[a]Estimates based on proportional counts. Pestlelike tansient stages were counted as coccoids.
[b]Log phase cell suspensions with $OD_{550} = 0.100$.
[c]Old culture suspension.
[d]Data represent the mean values of two independent culture experiments except where standard deviations are given for $n = 3$.

rather than those described by Felter et al. (1969). Since these cells also failed to show the same osmotic resistance rods when exposed to 20% sucrose (unpublished data), it appears justifiable to classify them as spheroplasts. This conclusion, however, need not be incompatible with the ability of these cells to form rods again. Although the conversion of spheroplasts to growing rod-shaped cells along with the resynthesis of the murein sacculus is rather unusual, it has been demonstrated in Gram-negative bacteria (Lark 1958; Hirokawa 1962; Schwarz and Leutgeb 1971). Whereas microcysts of *Sporocytophaga* are formed by conversion of the entire rod-shaped cells (Holt and Leadbetter 1967; Sudo and Dworkin 1973), pestlelike structures, similar to those described here for *C. johnsonae*, were described as part of the life cycle of *Sporocytophaga cauliformis*, a *species incertae sedis* (Graf 1962; Graf and Sturzenhofecker 1964; Leadbetter 1974). Our results show that *C. johnsonae* forms these pestlelike structures as transient forms during the starvation-induced development of coccoid stages. These, however, display the properties of spheroplasts and fail to match criteria established for the resting cells of gliding bacteria such as ultrasonic vibration and ultraviolet irradiation (Table 3) (Sudo and Dworkin 1969). Although the slightly enhanced resistance to heat of the coccoid stages of *C. johnsonae* is in contrast with the hypersensitivity of starved *Escherichia coli* cells to heat stress (Klein and Wu 1974), this would not be enough evidence to consider these coccoid stages as resting cells.

Exponentially grown rods starved in MB increased their DNA content as much as 65 and 32% at 15 and 25°C, respectively (Table 1). After reaching an advanced state of coccoid formation after 6 days at 25°C, concentrations of both cellular DNA and protein dropped rapidly. Continued starvation and increased formation of coccoid forms resulted in cellular DNA to protein ratios of 1.4 to 2.0 times higher than in rods harvested during exponential growth at 15°C, indicating DNA conservation. The maximal DNA to protein ratios were found in suspensions of 29°C, starvation-induced moribund rods.

Increases in cellular DNA and protein were noted during "fragmentation" of growing cultures of *Flexibacter* (Poos et al. 1972). A slight loss of protein was noted during unbalanced cell division of *C. johnsonae* at suboptimal growth temperatures. This was followed by a more pronounced loss of DNA and protein when the starvation period was extended for several weeks. The resulting increases of the DNA to protein ratios were not restricted to the formation of coccoid forms. These increases of the DNA to protein ratios reached maximum values in rods that had lost their viability during starvation at above optimal growth temperatures. Thus, DNA conservation alone would be an insufficient criterion to characterize the survival state.

Slight increases in the DNA content, which indicate the completion of replication cycles during the early stages of starvation, have been reported for other bacteria (Gronlund and Campbell 1963); Brdar et al. 1965) and may sometimes depend on the previously used growth substrate (Harrison and Lawrence 1963). As increases of viable counts peaked earlier than DNA (Table 1), the DNA synthesis occurring during starvation was not coupled with the observed peak of unbalan-

TABLE 2. Michaelis–Menten kinetic parameters of [U-^{14}C]glucose utilization at 15°C by *C. johnsonae* C-21 grown at 15°C

Cell type	Step[a]	Viable count, $10^9 \times$ CFU·L^{-1}	Glucose,[b] μM	K_m, μM	V_{max}, nmol·min^{-1}·mg protein^{-1}
Exponential phase	TR	107	41–333	448	28.1
($\mu = 0.04 \cdot h^{-1}$);	RE	76	49–392	2 456	35.3
motile rods	MI	22	200–40 000	4 012	101.8
Starvation, 4 days;	TR	322	200–800	399	2.9
mainly nonmotile	RE	50	12–100	137	2.0
short rods			200–40 000	8 144	83.7
	MI	50	25–100	401	1.9
			400–40 000	13 296	108.6
Starvation, 1 month;	RE	0	20–80	190	0.3
moribund rods[c]	MI	0	160–3200	2 936	4.5
Starvation, 2 months;	TR	17	41–667	82	8.8
~95% coccoids	RE	21	12–392	278	0.5
	MI	21	12–49	18	0.2

[a] TR = 3 min of transport, inhibited by 2 m*M* dinitrophenol; RE = 100 min of respiration (CO_2 evolution); MI = 100 min of incorporation into macromolecular compounds.
[b] Concentration ranges applicable for linear transformations of the Michaelis–Menten equation.
[c] Cell conversion inhibited at 29°C ; transport kinetics not determinable.

ced cell division. This would require the completion of one full cycle of replication (Clark 1968; Helmstetter and Pierucci 1968).

Within the ranges of glucose concentrations applicable to Michaelis–Menten kinetics, maximal rates of transport, respiration, and incorporation into macromolecular cell compounds sharply declined in starved cells when compared with cells assayed within 1 h after harvesting from the exponentially growing cultures (Table 2). Short rods, formed after 4 days of starvation at 15°C , displayed distinctly different kinetics for low and high ranges of glucose concentrations. Their K_m values for high levels of glucose were not significantly different from those determined for rods from the exponential growth phase.

Except for starved short rods in the upper concentration ranges (0.2 to 40 m*M*) (Table 2), starvation of viable cells was generally characterized by decreasing half-saturation constants (K_m) values for all three levels of glucose concentration. For example, K_m values obtained for incorporation indicated a 223-fold increase in substrate affinity ($1/K_m$) of coccoid cells over the log phase rods. Moribund, nontransformed rods incorporated glucose with a K_m comparable with that of the exponentially grown rods. The most striking increase of substrate affinities for respiration and dinitrophenol-inhibited transport was noted in coccoid cells, especially when compared with log phase rods.

Substrate affinities expressed by Michaelis–Menten constants for transport and the consecutive steps in substrate utilization provided adequate measures to evaluate the competitive ability of an organism to survive nutrient limitation and starvation (Button 1978; Akagi and Taga 1980). The ability of starved rods to incorporate and respire glucose with a high affinity constant at low concentrations and a low affinity constant at high glucose levels suggested the presence of alternative pathways (Neijssel et al. 1975). Glucose metabolism of the coccoid cell forms was adjusted to low concentrations of glucose only.

The cell type dependent affinity changes corresponded with structural difference of the cell envelopes. Comparatively high K_m and V_{max} values were found for exponentially growing cells, whose envelopes showed all the structural details that are typical for gliding bacteria, including an intermediate peptidoglycan layer and an extensively convoluted outer membrane through which extracellular slime is extruded (Figs. 3B, 3E, 2C) (Follet and Webley 1965; Pate and Ordal 1967; Humphrey et al. 1979). The cells lost their gliding motility as well as the pronounced convolution of their outer membrane (Fig. 3E) as the starvation period increased. During the transition from rods to coccoid forms large parts of the intermediate layer identified as murein sacculus dissappeared (Figs. 3A, 3C).

Resistance of starved cell suspension (at least 95% coccoid cells) and exponentially grown rods to ultraviolet irradiation, ultrasonic vibration, and heat was described by death rate constants ($-k$). These constants were calculated from linear regressions employing the equation

$$\ln (n_t/n_0) = -kt$$

where n_0 = initial number of viable cells and n_t = number of viable cells at time t.

The data in Table 3 show no significant differences

TABLE 3. Death rates ($-k$) for rods and coccoids exposed to various forms of physical stress

Type of stress	Intensity	Total period of exposure	$-k$ Rods	$-k$ Coccoids
Ultraviolet light	17.5 J·m^{-2}	24 s	0.26·s^{-1} ($r = -0.94$)	0.29·s^{-1} ($r = -0.88$)
Ultrasonic	100 W	160 s	0.02·s^{-1} ($r = -0.99$)	0.02·s^{-1} ($r = -0.99$)
Heat	38.5°C	24 min	0.09·min^{-1} ($r = -0.99$)	0.07·min^{-1} ($r = -0.95$)
	40.5°C	24 min	0.58·min^{-1} ($r = -0.99$)	0.14·min^{-1} ($r = -0.91$)

NOTE: r = correlation coefficient.

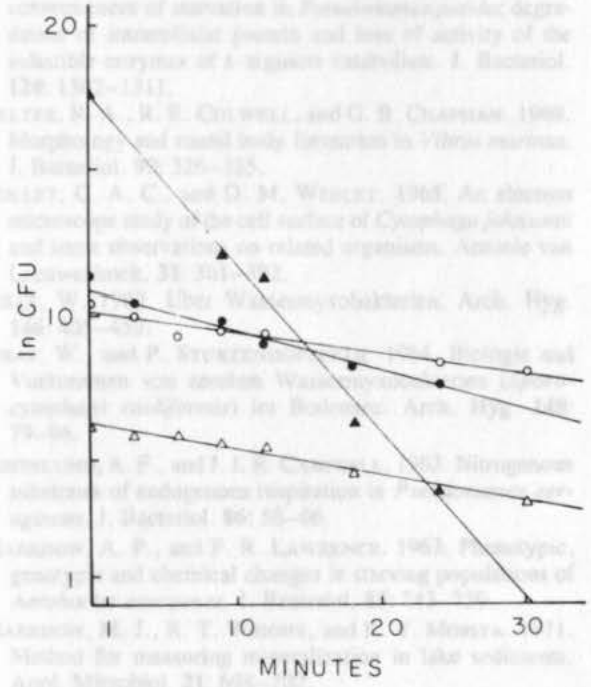

FIG. 4. Heat resistance of *C. johnsonae* C-21. Comparison of death rates derived from CFU determinations in suspensions of coccoids and exponentially grown rods. ○, coccoids at 38.5°C; ●, coccoids at 40.5°C; △, rods at 38.5°C, ▲, rods at 40.5°C.

between the death rates of two cell forms when treated with ultraviolet irradiation or ultrasonic vibration. However, thermal studies in a gradient temperature incubator indicated a significant difference of approximately 2° between the thermal death points of log phase rods (38.5°C) and coccoid cells (40.5°C). This was corroborated by thermal death kinetics (Fig. 4). At 40.5°C thermal death rates of the rods were approximately four times more rapid than those of the coccoid cells (Table 2).

The data presented in this paper present evidence for the first time that the coccoid form of *Cytophaga* should be considered spheroplasts, not resting cells or microcysts, but with the capacity to form rods again under the proper conditions. The data presented also demonstrate the morphogenetic flexibility of *Cytophaga* that has not received any attention in the past. The coccoid form is produced as a function of temperature and starvation. When extrapolated to the environment, the coccoid cell is probably the physiological and morphological form that provides the organism with its best survival state under the stress of lower temperatures and nutrient deprivation.

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Influence of Temperature Adaptation on Glucose Metabolism in a Psychrotrophic Strain of *Cytophaga johnsonae*†

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Influence of Temperature Adaptation on Glucose Metabolism in a Psychrotrophic Strain of *Cytophaga johnsonae*†

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Selective enrichment of yellow-orange-pigmented, gram-negative bacteria related to *Cytophaga johnsonae* from lake sediment was dependent on low temperatures (ca. 5°C). However, this temperature effect was abolished when excessive amounts of dissolved organic carbon (10 mM *N*-acetylglucosamine) were added. A psychrotrophic freshwater isolate of *C. johnsonae* was used to study the physiological versatility of this group. Exponential growth rates were found to be dependent on the temperature to which the cells used as the inocula were acclimated. Glucose incorporation and respiration were also dependent upon the acclimation temperature of the inocula. Patterns of $^{14}CO_2$ evolution obtained from position-labeled [^{14}C]glucose indicated that glucose was predominantly metabolized via the Embden-Meyerhof-Parnas pathway, which, however, was greatly reduced at 25°C when the concentration of glucose was as low as 5 μM/liter. Transport, respiration, and incorporation of glucose (0.2- to 20,000-μM/liter concentrations) into macromolecular cellular compounds were characterized by multiple K_m values which were a function of substrate concentration and temperature. It appeared possible that these multiple K_m values reflected the changing participation of the Embden-Meyerhof-Parnas pathway in glucose metabolism. These results may provide a physiological explanation for the selective enrichment of psychrotrophic freshwater cytophagas. Moreover, they exhibit the limits of interpreting kinetic data based on conventional heterotrophic potential measurements, especially when some complications may arise from temperature and substrate adaptations of the more versatile members of the chemoorganotrophic microflora such as *C. johnsonae*.

Yellow-orange-pigmented, gram-negative bacteria which can be readily isolated from various freshwater habitats on chitin agar are often flexuous rods with gliding motility. They form spreading colonies and produce a flexirubin-type pigment. They are mostly psychrotrophs, similar to the phenotype of *Cytophaga johnsonae* described by Stanier (21). They are abundantly found in permanently or temporarily cold freshwater habitats (18). These organisms are also degraders of various α- and β-linked polysaccharides in lakes (15). The combined effect of water temperature and availability of dissolved organic carbon on their growth has been noted (15, 16).

In an attempt to explain the results obtained in enrichment experiments, the physiological versatility of a representative, psychrotrophic isolate of *C. johnsonae* strain C-21 was examined, using temperature and substrate concentration gradients. This organism was isolated from a small eutrophic lake at 5°C (15). The techniques used to determine the incorporation and respiration of glucose by cells were similar to those usually applied to determine the kinetics of the heterotrophic potential in aquatic habitats (1, 5, 6).

Physiological versatility is apparently expressed by the existence of different metabolic pathways for glucose as a function of temperature or substrate availability (2, 7, 14, 23). Modified radiorespirometry experiments were carried out with *C. johnsonae* by using ^{14}C-differentially labeled glucose.

MATERIALS AND METHODS

Enrichment experiments. A 10-g portion of Lake Tahoe sediments (400-m depth) was suspended in 400 ml of surface water enriched with colloidal chitin (200 mg/liter). After the suspension had settled for 30 min, 5-ml portions were distributed (in triplicate) into culture tubes (20 by 150 mm) which were incubated at 5 and 25°C with and without 0.05 ml of 1 M *N*-acetylglucosamine solution. Half of the tubes were sealed with

† Technical paper no. 6506, Oregon Agricultural Experiment Station.

an overlay of 5 ml of sterile mineral oil to help exclude oxygen, and the other half were placed on a rotary shaker (100 rpm).

Colony-forming units (CFU) were determined at weekly time intervals for 3 weeks, using a double layer of chitin agar. Plate count agar (10 ml; Difco Laboratories, Detroit, Mich.) was placed in the bottom of a sterile petri dish and was overlaid with chitin agar containing agar (15 g/liter), colloidal chitin (10 g/ml), yeast extract (100 mg/liter; Difco), and *N*-acetylglucosamine (100 mg/liter) (16). Serial dilutions of 0.1 ml of the sediment suspension were made in 5 mM KH_2PO_4 buffer (pH 7.0) containing Tween 80 (10 mg/liter). Spread plates (in triplicate) were made on each dilution. After 14 days of incubation at 18°C, *C. johnsonae*-like colonies were counted and identified by the formation of chitinolytic halos resulting from the bathochromic color shift due to flexirubin pigments when KOH was added (20). Microscopic verification of the presence of long flexuous rods, often showing gliding motion, was also used.

Cultural experiments. *C. johnsonae* Stanier, strain C21, was isolated from freshwater (15). Its psychrotrophic properties have recently been reported by W. Reichardt, R. Y. Morita, and R. R. Colwell (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N63, p. 174). The organism was cultivated in a mineral medium (MM) consisting of the following (per liter): 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 3.0 mg of $CaCl_2$, and 0.3 mg of $FeCl_3$, buffered with 10 mM KH_2PO_4-$NaHPO_4$ buffer (pH 7.0; autoclaved separately) and supplemented with 10 mM membrane filter-sterilized glucose (0.10 μM).

Temperature-acclimated cells were produced by growing batch cultures of the organism at an incubation temperature of 5, 7, or 25°C in 1-liter Erlenmeyer flasks containing 500 ml of MM supplemented with 10 mM glucose, using a rotary shaker at 100 rpm. When the cultures reached their late exponential growth phase, 0.5 ml was transferred into fresh medium and the process was repeated for several months.

Growth rates. Exponential growth rates as a function of temperature were determined with a temperature gradient incubator (Scientific Industries, Mineola, N.Y.). The temperature gradient incubator was adjusted so that 30 different temperatures could be obtained at 1 to 2°C intervals. This permitted the incubation of 30 tubes (in duplicate) at 30 different temperatures simultaneously. Those tubes containing sterile medium (10 ml) were temperature equilibrated before being inoculated with 0.1 ml of acclimated log-phase culture suspensions (optical density at 500 nm [OD_{500}] = 0.50). Growth was measured by OD_{500} readings with a Bausch & Lomb Spectronic 20 spectrophotometer taken at 3- to 8-h intervals for periods up to 72 h.

Assays with D-[*U*-^{14}C]glucose. Cells were harvested by centrifugation (20 min, 10,000 × *g*) during their exponential growth phase and suspended in MM to an OD_{500} of 0.02. Sterile temperature gradient incubator tubes used with the same serum cap and bucket assembly as serum flasks for mineralization assays (5, 6) were filled with 4.9-ml portions of the cell suspension and incubated in the temperature gradient with 0.1 ml of a mixture containing 250 μmol of unlabeled glucose per liter and 0.3 μCi of D-[*U*-^{14}C]glucose (284 mCi/mmol). After an incubation period of 100 min, the assay was stopped by injecting 0.2 ml of 1 N H_2SO_4. At the same time 0.15 ml of phenethylamine (CO_2 absorbent) was injected into the bucket assembly containing fluted filter paper. The tubes were shaken for 1 h at 22°C. The fluted filter paper was removed and placed in a scintillation vial to which 10 ml of toluene-based Omnifluor was added. Radioactivity of the $^{14}CO_2$ produced was measured with a Beckman LS100 scintillation counter. CFU were determined at the initiation of each experiment. The acidified cell suspensions were kept at 2°C and within 100 min were filtered through membrane filters (0.22 μm; Millipore Corp., Bedford, Mass.) at 400 mmHg (ca. 53 kPa). The filters were rinsed three times with 5 ml of MM, dried at 50°C for 40 min, and then placed in scintillation vials each containing 10 ml of Omnifluor to measure the radioactivity of the incorporated glucose.

To determine the influence of the substrate concentration on rates of D-[*U*-^{14}C]glucose respiration and incorporation at 7 and 25°C, cells were grown at 16°C, harvested as above, resuspended in MM to an OD_{500} of 0.015, and subsequently starved for a period of 6 h at 16°C. The assays for incorporation and respiration of glucose were carried out in 60-ml serum vials with cap and bucket assembly (1, 5). A concentration gradient of glucose was produced by diluting a stock solution of filter-sterilized (0.22 μm; Millipore) unlabeled glucose (200 mM; 1:1) with distilled water. Portions, 0.5 ml, of the resulting concentration were pipetted into serum flasks (in triplicate), followed by 0.1 ml of D-[*U*-^{14}C]glucose (0.15 μCi; specific activity, 329 mCi/mmol). The assay suspension (4.4 ml) was incubated for 150 min at 7°C and 30 min at 25°C on a rotary shaker at 120 rpm. After the assay was stopped by acidification, all flasks were shaken (150 rpm) at 5°C before measuring the radioactivity of the $^{14}CO_2$ absorbed to phenethylamine and the ^{14}C incorporated in the 0.22-μm membrane-filtered cells, using the techniques described above.

To determine the influence of substrate concentrations on transport, 0.5 ml of each of 20 unlabeled glucose concentrations (see above), 0.1 ml of D-[*U*-^{14}C]glucose (0.15 μCi; specific activity, 329 mCi/mmol), and 0.4 ml of the culture suspension (OD_{500} = 0.45; grown at 16°C) were mixed in test tubes and incubated in a water bath at 7 or 25°C. Exactly 5 min after the cell suspensions had been added to the above mixture, duplicate 0.4-ml portions were filtered through Millipore membrane filters (0.22 μm), using a 400-mmHg vacuum. Samples poisoned with 2 mM dinitrophenol served as controls. The filters were rinsed three times with 5 ml of MM and subsequently treated as described above for incorporation assays.

Modified radiorespirometric assays were carried out with acclimated cells at 7 and 25°C with equimolar amounts of differentially labeled [^{14}C]glucose in 60-ml serum flasks containing either 4.8 or 1.8 ml of the cell suspension (OD_{500} = 0.100) and 0.1 ml of 250 μM or 25 mM unlabeled glucose to give final concentrations of 5 μM or 1.25 mM, respectively. Also added to the reaction mixture was 0.1 ml of a 25 mM solution of one of the following position-labeled [^{14}C]glucose preparations: D-[1-^{14}C]glucose (8.2 mCi/mmol); D-[2-^{14}C]glucose (6.0 mCi/mmol); D-[3,4-^{14}C]glucose (10.32 mCi/mmol); D-[6-^{14}C]glucose (9.0 mCi/mmol). The reaction mixture was shaken at 150 rpm. At 1-h intervals, the

TABLE 1. Enrichment of *Cytophaga*-like bacteria from Lake Tahoe sediment suspension[a]

Enrichment conditions	Without *N*-acetylglucosamine			With *N*-acetylglucosamine		
	10^6 CFU/liter	% CFU on chitin agar	% Chitinoclastic CFU	10^6 CFU/liter	% CFU on chitin agar	% Chitinoclastic CFU
5°C, aerobic	137	47	74	283	71	88
5°C, anaerobic	130	54	84	73	63	79
25°C, aerobic	<2	0	0	62	70	98
25°C, anaerobic	3	1	2	190	40	99

[a] Maximum counts occurring during 3 weeks of enrichment after 2 weeks at 25°C and after 3 weeks at 5°C; the initial number of *Cytophaga*-like bacteria was $<10^6$ CFU/liter.

reaction mixture was removed and the rates of incorporation and respiration were determined. All runs were made in triplicate. All radioactive substrates were obtained from New England Nuclear, Boston, Mass.

RESULTS AND DISCUSSION

Enrichment of *Cytophaga*-like freshwater bacteria. When colloidal chitin and *N*-acetylglucosamine were added to Lake Tahoe sediment, the numbers of bacteria resembling the phenotype of *C. johnsonae* Stanier increased regardless of the temperature of incubation (5 or 25°C) or the availability of oxygen (Table 1). Colloidal chitin enrichment for these bacteria was restricted to the lower temperature. Additional dissolved organic carbon compounds or *N*-acetylglucosamine was necessary to obtain a similar enrichment of the organism at 25°C. Under substrate conditions most likely to prevail in natural freshwater environments, a low-temperature-controlled selection for chitin-degrading cytophagas may be anticipated. Similar conclusions have been drawn from studies of *Cytophaga*-like bacteria in cold freshwater habitats (10, 15, 17). Since dissolved organic compounds of *N*-acetylglucosamine was necessary for enrichment for cytophagas at 25°C, this indicated a competitive capacity for substrate utilization by these organisms in the upper temperature range tested.

The exponential growth rates of 25°C acclimated cells (inoculum of *C. johnsonae* strain C21), when grown at different temperatures, showed a steady increase until reaching their optimum at between 27 and 30°C (Fig. 1). Slightly higher growth rates were obtained when 7°C acclimated inocula were used at the lower temperature range. The growth rates of cells acclimated to 7°C were maximal at around 25°C, but no significant differences were found over a fairly large range of near-optimal growth temperatures ranging from 15 to 30°C. It is known that the temperature characteristic (μ) can be different for cells acclimated at different temperatures (19).

Cells acclimated to 25°C kept their rates of glucose respiration at a relatively constant level between 5 and 27°C. On the other hand, cells acclimated to 5°C showed minimal respiration rates in the lower temperature range (Fig. 2). At around 10°C the respiration rates of 5°C acclimated cells started to increase. Between 18 and 31°C the respiration rates were more than twice the level observed for the 25°C acclimated cells. Glucose incorporation into cellular material was

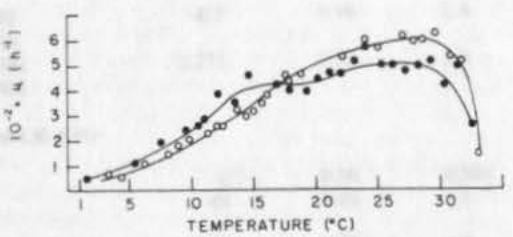

FIG. 1. Influence of temperature on exponential growth rates (μ) of psychrotrophic *C. johnsonae* strain C21, when acclimated to 7°C (○) and 25°C (●).

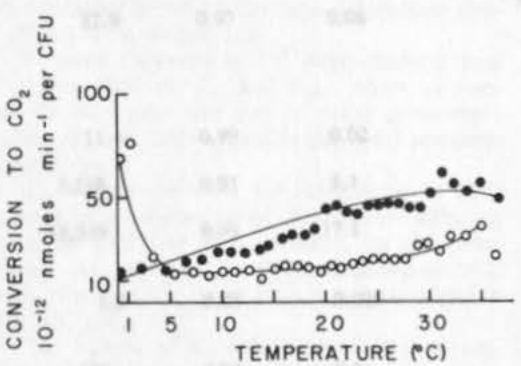

FIG. 2. Influence of temperature on specific respiration rates of glucose per CFU of *C. johnsonae* strain C21. Initial substrate concentration was 5 μM/liter after temperature acclimation to 5°C (●) and 25°C (○).

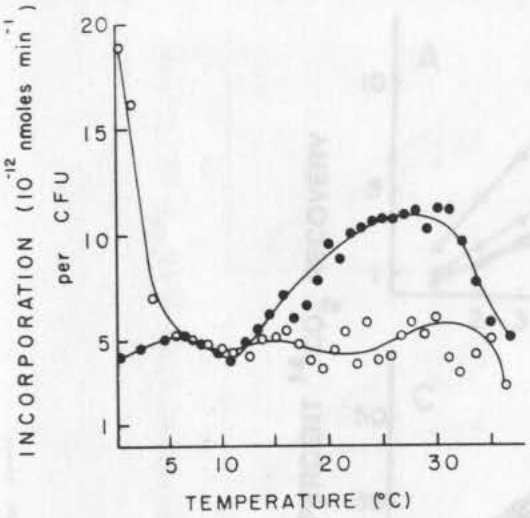

FIG. 3. Influence of temperature on specific rates of glucose incorporation into macromolecular cell compounds per CFU of *C. johnsonae* strain C21. Initial substrate concentration was 5 μM/liter after temperature acclimation to 5°C (●) and 25°C (○).

greater with 5°C acclimated cells between ca. 12 to 31°C (Fig. 3). There appears to be excessive glucose incorporation with 25°C acclimated cells when temperatures of <5°C are used. A comparison of Fig. 2 and 3 with Fig. 1 shows that the growth and incorporation rates started to decline at approximately the same temperature range (above 30°C), whereas respiration rates did so only above 35°C.

The apparent impact of temperature acclimation in growth and glucose utilization by 7 and 25°C acclimated cells was considered an indicator of extreme metabolic versatility of the organism. When 7 and 25°C acclimated cells were subjected to a sudden temperature shift, complete acclimation of the cells to the new temperature required at least two generations (data not shown). Apparently, this time lag was in contrast to the immediate response noted for exponential growth rates for *Escherichia coli* for temperature ranges with a constant-temperature characteristic (12). However, it resembled the pattern of temperature and substrate adaptation reported for psychrotrophic *Pseudomonas* spp. (8, 13).

The respiration pattern of 7 and 25°C acclimated cells at various temperatures of incubation may appear anomalous, but the 7°C acclimated cell curve may be the result of temperature affecting the fluidity of the membrane, which in turn affects transport, respiration, and incorporation. Previously, Haight and Morita (4) demonstrated that *Vibrio marinus* grown at the temperature at which it was isolated (ca. 5°C) demonstrated better oxygen uptake in the presence of glucose at 15°C (organism's optimum growth temperature) than did cells grown at

TABLE 2. Michaelis-Menten constants for transport, respiration, and incorporation of glucose at different concentration ranges by 16°C acclimated cells[a]

Concn range (μM/liter)	7°C			25°C		
	K_m (μM/liter)	r^2 [a]	V_{max} (10^{-9} nmol/min per CFU)	K_m (μM/liter)	r^2	V_{max} (10^{-9} nmol/min per CFU)
Transport (5 min)						
0.03–0.07	1.4	0.98	0.001			
5–39				82.9	0.97	0.06
156–1,250	1,212	0.92	0.5			
2,500–10,000	14,300	0.99	3.9			
Respiration (30 min)						
0.04–2.5				11.4	0.99	0.02
39–1,250	427	0.98	2.4			
156–2,500				5,118	0.91	8.1
2,500–20,000	2,273	0.95	3.0			
5,000–20,000				18,559	0.89	17.1
Incorporation (30 min)						
0.1–0.4				1.0	0.88	0.005
0.3–0.7	0.7	0.94	0.004			
2.5–20	40	0.99	0.1			
20–78				129	0.99	7.6
1,250–5,010	1,077	0.99	4.8			

[a] r^2 = Goodness of fit of linear transformation according to Eadie (3).

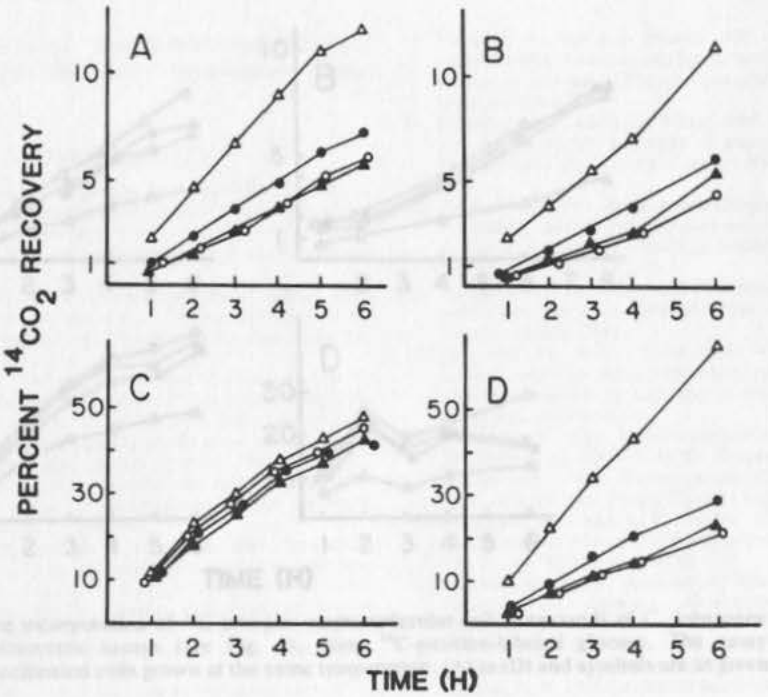

FIG. 4. Radiorespirometric pattern for *C. johnsonae* strain C21: percent cumulative recovery of $^{14}CO_2$ from equimolar concentrations of ^{14}C-differentially labeled glucose. The assay temperatures were run with acclimated cells grown at the same temperature. (A) 5 μM glucose at 7°C; (B) with 1.25 mM glucose at 7°C; (C) with 5 μM glucose at 25°C; (D) 1.25 mM glucose at 25°C. Symbols: ○, D-[1-^{14}C]glucose; ●, D-[2-^{14}C]glucose; △, D-[3,4-^{14}C]glucose; ▲, D-[6-^{14}C]glucose.

15°C. This was also true for endogenous respiration. They also demonstrated membrane differences between cells grown at two different temperatures as evidenced by the leakage of intracellular material with increased temperature. Because respiration on a substrate is better, the incorporation of glucose by 5°C acclimated cells is better (Fig. 3).

It is difficult to explain the "overshoot" of respiration and incorporation of glucose in Fig. 2 and 3 by the 25°C acclimated cells when the temperature of incubation is shifted to below 5°C. The data presented in Fig. 2 and 3 for this "overshoot" are indicated by 12 data points (each point on Fig. 2 and 3 is the average of duplicate samples). However, it should be pointed out that cold shock leads to a general increase in permeability of bacterial cells (9). Hence, the 25°C acclimated cells may undergo cold shock, whereas the 7°C acclimated cells do not. Increased permeability might be reflected in higher respiration and incorporation rates. In an effort to explain these results, studies on transport and radiorespirometric studies were initiated.

The kinetic rates of glucose transport, incorporation, and respiration were determined for a gradient of 20 different concentrations of glucose ranging from 0.2 to 20,000 μM/liter. Michaelis-Menten kinetics (3) proved applicable only within certain concentration ranges (Table 2). Both K_m and V_{max} values increased considerably towards higher concentration ranges. Ranges in which Michaelis-Menten kinetics were applicable differed as a function of the temperature at which the rates of glucose conversion were determined.

Transport kinetics at 7°C were characterized by three different K_m and V_{max} values as compared with only one pair of these parameters being determinable at 25°C in the lower temperature range.

K_m values obtained for respiration at 25°C indicated a broader range of substrate affinities than at 7°C. For incorporation, on the other hand, the maximal K_m value obtained at 25°C was considerably lower than that determined in the 7°C assay.

The variety of K_m values obtained for transport and incorporation of glucose suggests a greater physiological versatility of *C. johnsonae* at the lower temperature (7°C). This interpretation would help in understanding the selective

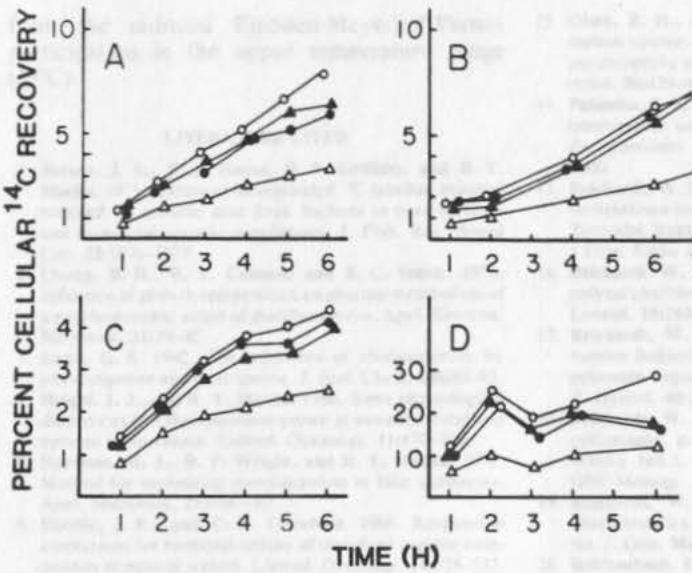

FIG. 5. Percent cumulative incorporation of ^{14}C into the macromolecular cell compounds of *C. johnsonae* strain C21 during radiorespirometric assays (see Fig. 4), using ^{14}C-position-labeled glucose. The assay temperatures were run with acclimated cells grown at the same temperature. (A) to (D) and symbols are as given in the legend to Fig. 4.

advantage of freshwater cytophagas in the lower temperature.

The patterns of sequence in which differentially labeled [^{14}C]glucose was respired or incorporated into cellular macromolecules did not reveal any significant alterations as a function of the temperature at which the cells had been acclimated and assayed (Fig. 4). At substrate concentrations corresponding to those used in the temperature gradient experiments, however, the relative predominance of D-[3,4-^{14}C] glucose as a major contributor to $^{14}CO_2$ evolution was markedly reduced at the elevated temperature (25°C). At the same time, mainly C-1 followed by C-6 and C-2 moieties were incorporated into macromolecular cell compounds irrespective of the temperatures and substrate concentrations chosen (Fig. 5).

The radiorespirometric data obtained for *C. johnsonae* C21 (Fig. 4) indicate major changes of glucose catabolism as a function of temperature. When the modified version of Wang's (23) technique was used, the maximal contribution of D-[3,4-^{14}C]glucose to the evolution of $^{14}CO_2$ indicated a predominance of the Embden-Meyerhof-Parnas pathway under all experimental conditions tested (22, 23). However, for the lower substrate concentration of 5 μM/liter, the relative conversion of D-[3,4-^{14}C]glucose to $^{14}CO_2$ was strikingly reduced at 25°C compared with the corresponding experiment at 7°C. According to Wang (22), this could indicate a shift to a less extensive participation of the Embden-Meyerhof-Parnas pathway in favor of the pentose phosphate and tricarboxylic acid cycle pathways. As far as the applicability of Wang's (22) methods is concerned, at a substrate concentration of only 5 μM/liter a possible interference by an excessive dilution of the labeled substrate in the endogenous cellular pools was least likely to occur.

Glucose catabolism via the Embden-Meyerhof-Parnas pathway seemed to be reduced only at substrate concentrations as low as 5 μM/liter but not at the much higher concentration of 1.25 μM/liter. It appears that the Embden-Meyerhof-Parnas pathway was favored at relatively high concentrations of glucose, irrespective of the temperature range chosen. This would also correspond to the observations made by Whiting et al. (24) that alternative pathways of glucose catabolism in *Pseudomonas fluorescens* were regulated by the available concentration of glucose.

The coexistence of concurrent metabolic pathways may also be indicated by multiple kinetic parameters (11). The triplicate K_m values obtained for glucose respiration by *C. johnsonae* at 25°C covered a substrate concentration range of more than three orders of magnitude. It is possible that these K_m values reflected the complex pattern of metabolic pathways resulting

from the reduced Embden-Meyerhof-Parnas participation in the upper temperature range (25°C).

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MICROBIAL ECOLOGY

Ecology and Taxonomy of Chitinoclastic *Cytophaga* and Related Chitin-Degrading Bacteria Isolated from an Estuary

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Abstract. A total of 103 strains of estuarine, chitinoclastic bacteria isolated from water, and sediment samples collected from the upper Chesapeake Bay, including 17 freshwater and 11 seawater isolates, were subjected to numerical taxonomy analysis. The isolates included 44 yellow-orange pigmented strains classified as *Cytophaga*-like bacteria (CLB) of the *Cytophagaceae*. Salt requirement of the strains ranged from tolerance to ≤1% NaCl to an absolute requirement for NaCl, with 1% NaCl satisfying this requirement. The largest phenon consisted of facultatively anaerobic, oligo-nitrophilic, and flexirubin pigment-producing freshwater and estuarine isolates, and included reference strains of both *Cytophaga johnsonae* Stanier and *Cytophaga aquatilis* Strohl and Tait. Other phena, containing a smaller number of strains, comprised marine and estuarine isolates which did not produce flexirubin pigments, and required organic nitrogen for growth and for production of chitinolytic enzymes. Salt-requiring, flexirubin pigment-producing, chitin-degrading strains were, on occasion, isolated from estuarine samples and represented phena found in estuaries. Most of the *Cytophaga* isolates, as well as chitin-degrading species not of the genus *Cytophaga* that were isolated from Chesapeake Bay, clustered in phena representing previously described species of aerobic, zymogenic, chitinoclastic bacteria. When the frequency of occurrence of features related to environmental parameters, viz., pH, salinity, temperature range of growth, and growth on media lacking organic nitrogen, was calculated, ecological groupings of strains in the 2 major phena of CLB could be distinguished among the estuarine, chitin-degrading bacteria.

Introduction

The effect of salinity on the population dynamics of freshwater bacteria discharged into estuaries has rarely been studied, with regard to physiological effects on the organisms or selection for specific taxonomic groups [39, 42, 43, 46], despite the fact that salt adaptation and/or requirement is considered an

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important taxonomic criterion [7]. The species composition of bacterial populations responsible for selected biogeochemical transformations in the freshwater environment can be expected to undergo profound changes when water carrying these microorganisms enters an estuary via run-off or riverine flow. Some species of freshwater and marine bacteria, however, are not affected by salinity changes. In fact, reports of plasmid-mediated salt resistance, or requirement, suggest that salt adaptation may be acquired relatively easily by certain freshwater bacteria [23, 24].

At the present time, chitin-utilizing *Cytophaga* are included in a single species, *Cytophaga johnsonae* Stanier [18]. The objective of the study reported here was to provide a taxonomic analysis of aerobic, zymogenic, chitinoclastic bacteria isolated from estuaries. In addition, it was intended that an understanding be gained of *Cytophaga* and related chitinoclastic species occurring in freshwater habitats and of their relationships to estuarine species [25–27]. Numerical taxonomy was used to identify strains of *Cytophaga johnsonae* among the gram-negative, yellow-pigmented, and (often) gliding bacteria of the *Flavobacterium-Cytophaga-Flexibacter-Lysobacter* complex [8, 13, 25, 30, 36, 45]. None of the reports published to date concerning *Cytophaga* describes the occurrence of *Cytophaga johnsonae* in the estuarine or marine environment. Thus, *Cytophaga* spp. and related chitin-degrading bacteria are of great interest. Of special interest is the persistence and phenotypic stability of freshwater chitinoclastic bacteria entering the estuarine and/or marine environment.

Materials and Methods

Isolation

A total of 103 strains of chitinoclastic bacteria, including 44 strains identified as *Cytophaga* spp., were isolated from surface water, bottom water, and sediment samples collected from sampling sites at Jones Falls, Chester River, Eastern Bay, Chesapeake Beach, and Hooper's Island in Chesapeake Bay during October 1978 through March 1979. Samples were subjected to bacteriological analysis aboard the R/V Ridgely Warfield immediately after collection.

Appropriate dilutions in ¼ strength artificial sea water [11], supplemented with 0.5 mM KH_2PO_4/Na_2HPO_4 buffer adjusted to pH 7.4 and containing 10 ppm Tween 80 were used. Four agar media were used, including double-layer agar plates, prepared with 15 ml Plate Count Agar (Difco Labs., Detroit, MI) and Marine Agar 2216 (Difco Labs., Detroit, MI), respectively, as the bottom layer and a top layer of 1) mineral agar amended with 1.0% reprecipitated chitin [26] suspended either in distilled water or ½ strength artificial seawater [11]; and 2) the same medium amended with 2 g/liter *Micrococcus lysodeicticus* cells (Difco Labs., Detroit, MI) as a substitute for chitin. Incubation of inoculated media was at 2, 15, and 36°C for 2–21 days.

Chitinoclastic bacteria were identified by a halo around colonies on chitin agar. Each yellow- or orange-pigmented, chitinoclastic bacterial colony observed at the highest dilution used for a given sample was isolated, while other nonpigmented, chitinoclastic colonies were picked at random. About 40% of the isolates appearing on the original plates did not remain viable during the time in which the steps of purification, transfer, and maintenance on Plate Count Agar, were followed. Nor did they remain viable during the time of subsequent selection of relatively fast-growing strains, that is, strains producing colonies within 4 days at 20°C.

For numerical taxonomy analysis, the estuarine, chitinoclastic bacteria, obtained as described above, were included with isolates of *Cytophaga*-like bacteria obtained from other aquatic environments and seven reference strains. Seventeen strains were from freshwater sources, including

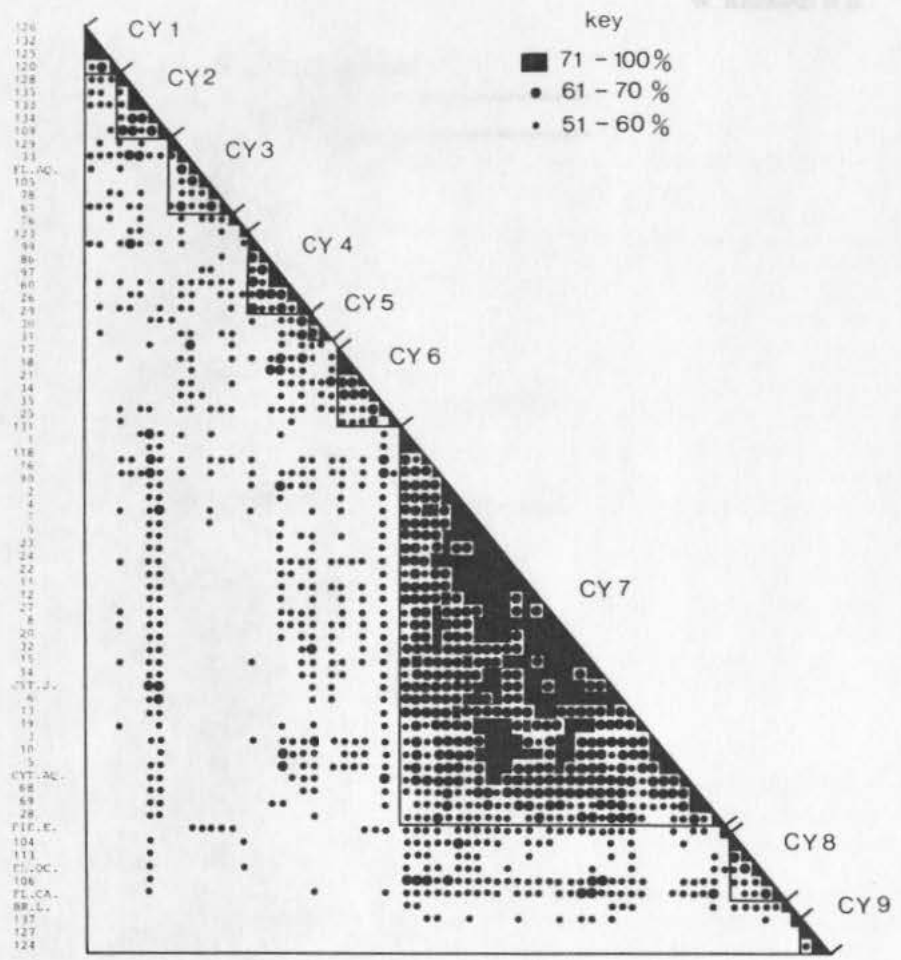

Fig. 1. Similarity matrix, based on the S_J similarity coefficient, for *Cytophaga* and related strains. Formation of clusters ≥60% similarity is indicated by the symbols shown. The ordinate provides strain numbers.

Mindel Lake, SW Germany; Lake Tahoe, California; Crater Lake and Odell Lake, Oregon; Kealia Lake, Maui, Hawaiian Islands; and the Willamette River, Oregon. Eleven marine strains included in the analysis were isolated from near-shore waters of the Pacific Coast off Oregon. Reference strains included in the study were yellow-orange pigmented, but only partly chitinoclastic, that is, *Cytophaga johnsonae* (DSM 425), *Flavobacterium capsulatum* (DSM 30196), *Flavobacterium aquatile* (DSM 1132), *Flexibacter elegans* (DSM 527), *Brevibacterium linens* (DSM 20158), (DSM = German Collection of Microorganisms, Goettingen); and *Flavobacterium oceanosedimentum* 31337 and *Cytophaga aquatilis* 29551 from the American Type Culture Collection, Rockville, Md.

All strains were maintained, by serial transfer, on Difco Plate Count Agar (PCA) slants prepared with, and without, addition of 0.2% chitin, and either with distilled water or ½ strength artificial seawater (ASW, *vide infra*. Stock cultures were stored at 2°C.

Media used for testing nonmarine strains (Strain Nos. 1–105 in Fig. 1) were prepared with distilled water. For those strains of marine or estuarine origin that showed salt requirement or

Table 1. List of features included in the study[a]

No.	Feature
1	Length >5 μm
2	Length variable
3	Length:Width ≥ 6
4	Shape: straight
5	Shape: curved/spiral
6	Shape: cocci
7	Coccoid stages
8	Branching
9	Flexuous rods
10	Endospores
11	Single cells
12	Pairs
13	Chaining
14	Star-shaped aggregates
15	Fruiting bodies
16	Motility
17	Gliding motility
18	Gram-positive or variable
19	Margin spreading
20	Margin entire
21	Colony translucent
22	Colony fluorescent
23	Colony iridescent
24	Colony shining surface
25	Colony viscous
26	Colony leathery
27	Colony white
28	Colony creme/grey
29	Colony orange
30	Colony dark-orange/brown
31	Colony yellow
32	Colony greenish
33	UV-fluorescent pigment
34	Diffusible pigment
35	Violacein type of pigment
36	Flexirubin type of pigment
37	Growth on 1% NaCl
38	Growth on 2% NaCl
39	Growth on 3% NaCl
40	Growth on ¼ strength ASW
41	Growth on ½ strength ASW
42	Growth on ¾ strength ASW
43	Salt required for growth
44	NaCl requirement only
45	UV sensitive
46	Growth at 2°C
47	Growth at 8°C
48	Growth at 15°C
49	Growth at 20°C
50	Growth at 25°C
51	Growth at 30°C
52	Growth at 35°C

Table 1. Continued

No.	Feature
53	Growth at 45°C
54	Salinity-induced G-T shift up
55	Salinity-dependent G-T shift down
56	Growth at pH 4.5
57	Growth at pH 5
58	Growth at pH 6
59	Growth at pH 8
60	Growth at pH 9
61	Growth at pH 10
62	Sal.-dep. shift up in pH range
63	Sal.-dep. shift down in pH range
64	Anaerobic growth with NO_3^- as sole *N* source
65	NO_3 reduction in NO_3^- and glucose
66	Growth in NH_4Cl and glucose
67	Growth on N-free media
*68	Swarming specific on Carbohydrate Min. Agar
*69	Heat stable alkaline phosphatase
*70	pNP-acetylglucosaminidase
71	Growth on sole C-source: galactose
72	Growth on sole C-source: ribose
73	Growth on sole C-source: xylose
74	Growth on sole C-source: arabinose
75	Growth on sole C-source: cellobiose
76	Growth on sole C-source: lactose
77	Growth on sole C-source: raffinose
78	Growth on sole C-source: cellulose
79	Hydrolysis of cellulose
80	Growth on CM-cellulose as sole C-source
81	Growth on starch as sole C-source
82	Growth on chitin as sole C-source
83	Hydrolysis of chitin
84	Growth on alginate as sole C-source
85	Growth on acetate as sole C-source
86	Growth on succinate as sole C-source
87	Growth on *L*-alanine as sole C-source
88	Growth on asparagine as sole C-source
89	Growth on glucosamine as sole C-source
90	Growth on acetylglucosamine as sole C-source
91	Hugh-Leifson-Test: growth
92	Hugh-Leifson-Test: oxidation
93	Hugh-Leifson-Test: anaerobic growth
94	Hugh-Leifson-Test: alkaline reaction
95	Lactose utilized (Kligler)
96	Citrate utilized (Simmons)
97	Agar hydrolyzed
98	Gelatine liquefied
99	Hugh-Leifson test: fermentation
100	Oxidase
101	Catalase
102	Alkaline phosphatase
103	NO_3^- reduction
104	Denitrification (gas)

Table 1. Continued

No.	Feature
105	Lytic activity
106	Arginine decarboxylase
107	Ornithine decarboxylase
108	Lysine decarboxylase
109	Phenylalanine deaminase
110	Urease
111	Indol production
112	Aesculin hydrolysis
113	H_2S-production
114	Methyl Red test
115	Voges Proskauer test
116	Hydrolysis of: Tween 20
117	Hydrolysis of: Tween 40
118	Hydrolysis of: Tween 60
119	Hydrolysis of: Tween 80
120	Sensitive to SDS
121	Sensitive to penicillin G
122	Sensitive to polymyxin B (in Agar)
123	Sensitive to polymyxin B (disc)
124	Sensitive to vibriostatic agent 0/129

[a] Tested only for *Cytophaga* and related strains

growth in full strength marine media during preliminary tests (strains No. 106–138, Fig. 1), media were prepared using ½ strength artificial seawater (ASW) adjusted to pH 7.4. Full strength ASW consisted of 24.0 g/liter NaCl; 7.0 g/liter $MgSO_4 \cdot 7H_2O$; 5.3 g/liter $MgCl_2 \cdot 6H_2O$, and 0.7 g/liter KCl [11].

The mineral basal medium (MB) used for preparing ASW-free media (MB 1) consisted of 10 mM/liter KH_2PO_4-Na_2HPO_4-buffer, pH 7.0; 1.0 g/liter $(NH_4)_2SO_4$; 0.2 g/liter $MgSO_4 \cdot 7H_2O$; 3 mg/liter $CaCl_2$; and 0.3 mg/liter $FeCl_3$. Mineral basal medium, in the case of media containing ASW (MB 2), had the same composition, except for a lower PO_4^{3-} content, that is, 1 mM/liter of phosphate-buffer. Difco-Bacto Agar and Difco Purified Agar, when used, were used in concentrations of 1.5%, unless specified otherwise.

Test media were inoculated from suspensions of colonies grown on agar media with mineral base (MB 1) or with ¾ strength ASW (MB 2) for 24–48 h. Agar media were inoculated using a multipoint inoculator. However, if cross-feeding was detected or expected, divided petridishes (multidishes) were used in combination with the multipoint inoculator described by Lovelace and Colwell [20]. Otherwise, nondivided agar plates of 9 cm diameter were used for testing for pH dependence, as well as utilization of monosaccharides as sole carbon sources. A 32 point "replicator" device, manufactured by Melrose Machine Shop, Woodlyn, PA, was used for inoculation. Incubation periods were 4–5 days at 22 ± 2°C, if not specified otherwise, with extensions up to 3 weeks for selected media when negative reactions were observed.

The choice of test characters for numerical taxonomy was based mainly on features that had proved useful in taxonomical studies of the genus *Cytophaga* [7]. Other nonauxotrophic, chitinoclastic bacteria were also included for comparison. Therefore, the tests were supplemented by including features frequently used in numerical taxonomy studies of estuarine bacteria [3, 11]. Characters used are listed in Table 1.

Cell morphology and motility, in the case of strains No. 1–17 (see Table 1), were examined by phase contrast microscopy at 1 day and 7 days at 20°C using Difco nutrient broth cultures. Gram staining was done using Hucker's modification of the Gram stain [11]. Colony morphology and pigmentation were recorded from Plate Count Agar under incident light.

Flexirubin pigments were verified by bathochromic color shift after adding a drop of 1 N NaOH (or KOH) to colonies grown on Difco Plate Count Agar [1].

Salt tolerance and salt requirement were measured from growth on nutrient agar consisting of 2 g Difco Bacto peptone; 0.5 g yeast extract; 0.5 g soluble starch; and 15 g Difco Bacto agar in 1 liter of mineral base (MB), to which was added 0, 1, 2, or 3% NaCl, and adjusted to pH 7. In addition, the same medium was made up to 1 liter of ¼, ½, or ¾ strength ASW, containing mineral base (MB) adjusted to pH 7.4.

For assay of sensitivity to UV light [21], suspensions of colonies on mineral basal medium incubated for 2–3 days were transferred to Difco Plate Count Agar plates, using sterile wooden applicator sticks for multiple inoculation. Immediately after inoculation of plate pairs, 1 plate of each pair was exposed to light from a 15 W UV-germicidal lamp for 90 sec at a distance of 55 cm (ca. 175 erg mm^{-2}). Colony growth was examined after 3 days at 20°C.

Temperature dependence for growth was examined using Mueller-Hinton Agar (Difco), or, where required, Plate Count Agar (PCA) prepared with distilled water or ¾ strength ASW. Growth at 2°C and 8°C was examined after incubation for 7 days. Growth at 15, 20, 25, 30, 35, and 45°C was examined after incubation for 2–4 days. "Growth temperature shift up" was recorded when growth on ASW agar was noted at a higher temperature than when ASW-free agar was used. "Growth temperature shift down" was recorded when the growth temperature range on ASW agar was lower than when ASW-free agar medium was used.

Dependence of growth on pH was tested using Mueller-Hinton agar (Difco Labs., Detroit, MI) or where required, Difco Plate Count Agar made up with appropriate buffer and adjusted to pH 4.5–10. Agar media were prepared with distilled water or ¾ strength ASW. Buffers used included 10 mM/liter Na-citrate + KH_2PO_4 (pH 4.5, 5); 10 mM/liter KH_2PO_4 (pH 6, 7); and 10 mM/liter glycine-NaOH (pH 8, 9, 10). Growth was examined after incubation for 4 days at 20°C. By comparison with growth on ASW-free media, "up- and down-shifts" of pH requirement for growth on ASW agar were also recorded.

Liquid media for testing NO_3^- as sole source of nitrogen contained a mineral basal medium, with $NaNO_3$ replacing $(NH_4)_2SO_4$, and amended with 0.1% membrane filter-sterilized glucose. Tubes were prepared using 5 ml aliquots of the medium added to small (4 × 0.5 inches) test tubes and addition of an overlay of 1 cm of sterile mineral oil after inoculation. After significant turbidity occurred, NO_3^- reduction was examined using the Griess-Ilosvay reagents [11]. Glucose-NH_4Cl-mineral agar consisted of 0.1% membrane filter-sterilized glucose in a mineral base (MB 1 or MB 2), with NH_4Cl as N-source and 1.5% purified agar (Difco Labs., Detroit, MI). Growth on N-free media was examined using a glucose mineral agar of the same composition, except for inorganic nitrogen source.

Growth on agar media amended with a sole organic carbon source, except for the agar, was examined using MB 1 or MB 2, with 1.5% of "purified" agar (Difco Labs., Detroit, MI) containing 1) 0.1% of membrane filter-sterilized and, where necessary, neutralized, soluble carbon source, that is, glucose, galactose, ribose, xylose, arabinose, cellobiose, lactose, raffinose, glucosamine, *N*-acetyl, glucosamine, asparagine, alanine, succinate, or acetate; or 2) insoluble polysaccharides, that is, cellulose (2%, Nutritional Biochem. Corp.), carboxy-methylcellulose (1%, Nutritional Biochem. Corp.), starch (5%, "soluble" potato starch, Baker Chemicals), alginate (10%, sodium alginate, Matheson, Coleman, and Norwood), or chitin 0.5%, purified, reprecipitated preparation made from commercial "pure" chitin (Roth Chemicals, W. Germany) [28]. Degradation of insoluble or colloidal polysaccharides was detected when the agar medium around and/or underneath the colony was translucent. Degradation of agar was recorded when pits formed on Difco Plate Count Agar medium. Growth on and utilization of glucose, using Hugh-Leifson [14] and Leifson [19] media, were examined using the following criteria: aerobic growth, anaerobic growth, oxidation, fermentation, and alkaline reaction. Lactose fermentation was examined using Kligler agar slants (Difco Labs., Detroit, MI). For detection of citrate utilization, Simmons citrate agar was used (Difco Labs., Detroit, MI).

Gelatin liquefaction was detected on 12% Difco nutrient gelatin adjusted to pH 6.8, after incubation at 18°C.

Tests for lytic enzyme activity were carried out using nutrient agar plates prepared with Plate Count Agar (Difco) or ½ strength Marine Agar 2216 (Difco) containing 2 g/liter lyophilized cells

Table 2. Origin and phenetic clusters of 131 chitinoclastic bacterial isolates from predominantly estuarine habitats including 65 *Cytophaga*-like bacteria, and 7 named yellow-pigmented reference strains

Strain no.	Cluster (S_J) of the total	Clusters of CLB[a]		Habitat[c]	Salinity (‰)	Temperature (°C)	Isolated at (°C)
		S_J	S_M				
Named reference strains:							
36 *C. johnsonae*	10	Cy 7	C 7	–	–	–	–
37 *F. capsulatum*	11	Cy 8	C 8	–	–	–	–
38 *C. aquatilis*	10	Cy 7	C 7	–	–	–	–
39 *F. oceanosedimentum*	S	Cy 8	S	–	–	–	–
54 *F. aquatile*	1C	Cy 3	C 2	–	–	–	–
58 *Fl. elegans*	S	S	S	–	–	–	–
59 *B. linens*	11	S	S	–	–	–	–
Freshwater isolates							
1	10	Cy 7	C 7	W, ML	–	4	2
13	10	Cy 7	C 7	S, LT	–	4	15
14	10	Cy 7	C 7	W, OL	–	16	10
15	10	Cy 7	C 7	W, KL	–	27	10
16	10	Cy 7	C 7	S, LT	–	4	10
17	1F	Cy 6	C 4	W, CL	–	13	10
18	1F	Cy 6	C 4	W, CL	–	13	10
19	10	Cy 7	C 7	S, LT	–	4	10
20	10	Cy 7	C 7	S, LT	–	4	10
21	1F	Cy 6	C 4	W, CL	–	13	10
25	1F	Cy 6	C 4	W, CL	–	13	10
27	10	Cy 7	C 7	S, LT	–	4	10
28	11	Cy 7	S	S, LT	–	4	10
31	1F	S	C 4	S, LT	–	4	10
32	10	Cy 7	C 7	S, LT	–	4	10
33	1C	Cy 3	C 2	W, WR	–	17	10
34	1F	Cy 6	C 4	S, LT	–	4	10
Marine isolates							
120	1B	Cy 2	C 1	W	33	12	10
124	S	Cy 9	C 3	W	33	12	10
125	1A	Cy 1	C 1	W	33	12	10
126	1A	Cy 1	C 1	W	33	12	10
127	S	Cy 9	C 3	W	33	12	10
128	1B	Cy 2	C 6	W	33	12	10
134	1B	Cy 2	S	W	33	12	10
135	1B	Cy 2	C 6	W	33	12	10
136	6	–	–	W	30	12	25
137	S	S	S	W	30	12	25
138	S	–	–	W	30	12	25
Estuarine Isolates							
2	10	Cy 7	C 7	W, CB	16	8	2
3	10	Cy 7	C 7	W, JF	11	10	2
4	10	Cy 7	C 7	W, JF	10	15	2
5	10	Cy 7	C 7	W, JF	11	10	15
6	10	Cy 7	C 7	W, JF	10	15	2
7	10	Cy 7	C 7	W, JF	10	15	2

Table 2. Continued

Strain no.	Cluster (S_J) of the total	Clusters of CLB[b]		Habitat[c]	Salinity (‰)	Temperature (°C)	Isolated at (°C)
		S_J	S_M				
8	10	Cy 7	C 7	W, CR	2	5	15
9	10	Cy 7	C 7	W, CR	2	5	15
10	10	Cy 7	C 7	S, CB	6	4	15
11	10	Cy 7	C 7	S, CB	6	4	15
12	10	Cy 7	C 7	W, JF	11	10	2
22	10	Cy 7	C 7	W, CB	5	4	2
23	10	Cy 7	C 7	W, CB	6	4	2
24	10	Cy 7	C 7	W, EB	8	6	2
26	1D	Cy 4	C 5	W, EB[a]	9	5	2
29	1E	Cy 5	C 5	W, EB[a]	9	5	2
30	1E	Cy 5	C 5	W, EB[a]	9	5	2
35	1F	Cy 6	C 3	W, EB[a]	9	5	2
60	1D	Cy 4	C 5	W, JF	11	17	15
61	1C	S	C 2	W, EB[a]	9	5	15
68	10	Cy 7	C 7	W, JF	10	15	2
69	10	Cy 7	S	W, JF	10	15	2
76	S	S	S	W, HI	·	·	15
78	1C	Cy 3	C 2	W, EB[a]	9	5	15
86	1D	Cy 4	C 5	S, EB[a]	9	5	15
90	10	Cy 7	C 7	W, CR	2	5	15
97	1D	Cy 4	C 5	W, EB	8	6	15
99	1D	Cy 4	C 5	S, CR	8	15	15
104	2	Cy 8	S	W, JF	5	6	36
105	1C	Cy 3	C 2	W, EB[a]	9	5	15
106	6	Cy 8	C 8	W, CR	2	4	36
109	S	Cy 3	C 1	S, JF	11	15	2
113	6	Cy 8	S	W, JF	11	10	36
118	10	Cy 7	C 7	W, JF	10	14	2
123	1D	Cy 4	C 5	S, CB	6	4	15
129	1B	Cy 3	C 1	W, EB[a]	9	5	2
131	10	Cy 7	C 7	W, EB[a]	9	5	15
132	1A	Cy 1	C 1	W, EB[a]	9	5	15
133	1B	Cy 2	S	W, EB[a]	9	5	15
40	3E	–	–	W, CR	13	15	36
41	3E	–	–	W, JF	10	15	2
42	5	–	–	W, JF	11	17	15
43	S	–	–	W, JF	11	17	15
44	5	–	–	S, JF	11	15	2
45	4	–	–	W, EB	9	14	2
46	4	–	–	W, JF	10	15	2
47	4	–	–	W, JF	10	15	2
48	4	–	–	W, JF	10	15	2
49	4	–	–	W, JF	10	15	2
50	4	–	–	W, JF	10	15	2
51	3E	–	–	W, JF	10	15	2
52	3E	–	–	W, JF	10	15	2
53	5	–	–	W, CB	16	8	15
55	C3	–	–	S, CB	16	8	15
56	C3	–	–	S, CB	16	8	15

Table 2. Continued

Strain no.	Cluster (S_J) of the total	Clusters of CLB[b]		Habitat[c]	Salinity (‰)	Temperature (°C)	Isolated at (°C)
		S_J	S_M				
57	7	–	–	S, CB	16	8	15
62	3E	–	–	W, JF	10	15	15
63	C3	–	–	W, JF	11	10	2
64	3E	–	–	W, CR	13	8	15
65	3E	–	–	W, CR	8	14	15
66	3F	–	–	W, JF	10	15	2
67	10	–	–	W, JF	10	15	2
70	3F	–	–	S, JF	11	15	2
71	3C	–	–	S, JF	12	11	2
72	3F	–	–	S, JF	12	11	2
73	3A	–	–	S, JF	12	11	2
74	5	–	–	W, JF	11	10	36
75	3A	–	–	W, JF	11	10	2
77	3C	–	–	W, HI		4	36
79	2	–	–	W, JF	10	15	2
80	3C	–	–	W, JF	10	15	2
81	3A	–	–	S, JF	12	11	2
82	2	–	–	S, JF	12	10	2
83	3B	–	–	S, JF	12	11	2
84	3C	–	–	S, JF	12	11	2
85	3D	–	–	S, JF	12	11	2
87	5	–	–	W, JF	11	10	2
88	3D	–	–	W, JF	11	10	15
89	3D	–	–	W, JF	11	10	36
91	5	–	–	W, CR	2	5	15
92	8	–	–	S, CR	14	1	15
93	S	–	–	S, CR	14	1	15
94	8	–	–	W, JF	5	6	15
95	5	–	–	W, JF	5	6	15
96	2	–	–	W, JF	5	6	15
98	3A	–	–	S, JF	6	5	15
100	5	–	–	W, CR	2	5	36
101	5	–	–	S, CR	14	1	36
102	5	–	–	S, JF	6	5	36
103	8	–	–	S, JF	6	5	15
107	9	–	–	S, EB	9	5	15
108	9	–	–	S, CR	13	17	36
110	3F	–	–	S, JF	11	15	2
111	9	–	–	W, CB	16	8	15
112	9	–	–	W, EB	15	7	15
114	7	–	–	S, EB	15	7	36
115	2	–	–	W, JF	10	15	36
116	2	–	–	W, JF	11	10	2
117	3B	–	–	W, EB	15	7	2
119	S	–	–	W, JF	5	6	36
121	7	–	–	W, EB	8	6	15
122	2	–	–	S, EB	9	5	15
130	2	–	–	W, EB	9	5	2

of *Micrococcus lysodeicticus* (ATCC 4696) (Serva Chemicals, Heidelberg, West Germany). Colonies surrounded by translucent zones (halos) were recorded as positive.

Aesculin hydrolysis [34] was examined using plates prepared with 1.5% Bacto agar (Difco). Black zones around colonies indicated a positive reaction. In most cases, the test could be read within a few hours of incubation.

Hydrolysis of Tweens 20, 40, 60, and 80 (polyoxyethylene monolaureate, monopalmitate, monostearate, monooleate) was detected using agar plates containing 1 g/liter yeast extract (Difco), 10 g/liter of Bacto peptone (Difco), 5 g/liter of NaCl, 0.1 g/liter of $CaCl_2 \cdot H_2O$, and 15 g/liter agar (Difco) made up in a basal mineral medium (MB 1 or MB 2). One ml aliquots of the test compounds (Tweens 20, 40, 60, 80) were autoclaved separately and incorporated into 100 ml portions of the liquid agar medium. Precipitates of calcium salts surrounding a colony indicated the presence of lipase [32].

Alkaline phosphatase was detected by release of phenolphthalein from 0.01% membrane filter-sterilized phenolphthalein phosphate incorporated in Difco Plate Count Agar or Difco Marine Agar [4].

The cytochrome oxidase test was carried out by picking colonies from plate count agar (Difco Laboratories, Detroit, MI) and testing with tetramethyl-*p*-phenylene-diamine-hydrochloride reagent [17]. A positive reaction was indicated by a purple color appearing within 10 sec after transfer of a colony, using a small glass rod, to filter paper soaked with 1% aqueous solution of the reagent. The catalase test was considered positive when gas bubbles formed immediately after pipetting 3% H_2O_2 onto the surface of cultures on Difco Plate Count Agar incubated for 1–2 days.

Nitrate reduction and denitrification were detected using the assay for NO_2^- Griess-Ilosvay reagent [11] and production of gas in Durham tubes after growth in 1% peptone media containing 0.2% KNO_3 [33].

Assays for arginine dihydrolase were carried out according to Thornley [41]. Lysine and ornithine decarboxylase were detected using Møller's [22] method and Difco basal medium.

Urease assays followed the method described by Christensen [10].

Phenylalanine deaminase was determined on agar slants according to Ewing et al. [33], using 10% $FeCl_3$ solution as reagent.

Production of indole was detected with the Ehrlich Boehme dimethylaminobenzaldehyde reagent [33], after growth in 1% trypticase peptone (BBL) broth was observed.

Production of H_2S was indicated by black precipitate formed after stab-inoculation of Kligler agar (Difco) slants.

The Voges-Proskauer test for acetoin production (O'Meara modification) and methyl red test were carried out, using cultures incubated for 6 days at 24°C [33].

Sensitivity to sodium dodecylsulphate (SDS) was detected on Difco Plate Count Agar containing 0.1% of sodium dodecylsulphate [8].

Antibiotics were incorporated in Mueller-Hinton Agar or Plate Count Agar (Difco) for testing susceptibility to 10 μg/ml of Penicillin G (Sigma Chemicals) and 38 μg/ml (= 300 i.u./ml) polymyxin B (Sigma Chemicals). In addition, filter discs containing 300 i.u./ml polymyxin B (BBL Products) were used on a pre-seeded agar medium, as in the case of the 0/129 reagent. Sensitivity to the "vibriostatic" compound 0/129 2,4-diamino-6, 7-diisopropylpteridine [31] was detected by

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←

Clusters of *Cytophaga*-like bacteria based on similarity coefficients S_M and S_J, clusters of the total based on S_J only

[a] Water samples with delayed incubation (kept at 5°C for up to 3 weeks) are marked

[b] CLB = *Cytophaga*-like bacteria

[c] Abbreviations for habitats and sampling sites: W = water sample, S = sediment sample, ML = Mindel Lake, LT = Lake Tahoe (400 m), OL = Odell Lake (surface), K = Kealia Lake (surface), CL = Crater Lake (littoral). WR = Willamette River; marine samples were isolated from surface water at the Oregon Pacific Coast near Newport. Estuarine sampling sites in the Chesapeake Bay were CR = Chester River, JF = Jones Falls, CB = Chesapeake Beach, EB = Eastern Bay, and HI = Hooper's Island

applying a few crystals of this compound on the surface of agar plates after inoculation with a bacterial suspension of optical density (O_D, 550 nm, Bausch and Lomb Spectronic 20) ≥.200.

Heat tolerance of alkaline *p*-nitrophenyl-phosphatase activity was determined for 1–2 day culture suspensions exposed to 70°C for 20 min, using a water bath. The test was used exclusively for *Cytophaga*-like strains. Assay conditions were as follows: 1 ml of a cell suspension in 0.1 M/liter tris-HCL-buffered mineral medium (MB 1), pH 8.5, of the heated sample or the nonheated control and 2 ml of a solution of 3 mM *p*-nitrophenylphosphate/liter (Serva Chemicals, Heidelberg, W. Germany) in 0.1 M/liter tris-HCL, pH 8.5, were incubated for 30 min at 36°C. Development of a deep yellow color, arising from formation of *p*-nitrophenol, indicated a positive result.

P-nitrophenol-*N*-acetylglucosaminidase activity was determined by mixing 1 ml of a culture suspension in mineral base MB 1 (O_D, 550 nm, 1 cm ≥ .300) and 2 ml of a solution of 1.5 mM/liter *p*-nitrophenol-*N*-acetyl glucosamide (Serva Chemicals, Heidelberg, W. Germany) in 0.05 M/liter Na-K-phosphate buffer, pH 5.5. After incubation for 2 h at 36°C, 0.2 ml of 10 N NaOH was added. Development of a deep yellow color, when *p*-nitrophenol formed, indicated a positive result.

The data were coded, entered into the computer, and analyzed using methods and programs developed in our laboratory. The S_J and S_M coefficients were used to calculate phenetic similarity. The S_M, the "matching" coefficient, includes negative matches, with the result that overall similarities tend to be higher than when the S_J is used [35]. Both sets of data, that is, S_J and S_M computations, are presented in this study.

Results

Chitinoclastic Cytophaga *and Related Bacteria*

Phenetic relationships among the yellow-pigmented isolates examined in this study are illustrated in Figs. 1 and 2. Results obtained using both the S_J and S_M similarity indices yielded a major cluster, phena Cy 7 and C 7, respectively, which included 31 [29] strains, among them the reference strains of *Cytophaga johnsonae* Stanier and *Cytophaga aquatilis* Strohl and Tait. All strains of phenon Cy 7 were either of freshwater or estuarine origin and none required NaCl for growth. Only 32% of the strains tolerated 3% NaCl, but 97% produced one or more pigments of the flexirubin type and 100% were able to grow on glucose as a sole source of organic substrate. Ninety-four percent of the strains grew under anaerobic conditions in soft agar, prepared with the medium of Hugh and Leifson [14], and produced lysis of *Micrococcus lysodeicticus* cells (Table 3).

Growth on mineral medium, with glucose as sole organic substrate, was also characteristic of strains comprising the smaller phenon, Cy 8, which included 3 estuarine isolates and the reference strains of *Flavobacterium oceanosedimentum*, which was chitinoclastic, and *Flavobacterium capsulatum*, which was not chitinoclastic. Strains of this phenon, however, did not produce pigment of the flexirubin type and, in addition, were capable of growth in the presence of 3% NaCl.

Nearly all remaining isolates included in this study grouped into smaller clusters and comprised either freshwater or salt-adapted strains lacking the ability to grow on a mineral medium with glucose as the sole organic substrate. Phenon Cy 6, based on results of the S_J calculations, comprised predominantly the flexirubin pigment-producing, freshwater-adapted-isolates, that is, a total

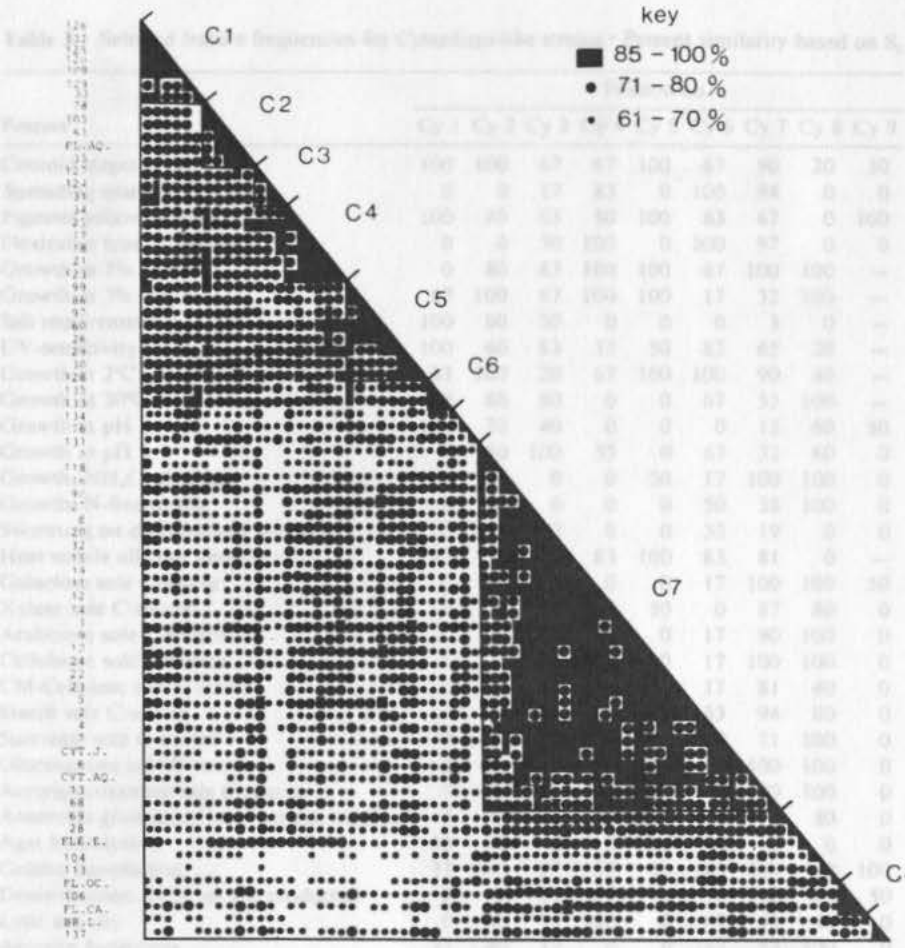

Fig. 2. Similarity matrix, based on S_M similarity coefficient, for *Cytophaga* and related strains. Formation of clusters ≥80% similarity is indicated by the symbols shown. The ordinate provides strain numbers.

of 6 strains, demonstrating a very low salinity tolerance of ≤1% NaCl. Sixty-seven percent of the strains were capable of producing enzymes lytic for *Micrococcus lysodeicticus*. However, phenon Cy 4, based on results obtained using the S_J coefficient, was represented exclusively by estuarine, flexirubin-pigment-producing strains, 6 in total, which demonstrated salinity tolerance for 3% NaCl and were lytic and, in general, aerobic.

Phenon Cy 3 (S_J) included *Flavobacterium aquatile* and comprised 6 isolates, predominantly estuarine, with half of the strains being strictly aerobic or producing pigments of the flexirubin type. None of the estuarine strains in this phenon were lytic, but 3 required salt for growth.

The remaining phena, Cy 1, Cy 2, Cy 5, and Cy 9, derived from analyses using the S_J coefficient, did not possess pigment of the flexirubin type. Among

Table 3. Selected feature frequencies for *Cytophaga*-like strains.[a] Percent similarity based on S_j

Feature	Phenon no.								
	Cy 1	Cy 2	Cy 3	Cy 4	Cy 5	Cy 6	Cy 7	Cy 8	Cy 9
Coccoid stages present	100	100	67	67	100	67	90	20	50
Spreading margin	0	0	17	83	0	100	94	0	0
Pigment yellow-orange	100	80	83	50	100	83	67	0	100
Flexirubin type of pigment	0	0	50	100	0	100	97	0	0
Growth in 1% NaCl	0	80	83	100	100	67	100	100	–
Growth in 3% NaCl	67	100	67	100	100	17	32	100	–
Salt requirement	100	80	50	0	0	0	3	0	–
UV-sensitivity	100	60	83	33	50	83	65	20	–
Growth at 2°C	33	100	20	67	100	100	90	40	–
Growth at 30°C	0	80	80	0	0	67	55	100	–
Growth at pH 5	100	20	40	0	0	0	13	60	50
Growth at pH 9	0	60	100	33	0	67	32	60	0
Growth, NH_4Cl-glucose	0	20	0	0	50	17	100	100	0
Growth, N-free media	0	20	0	0	0	50	58	100	0
Swarming on carbohydrate medium	0	0	0	0	0	33	19	0	0
Heat stabile alkaline phosphatase	33	75	67	83	100	83	81	0	–
Galactose sole C-source	0	20	0	0	0	17	100	100	50
Xylose sole C-source	0	40	17	0	50	0	87	80	0
Arabinose sole C-source	0	20	0	0	0	17	90	100	0
Cellobiose sole C-source	0	20	17	0	50	17	100	100	0
CM-Cellulose sole C-source	0	40	0	0	0	17	81	40	0
Starch sole C-source	33	40	17	100	50	33	94	80	0
Succinate sole C-source	0	20	33	0	50	17	71	100	0
Glucosamine sole C-source	0	20	17	0	50	0	100	100	0
Acetylglucosamine sole C-source	0	40	17	0	0	0	100	100	0
Anaerobic growth in Hugh-Leifson medium	0	40	50	17	100	50	94	80	0
Agar hydrolysis	33	60	0	0	0	0	0	0	0
Gelatin liquefaction	33	80	67	83	50	83	100	60	100
Denitrification (NO_2^- or gas produced)	0	0	17	67	0	0	42	60	50
Lytic activity	0	80	33	100	0	67	94	40	0
Aesculin hydrolysis	33	40	17	0	0	33	84	100	0
Tween 40 hydrolysis	33	60	100	50	0	0	19	80	0
Penicillin sensitivity	100	0	100	50	100	67	0	20	100
Polymyxin B sensitivity	0	25	67	40	0	0	23	25	100
0/129-sensitivity	0	100	0	33	50	20	31	25	–
Stabile chitinolytic activity	67	100	50	100	50	100	100	80	100
Number of strains comprising the phenon	3	5	6	6	2	6	31	5	2

[a] *Cytophaga* spp. and related gram-negative yellow-pigmented nonflagellated bacteria; see also Fig. 1

them, phena Cy 1 and Cy 2 were predominantly marine, that is, salt-requiring strains, with lytic activity associated only with cluster Cy 2. None of the strains in clusters Cy 1, Cy 2, and Cy 5 were sensitive to polymyxin B.

The reference strain of *Flexibacter elegans* did not cluster with any of the chitinoclastic *Cytophaga* isolates, although it proved to be related to strains of the *Cytophaga johnsonae* phenon (Cy 7).

When the simple matching coefficient S_M was used, the major cluster ob-

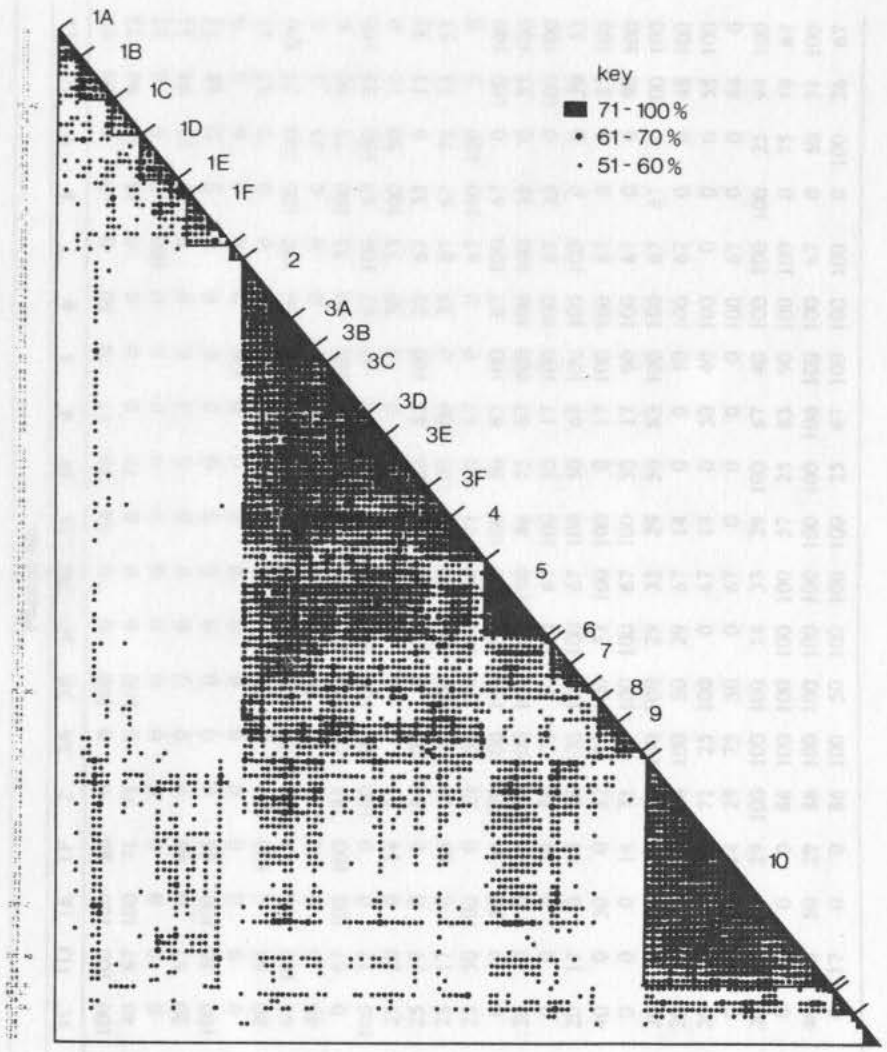

Fig. 3. Similarity matrix, based on the S_J similarity coefficient, for aerobic, zymogenic, chitinolytic bacteria, including *Cytophaga* and related spp. Clusters formed at similarity of ≥60%, as indicated by the symbols shown. Strain numbers are given on the ordinate (see Table 2).

tained, C 7, was found to be identical with the major cluster Cy 7, obtained using the S_J coefficient, except for 2 strains that did not cluster at ≥80% similarity (Fig. 2). Among the smaller phena, S_M-derived clusters, C 1 and C 3, were identical with S_J-derived clusters Cy 1 and Cy 9, respectively. S_J-derived clusters, Cy 4 and Cy 5, both were contained in the S_M-derived cluster, C 5, whereas only part of the strains in the S_J-derived clusters, Cy 2, Cy 3, Cy 6, and Cy 8, also appeared in the corresponding S_M-derived clusters C 6, C 2, C 4, and C 8, respectively (Figs. 1 and 2; Table 2).

Table 4. Selected feature frequencies for all strains examined in this study

Feature	Phenon no.																				
	1A	1B	1C	1D	1E	1F	2	3A	3B	3C	3D	3E	3F	4	5	6	7	8	9	10	11
Length: Width 6 μ	100	100	100	100	100	86	0	0	100	0	0	14	50	17	0	50	0	0	0	100	67
Coccoid stages	100	100	40	67	100	71	71	0	100	0	0	0	75	0	0	0	0	33	50	94	33
Gram-positive	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	33
Spreading margin	0	17	20	67	0	86	0	0	0	0	0	0	0	0	0	0	0	0	25	94	33
Pigment yellow-orange	100	83	100	50	100	86	0	0	0	0	0	0	0	0	0	0	0	0	0	68	33
Violacein type pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0
Flexirubin type pigment	0	17	80	83	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	97	33
Growth in 3% NaCl	67	100	60	100	100	14	100	100	100	100	100	100	100	100	30	100	100	100	100	29	100
Salt requirement	100	67	40	0	0	0	0	0	50	0	0	14	0	0	0	0	0	0	25	3	0
Growth at 2°C	33	100	0	67	100	100	86	100	100	100	100	100	100	100	100	50	33	100	75	90	0
Growth at 35°C	0	67	100	17	0	0	100	100	100	100	100	100	100	100	40	67	100	67	100	23	100
Growth temp. shift up	0	33	25	50	0	14	57	0	0	0	0	0	0	0	0	50	33	100	50	10	0
Growth at pH 5	100	17	25	17	0	0	86	100	0	100	100	100	100	83	100	33	67	33	0	13	33
Growth at pH 10	0	17	25	17	0	43	100	100	100	100	100	100	100	100	0	33	67	67	75	13	33
Growth pH shift down	0	17	25	50	100	0	29	50	50	100	100	71	100	67	0	0	67	100	100	3	0
Growth, NH_4Cl-glucose	0	17	0	0	50	14	100	100	100	100	100	100	50	67	100	67	100	67	0	100	100
Growth, N-free media	0	17	20	0	0	43	100	100	100	100	100	86	75	67	100	100	100	33	0	55	100
Galactose, sole C-source	0	17	0	0	0	14	86	75	50	100	67	100	50	17	100	100	67	33	0	100	100
Ribose, sole C-source	0	33	20	17	0	14	86	100	100	100	67	100	50	67	100	100	100	0	0	29	33
Xylose, sole C-source	0	33	40	0	50	0	57	75	100	57	100	100	0	17	100	100	67	0	0	87	100
Arabinose, sole C-source	0	17	0	0	0	14	71	75	100	100	67	100	50	17	90	100	67	0	0	90	100
Cellobiose, sole C-source	0	17	20	0	50	29	86	100	100	29	33	29	50	83	100	100	67	67	0	100	100
Lactose, sole C-source	33	33	20	0	0	0	86	100	50	29	67	14	0	0	10	100	67	0	0	48	100
Raffinose, sole C-source	33	33	20	0	0	14	71	25	100	0	67	14	0	50	40	100	0	0	0	55	100
CM-cellulose, sole C-source	0	33	0	0	0	14	29	75	50	0	67	0	0	0	0	100	67	0	0	84	0
Starch, sole C-source	33	50	20	83	50	29	100	100	100	14	33	29	100	67	40	100	100	100	25	94	100
Acetate, sole C-source	33	17	0	0	0	0	86	100	100	100	100	57	25	83	90	100	100	0	75	19	67
Succinate, sole C-source	0	17	40	0	50	29	86	100	100	100	100	100	100	100	100	100	67	0	50	71	100
L-alanine, sole C-source	0	17	0	17	0	0	86	100	50	100	100	100	25	67	100	100	100	0	100	26	67

Table 4. Continued

Feature	Phenon no.																				
	1A	1B	1C	1D	1E	1F	2	3A	3B	3C	3D	3E	3F	4	5	6	7	8	9	10	11
Acetylglucosamine, sole C-source	0	33	20	0	0	0	100	100	100	100	67	100	100	100	80	100	100	33	50	100	100
Hugh-Leifson, anaerobic	0	50	40	0	100	57	100	25	50	29	0	29	25	83	60	67	100	100	25	90	67
Hugh-Leifson, alkaline	0	17	0	33	50	14	0	0	0	14	0	0	50	100	20	0	0	0	25	7	33
Hugh-Leifson, fermentation	0	0	0	0	0	0	100	0	0	14	0	0	0	0	0	33	100	100	0	0	0
Simmons citrate	0	17	0	0	0	0	57	100	50	100	100	100	0	100	60	0	0	0	75	13	0
Agar hydrolysis	33	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oxidase	100	83	20	100	0	57	100	100	100	100	0	100	100	17	100	67	67	100	100	87	0
Catalase	33	100	0	50	0	57	43	50	100	86	100	86	25	0	100	67	100	0	50	94	33
Nitrate reduction	0	0	0	0	0	0	86	75	50	71	33	14	100	83	50	100	100	100	50	42	33
Lytic activity	0	83	40	83	0	71	57	25	50	29	0	29	25	17	0	33	33	0	0	94	33
Arginine decarboxylase	0	17	0	50	50	0	71	25	50	86	100	86	0	17	30	0	33	0	75	19	0
Ornithine decarboxylase	0	0	0	67	0	0	100	100	100	71	100	100	100	100	60	33	0	0	50	36	33
Lysine decarboxylase	0	17	0	33	0	0	100	100	100	71	100	71	75	100	70	0	0	0	50	29	0
Aesculin hydrolysis	33	33	40	0	0	43	71	25	0	14	0	0	25	0	100	100	67	0	25	84	67
Voges Proskauer	0	0	0	0	0	0	14	0	0	0	0	0	0	0	90	0	0	0	0	3	0
Tween 20 hydrolysis	67	67	60	67	0	29	100	100	100	86	67	71	100	100	40	67	67	100	100	45	0
Tween 40 hydrolysis	33	50	80	67	0	0	100	100	100	43	0	71	100	100	30	100	67	100	100	19	0
Tween 60 hydrolysis	0	33	80	17	0	0	100	100	50	100	0	43	75	67	0	67	67	100	100	16	0
Tween 80 hydrolysis	33	0	80	50	0	0	100	100	100	100	0	43	100	100	30	67	33	100	100	7	0
SDS-sensitive	100	100	100	100	100	100	0	0	0	0	0	0	0	0	80	67	100	67	0	94	100
Penicillin G sensitive	100	100	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0	25	0	67
0/129-sensitivity	0	100	0	20	50	20	0	25	0	0	0	0	25	83	0	0	0	0	0	31	0
Stabile chitinolytic activity	67	100	50	100	50	100	100	100	100	100	100	100	100	100	100	100	100	67	50	100	33
Number of strains comprising phenon	3	6	5	6	2	7	8	4	2	7	3	7	4	6	10	3	3	3	4	31	3

Percent based on S_j. See also Fig. 3

Aerobic, Zymogenic, Chitinolytic Phena

From results of analyses using the S_j coefficient, which also included all of the non-*Cytophaga*, zymogenic, chitin degraders isolated from Chesapeake Bay, the *Cytophaga* strains formed 2 major clusters consisting of phena 1 A–1 F (including subphena Cy 1 through Cy 6) and phenon 10 (representing Cy 7) (Fig. 3, Table 4). The remaining non-*Cytophaga* strains of chitin-degrading, estuarine bacteria included the following phena: 2 (Vibrionaceae); 3 (pseudomonads); 4 (0/129-sensitive organisms related to *V. metschnikovii*); 5 (*Chromobacterium*); 6 (gram-negative, nonauxotrophic CM-cellulolytic strains; 7 (gram-positive facultatively anaerobic bacteria); 8 (gram-negative fermentative bacteria); 9 (glucose-oxidizing bacteria); and the reference strains of *Brevibacterium linens* and *Flavobacterium capsulatum* in phenon 11.

Significant relationships between salinity and temperature of sampling site at the time of isolation (Table 2) and phena isolated from that site were not observed.

Salinity-induced "shifts up" of growth temperature ranges were observed most frequently (10 out of 44 strains) for *Cytophaga* and related strains of phena other than phenon 10, that is, phenon Cy 7, as well as among fermentative chitin degraders of phenon 2 (4 out of 8 strains), but not among pseudomonads of phena 3, 4, or 5 (Table 4).

Discussion

According to previously published studies dealing with the taxonomy of *Cytophaga* spp., only *Cytophaga johnsonae* [37] and, less significantly, *Cytophaga aquatilis* [40], have been recognized as chitin-degrading species of *Cytophaga*. It is not clear whether *C. aquatilis* is, in fact, an actively chitin-degrading species. Both species are considered freshwater forms. Unfortunately, chitin-digestion is not a clear-cut characteristic readily or easily defined. Some of the simple assays for chitin degradation may produce false negative reactions. Thus, when the property of chitin digestion, hydrolysis, or utilization is used as a "key" character for identification, detection methods for chitin degradation which yield false-negative reactions, as in the case of the reference strain of *Flexibacter elegans*, can cause considerable confusion in the taxonomy of the genus. Clearly, a negative reaction, based only on a single type of assay, cannot be considered sufficient for a reliable identification. Catabolite repression can mask the results of tests for chitinolytic activity, as has been shown for several isolates of *Cytophaga johnsonae* [25, 26]. Cloning of the chitinase gene is presently under way in our laboratory in the hopes that the molecular genetic approach will alleviate this taxonomic problem.

In the study reported here, a group of predominantly estuarine and marine *Cytophaga* isolates, including phena Cy 1 through Cy 5 (Fig. 1) gave positive results only when tested on an agar medium containing a complex source of dissolved organic nitrogen (Reichardt and Colwell, manuscript in preparation). Furthermore, results of quantitative assays for chitinase in various *Cytophaga* strains examined in this study indicated that chitinase activity depends largely

on induction and is dependent on the type of chitin preparation used in the assay, that is, conformational properties of the chitin and the degree of acetylation.

The major cluster, phenon Cy 7, which included freshwater and estuarine strains, together with reference strains of *C. johnsonae* and *C. aquatile*, could be readily identified as a chitin-degrading *Cytophaga* species. The other phena demonstrated intrinsic differences among strains, with regard to salinity requirement and/or response to salt, notably in the case of phena Cy 6 and Cy 1 and 2, respectively. These 3 phena represented the extremes of low salinity tolerance, on one hand, and requirement for salt for growth on the other. Based on available reports in the literature, salt-requiring estuarine and marine *Cytophaga* spp. capable of degrading chitin have not been described, even in the case of marine isolates studied by numerical taxonomy and clustered on the basis of production of a yellow pigment [12]. The majority of the estuarine and marine strains included in the study reported here, which showed tolerance to $\geq$ 3% or requirement for NaCl for growth, were completely separated from the facultatively anaerobic, predominantly oligonitrophilic, freshwater reference strain of *C. johnsonae*.

The marine and part of the estuarine strains included in our study grew, in general, only under aerobic conditions and required a complex source of organic nitrogen, that is, peptone, for growth and degradation of chitin. The strains demonstrated slightly higher growth temperature maxima than the majority of the isolates of *C. johnsonae*, that is, 34°C vs 31°C, and failed to produce lytic enzymes or the flexirubin type of pigment. Strains of some phena, notably Cy 1 and Cy 3, were also resistant to polymyxin B.

The significant differences in phenotype observed in this study, between marine chitin-degrading bacteria and *Cytophaga johnsonae*, the commonly occurring chitinoclastic bacterium in freshwater systems [26], should have made it easy to detect and quantitate the incidence of freshwater *Cytophaga* spp. in estuaries and coastal waters. However, from the results of this study, some of the phenotypes included in phena Cy 3 and Cy 4 represented neither of the 2 prototypes, that is, *C. johnsonae* or marine chitin degraders. The most striking case is that of salt-requiring strains which were almost identical to *C. johnsonae* in phenotype, including production of flexirubin-type pigment. Thus, the salinity responses of those isolates examined in this study that possessed characteristics of *C. johnsonae* [37], varied from tolerance to concentrations of NaCl $\leq$1% all the way to requirement for 1% NaCl for growth, a broad range of phenotype, that can be explained by plasmid-mediated regulation of salt resistance [23, 24]. However, analysis for the presence of plasmids in these strains has not yet been done. Such studies are planned.

With respect to occurrence and distribution of chitin-degrading bacteria in Chesapeake Bay, those phena comprising the aerobic, nonauxotrophic isolates matched the published descriptions of marine and nonmarine species that have been identified and classified by traditional, that is, "alpha," taxonomy methods [35]. Strains of *Cytophaga johnsonae*, *Chromobacterium* spp. and several members of the *Actinomycetales* were isolated during this study, representing taxa comprising the predominant chitin-degrading bacteria of terrestrial and freshwater environments (2, 26, 44). The *Vibrionaceae*, in particular, are well es-

tablished as marine and estuarine chitin degraders, as are *Pseudomonas* spp. [5, 6, 15, 16, 29]. Thus, an abundance of taxa, as well as numbers of chitin-degrading bacteria occur in the aquatic environment, both freshwater and marine.

Some of the phena observed in this study were characterized by features of, as yet unrecognized taxonomic value, but of great potential significance in microbial ecology. For example, the phenomenon of temperature-induced "shifts up" of growth temperature range, in the case of marine bacteria [38], was observed to occur in 16% of the estuarine isolates, but was not found to occur in certain phena, such as pseudomonads of phenon 3 and *Chromobacterium* of phenon 5. Although rarely encountered in, and never before reported for *Cytophaga* strains isolated from freshwater and/or marine sources, it was observed to occur far more frequently among estuarine *Cytophaga*-like isolates. The ubiquity of this regulatory phenomenon is not known at all, but could be a major factor in the dispersal and survival of bacteria in estuaries, since estuaries represent a constantly fluctuating and shifting physical and chemical milieu for microorganisms.

The 2 physiological groupings of *Cytophaga* isolates observed in this study, Cy 7 and Cy 4, for example, representing freshwater and marine forms, respectively, differed in characteristics which may have major ecological significance not necessarily related to salinity adaptation. Logically, one might ask the question whether any of the differences noted can be interpreted as an expression of an inherently variable pattern of selective forces operating in an estuary, thereby affecting phenotypic properties, as well as distribution, of chitinoclastic bacteria in freshwater and/or marine habitats. Unfortunately, information available concerning habitat preference of chitin-degrading bacterial species in the aquatic environment is fragmentary and is, therefore, unable, at present, to support a satisfactory answer to this important question.

Although several studies dealing with the taxonomy of chitinoclastic bacteria have been published, distinctly different characteristics of marine species have not been described which, when compared with those of nonmarine isolates, can be used to establish the ecological niche of given bacterial taxa in the natural environment. That is to say, features other than salinity tolerance and/or requirement, have not been found to be correlated with ecological relationships, per se. Clearly, from results of the studies reported here, the intrinsic advantage of numerical taxonomy, when applied to ecology, becomes obvious. Flexibility in selection of test features allows classification of members of a biogeochemically defined group of organisms, such as chitin-degrading bacteria, not only on a strictly taxonomic basis but also in ecological terms. That is, patterns of phenotypic properties can be detected and related to environmental parameters. Thus, in the future, applications of numerical taxonomy to microbial ecology will prove to be valuable in elucidating the structure and function of microbial communities.

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5

Growth and Production of the Seaweed, *Himantothallus grandifolius*, at King George Island

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Summary. Growth of *Himantothallus grandifolius* was measured *in situ* for 32 d during late summer in Admiralty Bay, King Georg Island, Antarctica. Plants grew at a mean rate of 6.0 mm d^{-1} which is comparable with that of laminarians in southern temperate waters for the same time of the year. Conversion of length to biomass showed that the plants increased their biomass by approximately 22% during this period. This increase in biomass was almost negated by loss of frond material, due to constant erosion at the frond tips which are colonized by bacteria which promote erosion. Eroded material enters the detritus food chain and probably supports a rich and diverse coastal fauna.

1 Introduction

In contrast to Antarctica's sparse land flora, the region's coastal waters support large and spectacular submarine "forests" of brown and red algae which have only begun to be explored (Zanefeld 1968; Moe and Silva 1977; Ramirez 1982). *Himantothallus grandifolius* is a large kelp-like alga which appears to have a circumpolar distribution (Skottsberg 1964; Skottsberg and Neushul 1960), but about which otherwise little is known. The species, especially when represented by single bladed specimens, resembles *Laminaria longicruris* and *L. saccharina* in gross morphological appearance , but attains larger dimensions and also differs in other respects (Neushul 1963; Moe and Silva 1981). Until recently, its taxonomic status was unclear and it was assigned a provisional place among the Laminariaceae by Skottsberg and Neushul (1960). However, Moe and Silva (1981) have placed it definitively in the Desmarestiaceae (Desmarestiales).

In temperate waters throughout the world, large kelps have been recognized as important primary producers surpassing the production of phytoplankton in the areas where they occur (Mann 1973). Kelp-beds appear to function as distinctive ecosystems with recognizable food chains which in many cases lead to economically important animals, such as mussels and lobsters (Field et al. 1977). These systems also export large amounts of organic matter (detritus) which settles in deeper water and provides much of the energy-rich material on which benthic communities depend (Field et al. 1980a; b; Stuart et al. 1981). Colonization of algae by bacteria, and a rich and diverse benthic animal community observed in association with the algae in the Antarctic, leads to the speculation that the Antarctic coastal ecosystems may be structured similarly to kelp-bed ecosystems elsewhere.

The potential significance of macrophytic algae in the Antarctic has been pointed out by several (Neushul 1968; Zanefeld 1968; Dell 1972), but very little is known about their roles in the coastal ecosystems of the Antarctic and Sub-Antarctic. Previous reports dealing with algae in the Antarctic have been mainly of a descriptive nature (Papenfuss 1964; Delepine et al. 1965; Neushul 1968; Zanefeld 1966, 1968; Moe and Silva 1977, 1981), since ecological and physiological studies are usually handicapped by the severe conditions encountered in these regions. Our study, using rather simple techniques, attempted to determine the patterns of growth and production in *H. grandifolius*, in Admiralty Bay, and to establish a foundation for future investigations of the roles of macrophytic assemblages in Antarctic coastal ecosystems.

2 Study Area, Materials, and Methods

A detailed description of the hydrology of Admiralty Bay (62° 09′S, 58° 28′W), on the eastern side of King George Island, is given by Rakusa-Suszczewski (1980).

Himantothallus grandifolius is a perennial, sublittoral alga which grows at depths between 30 and 100 m in Admiralty Bay where it seems to form dense beds. Next to the dominant *Desmarestia* species it is probably one of the most important algae, as far as biomass is concerned (Moe and Silva 1977). Gepp and Gepp (1912), Skottsberg and Neushul (1960) and Moe and Silva (1977, 1981) give detailed descriptions of the plant. In contrast to their observations, however, and those of Zinova (1959) who found

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multiple branched specimens, most (69%) of the some 200 plants collected during the course of our investigation, were single bladed. Twenty-two per cent of the plants had two, and nine had three, blades. No plant with more than three blades was found. The largest plant dredged had two blades, 16.7 and 17.5 m long, with a maximum width of 0.90 m and a combined mass of 33 kg. These dimensions surpass those recorded in the literature. All large intact plants, longer than approximately 6 m, were fructifying at the distal ends of the blades.

Specimens of *H. grandifolius* were obtained from depths between 20 and 60 m using an anchor dredge. Twenty-five whole, undamaged plants, each with a single blade 0.2–10 m long, were placed in large barrels filled with seawater and immediately transported into shallow water for processing. The holdfasts were secured to a thick (80 mm) nylon rope 15 m long, using plastic-coated wire to which tags had been attached. Two anchors and a buoy were tied to the rope.

Measurements and punching of the plants for growth studies were done according to the method of Mann et al. (1979), which is based on that of Parke (1948). Holes (Ø = 5 mm) were punched with a cork-borer at 0.5 m intervals along the central axis of the blade, beginning at the junction with the stipe. Care was taken not to expose the plants to air during handling. The rope, together with the plants, was towed carefully into the bay and lowered to 25 m where it remained for 32 d between February and the beginning of March. Subsequently, the plants were taken into the laboratory where the distances moved by the punched holes along the length of the blades were recorded. Length increments were converted to biomass increments, using a regression of length to fresh mass obtained from 106 plants.

To determine percentage dry mass and organic content of different sections of a plant, an intact 15 m specimen was fractionated into holdfast, stipe and 1 m blade portions. The first 1 m was divided into two parts. Dry mass of three replicate subsamples per fraction was obtained by freeze drying, and the organic content was determined after combusting the dried material in a muffle furnace at 550°C for 4 h.

3 Results

Of the 25 plants punched and measured, 17 were recovered after 32 d in situ. All the blades showed substantial growth increments. Elongation occurred from the base to approximately half way up the length of the blades, with the rate of elongation decreasing progressively towards the tip (Fig. 1). The sum of the growth increments, as indicated by the distal displacement of the holes, gives the amount of new growth in terms of length. Although the largest plant had the greatest length increment, growth rate of the other plants could not be related to plant size (Table 1). The mean growth rate (increment in length) was 6.0 mm d^{-1},

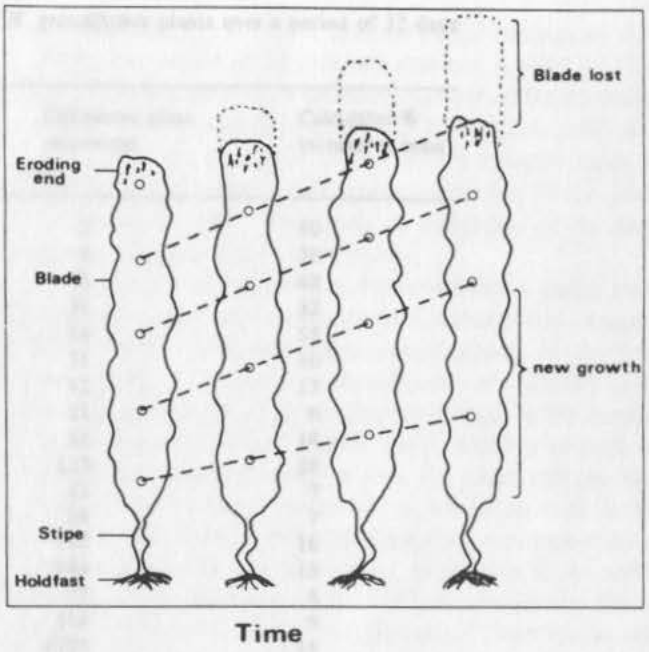

Fig. 1. General pattern of growth in mature *H. grandifolius*

which is the length that the plants would have grown had there been no erosion at the tips. Thus, it is possible to record the amount of tissue added, despite erosion of the ends having taken place. The amount of tissue lost can be determined by simple subtraction.

Changes in the percentage dry mass and organic matter in a mature plant give a further indication of the growth pattern observed (Fig. 2). Percentage dry mass and consequently organic matter were high in the stipe, as a result of its compact structure. It was not possible to determine the dry mass of the holdfast, because adhering particles could not be removed. The base of the blade showed a marked decrease in both dry mass and organic matter, compared to the stipe. This decrease in both parameters reached the lowest levels in the proximal 2–4 m of the blade. Both parameters increased up the blade, and with increasing age. The peak at the 5 m section (Fig. 2) is possibly due to some irregularity. Highest values were recorded near the distal end of the blade. At the tip, the area where the blade begins to senesce and disintegrate, dry mass and organic matter were again reduced; the organic matter decreasing prior to the dry mass.

As *H. grandifolius* blades increase in length, they become wider and thicker, so that the length to mass relationship is not linear. It is described by a power curve (Fig. 3). Using the equation

$$M = 0.0123L^{1.950}$$

(where M = Mass and L = Length), it is possible to convert each observation on initial length and subsequent length increment to a corresponding biomass increment. The amount of tissue lost by the plant can be quantified in a

Table 1. Length increments and calculated production of *H. grandifolius* plants over a period of 32 days during February and March 1983

Initial blade length mm	Length increment mm	Daily length increment mm	Calculated mass increment g	Calculated % increase in mass
200	50	2.0	2	50
400	70	2.0	6	38
860	240	8.0	45	62
1000	150	5.0	31	32
1030	160	5.0	34	55
1800	90	3.0	31	10
1830	120	4.0	42	13
1900	60	2.0	21	6
1930	140	4.0	51	14
2220	280	9.0	120	26
2600	90	3.0	43	7
3500	120	4.0	76	7
3600	280	9.0	187	16
3650	230	7.0	154	13
4000	100	3.0	72	5
4550	200	6.0	164	9
9100	640	20.0	1026	14
Mean	180	6.0	–	22

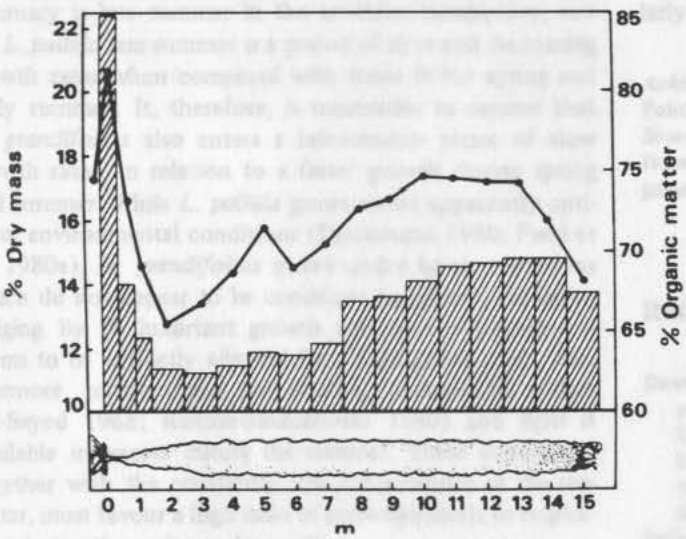

Fig. 2. Dry mass as a percentage of (wet wt.) mass (*histograms*), and organic matter as a percentage of (dry wt.) mass (*graph*) of a large *H. grandifolius* plant in relation to its length (*m*)

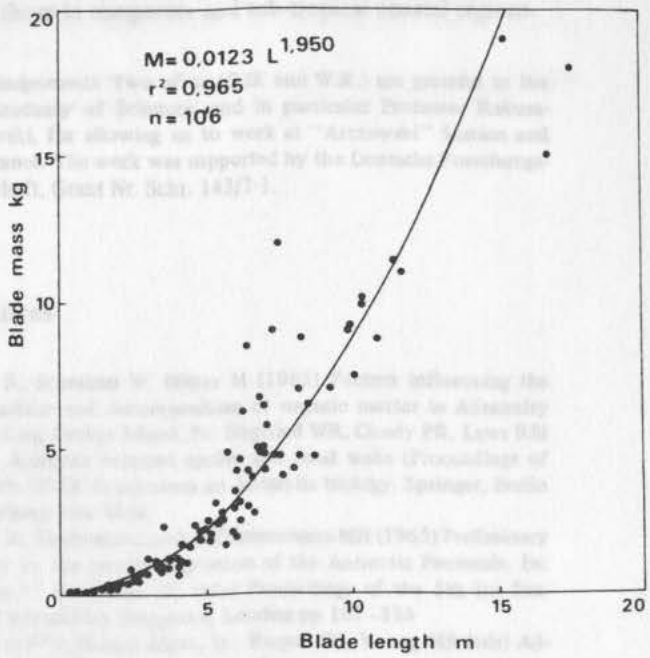

Fig. 3. Relationship between blade length (*m*) and (wet wt.) mass (*kg*) in *H. grandifolius*

similar way. Thus, the blades increased their biomass by a mean of 22% during the month of February (Table 1). The amount of tissue lost was not calculated.

4 Discussion

The pattern of growth in mature *H. grandifolius* plants is similar to that of laminarians, such as *L. digitata* and *L. longicruris* (Mann 1973) and *L. pallida* (Dieckmann 1980). There is a difference, however, in that the zone of elongation is not localized at the base of the blade, but is spread over approximately half of its length. Rate of elongation is fastest near the base and decreases progressively toward the tip. A similar growth pattern was observed in *Ecklonia maxima* by Mann et al. (1979).

The observed changes in percentage dry mass and organic matter in the blade are a clear reflection of the growth pattern observed. Thus, the base, because it has the intercalary meristem and is the area of active cell division and growth, has a higher organic content and, hence, higher dry mass than the adjacent blade tissue which represents the zone of cell enlargement and elongation. During this process water is taken up, resulting in a decrease in both parameters. The subsequent progressive increase in dry mass and organic matter towards the distal end of the blade is a result of cell maturation which is accompanied by accumulation of storage products and differentiation into reproductive tissue. The decrease at the tip is most likely caused by leaching of organic matter from senescing and disintegrating tissue (Newell et al. 1980; Stuart et al. 1981). Zieliński (1981) found a similar variation in the calorific value of different *H. grandifolius* sections.

Growth rates and production by *H. grandifolius* during the study period are surprisingly high, and are comparable with those of laminarians growing in temperate waters. *Laminaria pallida*, for example, has a frond elongation rate of 30–70 mm d^{-1} during February in South Africa, depending on depth of water (Dieckmann 1980). This represents a biomass increase of about 20–45%. However, February is late summer in the southern hemisphere, and for *L. pallida* late summer is a period of slow and decreasing growth rates when compared with those in the spring and early summer. It, therefore, is reasonable to assume that *H. grandifolius* also enters a late-summer phase of slow growth rates, in relation to a faster growth during spring and summer. While *L. pallida* grows under apparently optimum environmental conditions (Dieckmann 1980; Field et al. 1980a), *H. grandifolius* grows under harsh conditions which do not appear to be conducive to growth. However, judging by its luxuriant growth and large dimensions it seems to be perfectly adapted for a high growth rate. Furthermore, nutrients are not limiting in Antarctic waters (El-Sayed 1968; Rakusa-Suszczewski 1980) and light is available in excess during the summer. These conditions, together with the constantly low temperatures of the seawater, must favour a high ratio of photosynthesis to respiration during the spring and summer.

This investigation has answered certain questions attending the pattern of growth in *H. grandifolius*, but it has also revealed the paucity of information on environmental factors which influence algal growth in the Antarctic. Although the method applied to measure productivity of *H. grandifolius* is satisfactory, it is necessary to follow growth for at least 1 yr. Plants should be monitored continuously during the summer months and once before, and immediately after, winter. For data on standing stock, it will be necessary to carry out SCUBA surveys or ship-based underwater photographic surveys.

Despite the lack of information on the standing stock of *H. grandifolius*, it is interesting to calculate, as an example, the production by a single plant for the period of growth as measured. Since blades increased their biomass by 22% during the period of 32 d in late summer, a plant of 10 m length, with a mass of 10 kg (determined from the regression equation), increases its mass by 2.2 kg or 325.6 g (dry wt.) mass or 234.4 g organic matter with a calorific value of 4,000 kJ, as calculated from mean conversion factors given by Zieliński (1981). This gives an indication of the large production potential of these plants.

The occurrence of a rich and diverse benthic faunal community in association with algae in Admiralty Bay (Rakusa-Suszczewski 1980), and other coastal regions of the Antarctic (Dell 1972), points to the existence of a detritus-based food chain. Several of the macrophytic algae in the Antarctic are perennial. Thus, despite the probability of little or no growth occurring during winter, the plants still produce detritus due to constant erosion of the blade ends during months when little or no phytoplankton production occurs. Recent studies of the importance of detritus in Antarctic coastal waters (Dawson et al. 1985; Reichardt and Dieckmann 1985) have shown that the rate of bacterial decomposition of algal debris is comparable to that of temperate marine systems. Hence, it is probable that the Antarctic shallow coastal ecosystems is based at least in part on a detritus foodchain which is structured, and functions, similarly to those in temperate and sub-tropical coastal regions.

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6

Kinetics and Trophic Role of Bacterial Degradation of Macro-Algae in Antarctic Coastal Waters

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Summary. Aerobic formation of detritus from debris of the Antarctic Kelp, *Himantothallus grandifolius*, yielded amounts of carbon and nitrogen 2 and 4 times higher, respectively, than fresh algae, and a concomitant decrease of the carbon:nitrogen ratio from 19 to approximately 10. These changes were achieved only in the presence of the indigenous Antarctic bacterio-flora. During formation of detritus from the Antarctic red alga, *Leptosomia simplex*, C:N ratios may drop even below the Redfield ratio reported for phytoplankton. The increases in the C and N contents of the debris and its colonization by epiphytic bacteria were characterized by saturation kinetics, which showed an initial lag, subsequent steep increment, and a final stationary phase. During its exponential phase, rates of bacterial colonization of debris from brown and red algae were comparable to those calculated for kelp in areas of temperate climates. There is preliminary evidence that some of the enzymes, which participate in the decomposition of the particulate detrital matter, are optimally adapted to the temperature regime of a permanently cold environment. The results obtained favour the idea that the formation of macro-algal detritus in Antarctic coastal waters is as efficient as in temperate regions. Preliminary assessments indicated that partly degraded thalli from both the brown and red algae were preferred over fresh thalli, as a food source, by intertidal amphipods.

1 Introduction

Primary productivity at the edge of the seas often reaches maximal values that are comparable to the productivity of tropical rain-forests (Mann 1973). In temperate latitudes, the total production of sub-littoral seaweeds, such as kelp, may be three times that of the total production of phytoplankton, in certain coastal areas. More than 90% of these benthic macrophytes enter detritus food chains. Despite the abundance of macro-algae in coastal Antarctic waters (Dieckmann et al. 1985), there seems to be no indication as to whether the Antarctic region provides conditions adequate for detritus food chains to play an efficient role as a major pathway for nutrient cycles.

Generally speaking, formation of detritus is triggered by disruption and ageing of the macrophyte tissues, followed by leaching and stimulation of microbial growth in the leachates as well as subsequent colonization of the fragments. The conversion of macrophyte debris to detritus is often characterized by: a succession of epiphytic bacteria which have the capacity to decompose detrital bio-polymers; a decrease of the C:N ratio; and, an increase of nutritive value of debris as food for macro-heterotrophic consumer organisms. In Antarctic waters, it can be anticipated that formation of detritus from macro-algae, the only benthic macrophytes in this environment, is further influenced by: the unique grinding forces exerted by pack-ice; adaptation of growth rates and enzyme activities of the epiphytic bacteria to a permanently cold temperature regime; and, the endemic nature and possibly different degradability of most of the predominant macro-algae.

Our investigation aimed to determine whether conditions for the formation of detritus in Antarctic coastal waters were adequate to postulate that detritus-based food chains may play a similarly efficient role as those in lower latitudes. Hence, experiments were designed to simulate certain aspects of detritus formation from artificially generated debris from two of the most abundant macro-algae in Admiralty Bay (South Shetland Islands). The species involved were *Himantothallus grandifolius* (a brown alga) and *Leptosomia simplex* (a red alga).

Time-course studies dealt with the C and N contents of the debris, colonization by epiphytic bacteria, and selected enzyme activities involved in the breakdown of particulate bio-polymers. As a biomass equivalent, the ATP content of the total suspensions was measured. Intertidal amphipods were used to assess the food value of the macro-algae.

2 Materials and Methods

2.1 Experimental Design

Five kg (wet wt) of *H. grandifolius* dredged from 30–60 m in Ezcurra Inlet (Admiralty Bay, King George Island) was cut and shredded, employing a high-speed blender (Ultra-turrax) before final preparation. The debris was suspended in 6 l of 0°C Antarctic seawater and filtered through a 1.0 mm plankton net. The suspended matter in the filtrate was washed on a 200 μm-sieve and resuspended in 10 l of 0°C Antarctic seawater. Three kg (wet wt.) of *L. simplex*,

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collected from the upper sub-littoral of Admiralty Bay during low tide, was shredded, using an Ultraturrax blender for batches of 250 g in 500 ml 0°C Antarctic seawater, and subsequently filtered through 1.0 mm and 0.2 mm sieves to obtain the 0.2–1.0 mm particle fraction of suspension in 8 l of 0°C Antarctic seawater.

The suspended algal matter was incubated in the dark in aerated 3–1 or 5–1 flasks and 2–1 (*L. simplex*) or 4.5–1 (*H. grandifolius*) suspensions immersed in water baths at 0°C. At the beginning of each incubation a final blending (Ultraturrax) was carried out for 10 min. As controls, half of the flasks were irradiated (2 times for 1 h) with ultra-violet light using an immersible Hanau Sterisol lamp (TNN 15/35) to inactivate the micro-flora remaining on the debris and in suspension. The influence of dissolved nitrogenous organic matter was studied by adding Bacto-Peptone at a final concentration of 2.5 g l^{-1}.

The food value of the two algae was assessed by feeding 20 mm (diameter) discs, punched from fresh or partly degraded algal thalli, to 20 intertidal amphipods (Pontogeneiidae). These were kept in 250 ml perforated plastic containers in a 100 l tank with a continuous through-flow of fresh Antarctic seawater of ca. −1°C. At daily intervals, discs were taken out, blotted quickly between two layers of filter paper, and weighed in order to determine the daily consumption. At set time intervals, chosen according to the density of epiphytic bacteria in preliminary experiments, samples of the suspended debris were taken with a sterilized 50 ml syringe and processed immediately for ATP extraction, separation of the particulate fraction by centrifugation, and for enzyme assays.

2.2 Counting Epiphytic Bacteria

Five ml sub-samples of the suspension were poured into 10 ml centrifuge tubes, fixed with 0.5 ml of 38% formaldehyde and stored at 5°C. For acridine-orange (A.O.) epi-fluorescence microscopy, 0.5 ml of the staining solution (1,000 ppm acridine-orange in 0.15 M phosphate buffer, pH 4.7,) was added for 5 min, centrifuged (6,000 g), re-suspended in distilled water and again centrifuged. A drop of the re-suspended pellet (in 5 ml of distilled water) was used for counting the bacteria adhering to the debris, employing a Leitz Dialux 20 epi-fluorescence microscope with a 100 times NPL-Fluotar oil immersion objective. A 50 times 50 μm counting grid hat to cover algal surfaces in at least 20 different fields of vision. Results are given as numbers of adherent cells per 2,500 μm^2 unit surface area.

2.3 Chemical Analyses

Total adenosine triphosphate (ATP) was extracted immediately by heating 1 ml of a sub-sample and 1 ml of 0.4 M tris-HCL buffer, pH 7.7, for 5 min at 100°C. Extracts were stored frozen at −20°C. Assays based on the luciferin-luciferase reaction were carried out with a 50 μl sample volume and 2-ml 20 mM tris-HCL buffer, pH 7.7, containing 10 mM $MgSO_4$ and 1 mg ml^{-1} of FLE-250-luciferin-luciferase (Sigma), by measuring peak heights of bio-luminescence in a JRB-ATP-photometer.

The total particulate matter was collected by centrifugation, stored at −20°C and freeze-dried for the determination of dry wt., total C and N. Carbon and N were measured in triplicate sub-samples of approximately 100 μg in tin cups using a Carlo Erba elemental analyzer, model 1106, with automatic sampler and combustion at 1,020°C. Acetanilide was used as a standard.

2.4 Enzymatic Decomposition Potentials

To measure activities of bio-polymer–solubilizing enzymes, 5 ml sub-samples were extracted with 25 ml ice-cold Triton X100-buffer (0.02 M phosphate buffer, pH 7.0, in sterile seawater containing 2.5% Triton × 100 and 2 g l^{-1} polyvinylpyrrolidone as a stabilizer). Amylase, protease and cellulase activities were measured by incubating 3-ml extracts in 10-ml centrifuge tubes with 20 mg of the commercial particulate enzyme-substrates coupled to a blue dye (hide powder-, amylopectin-, and cellulose-azure, Sigma Chemicals). After 18-h incubation at 0°C (or employing a temperature gradient) the reactions were terminated by adding 1 ml of 16% formaldehyde in 0.1 M KH_2PO_4-Na_2HPO_4-buffer, pH 7.5. After 20 min of centrifugation (6,000 g) the absorbance was measured at 600 nm (10-mm path length using a Zeiss PMQ3 spectro-photometer. The absorbance was used as a relative unit.

3 Results and Discussion

3.1 Changes of Carbon and Nitrogen Contents in Macro-Algal Debris

Whereas the C:N atomic ratio of phytoplankton is 6.6, according to the Redfield ratio, the median C:N ratio of marine benthic macrophytes is as high as 18.3 (Atkinson and Smith 1983). Degradation of macrophyte debris is usually characterized by decreasing C:N ratios, which may be partly attributed to the build-up of micro-biota on the debris and subsequent immobilization of dissolved N (Thayer et al. 1977; Haines and Hanson 1979). On the other hand, decomposition experiments with particles derived from *Laminaria* suggest that N is conserved via formation of refractory polyphenol-complexed fractions in the plant debris (Robinson et al. 1982). Some authors even doubt that the contribution of micro-organisms to the N pools of detritus are significant (Christian and Wetzel 1978; Hanson 1982). Our experiments with detrital particles obtained from *H. grandifolius* showed a doubling of particulate C and a 4 times increase of particulate N after a week

at 0°C (Fig. 1). In suspensions irradiated with UV light to inactivate epiphytic bacteria, this increase was markedly delayed, indicating that biological processes contributed considerably to the %C- and %N-increases, while the debris was converted to detritus.

Saturation type kinetics characterized the C- and N-fluxes of debris from both *H. grandifolius* and *L. simplex*, the most abundant macrophyte in the shallow sub-littoral. In contrast to *H. grandifolius*, only minor differences were found between the UV-irradiated and the untreated samples for *L. simplex* (Fig. 2). The further decrease of C and N after extended incubation (over 20 d) in debris amended with peptone may indicate that dissolved organic N plays a rate-limiting role for the decomposition of detritus (Robinson et al. 1982).

Enrichment of particulate N in debris from *L. simplex* started at relatively high levels of ca. 4%, which coincided with the maximum levels reached by mature detritus from *H. grandifolius*. Although certain red algae contain more N than kelp, contents as high as 3%–5% may indicate storage (Bird et al. 1982).

Can C- and N-fluxes in these experiments be considered as a specific achievement of the indigenous Antarctic micro-flora at ambient temperatures around 0°C? When debris of the Antarctic *H. grandifolius* was suspended in Baltic seawater at 0°C, the increase of C and N again followed a saturation-type curve, yet the saturation value for N enrichment was only 50% of that noted under the influence of an Antarctic micro-flora (Figs. 1 and 3). This low N accumulation contributed to a final C:N ratio of 14 for mature kelp detritus. On the other hand, considerably lower C:N ratios were observed when the detritus had been allowed to form in water from the original habitat of the alga (Fig. 4). Thus, a direct relationship between the indigenous epiphytic micro-flora on the debris and its gain in N is indicated.

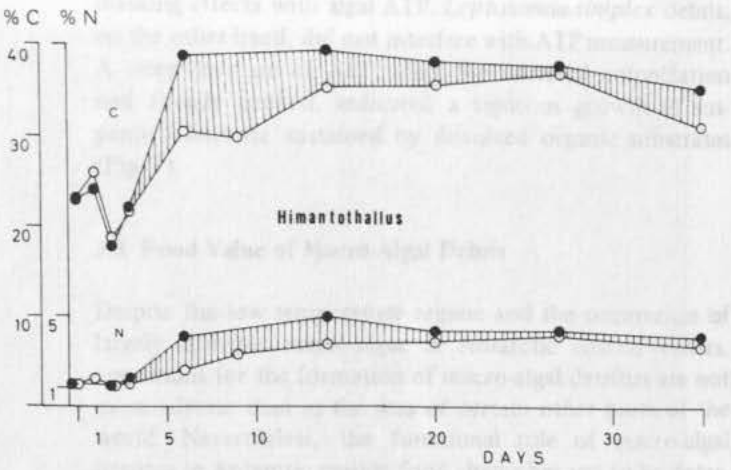

Fig. 1. Carbon and nitrogen during fluxes formation of detritus from *H. grandifolius* debris without (*dots*) and with UV-irradiation (*circles*)

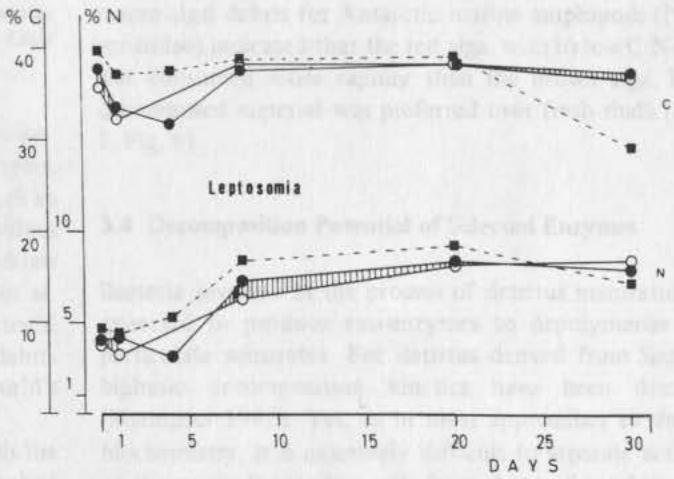

Fig. 2. Carbon and nitrogen fluxes during formation of detritus from *L. simplex* debris without (*dots*) and with (*circles*) UV-irradiation. *Broken line* traces results from a parallel experiment with the addition of peptone

3.2 Growth of Bacteria on Algal Debris

Experimental manipulations (viz, UV-irradiation) causing temporary inactivation of part of the epiphytic micro-flora could not replace the use of analytical controls. Hence, conversion of the particulate algal biomass into bacterial biomass had to be confirmed by direct counting. There was no visible evidence of protozoans in any of these experiments.

Fresh debris of *H. grandifolius* and *L. simplex* was colonized by relatively small, mostly coccoid, bacterial cells (0.1–0.4μm), Similarly to kelp in temperate latitudes, the thalli of *H. grandifolius* were predominantly colonized along the junctions of the epidermal cell walls (Linley et al. 1981; Koop et al. 1982; Fig. 5). After a lag of 2 to 3 days dividing cells became more and more frequent, together with a predominance (95%) of relatively large rods (Fig. 5).

Bacterial cells counted on debris with a surface area of at least 2,500 μm^2 revealed exponential growth for 4–7 d following an initial adaptation phase of 1–3 d (Fig. 6). During exponential growth, the apparent doubling times

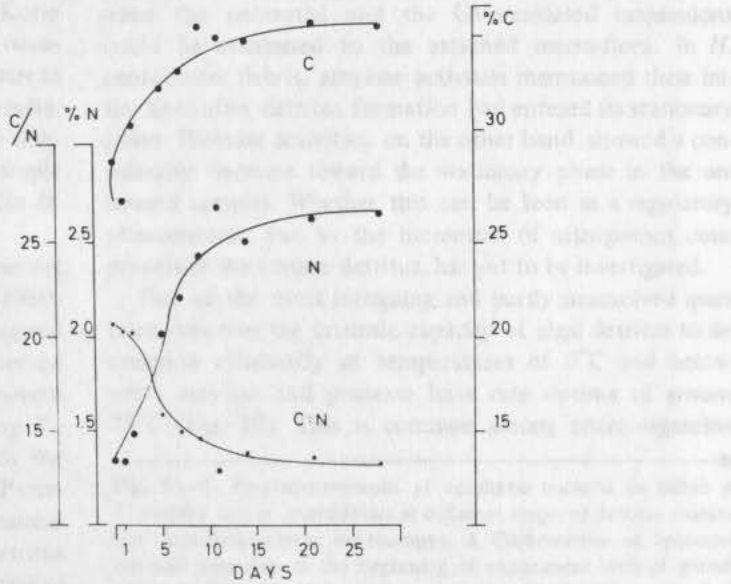

Fig. 3. Carbon and nitrogen in *H. grandifolius* debris during detritus formation in Baltic seawater. C:N ratios drawn on *left*

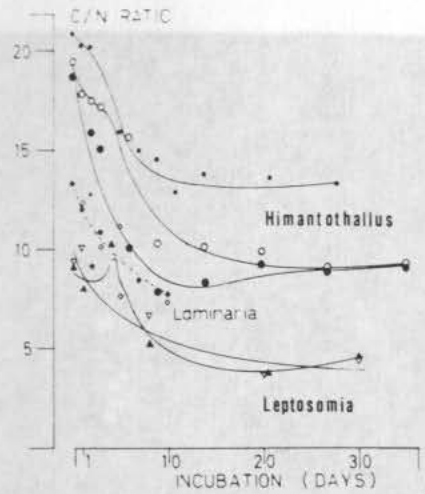

Fig. 4. Fluxes of C:N ratios in macro-algal debris under different treatments: *H. grandifolius*, in Antarctic seawater (●, o); *H. grandifolius* in Baltic Seawater (●); *L. simplex*, in Antarctic seawater (▲△); and, *Laminaria saccharina*, in Baltic seawater (■□). *Clear symbols* in all cases indicate UV-irradiation

ranged from 21.h for *L. simplex* to 27 h for *H. grandifolius*. These apparent doubling times must be viewed as a complex parameter based on the growth of adhering cells, as well as settling and detachment processes. Very similar values (24.8 h) were calculated for *Ecklonia maxima* debris from the South African coast, from data given by Koop et al. (1982). This indicates that the accumulation of a bacterial micro-flora on the surface of Antarctic macro-algal debris occurs at the same rate as in other parts of the world's oceans, in spite of the lower temperature regime.

Bacterial colonization of the debris correspond with the time course of %C and %N-increases (Figs. 1, 2, 6). Attached bacterial growth reached a maximum density of ca 400,000 cells mm^{-2} for detritus from both species. Densities of this order of magnitude also have been reported for other marine algae from other regions (Cundell et al. 1977; Koop et al. 1982), whereas bacterial densities on *Posidonia oceanica* apparently reach only 40,000 cells mm^{-2} (Velimirov et al. 1981). Bacterial counts indicated further that UV-irradiation had inactivated the epiphytic micro-flora only temporarily. The resulting time lag against the untreated sample disappeared after 1 day for *L. simplex* and 6 days for *H. grandifolius*, respectively.

So far only the particulate fraction of the experimental system has been considered. However, Newell et al. (1980) found that the conversion of kelp particles into bacterial biomass is much lower than bacterial growth on dissolved leachates. In our experiments, total dry wt. losses amounting to 37% in the first 6 days indicated intense leaching. To obtain a global biomass equivalent comprising both the algal debris and the suspended micro-organisms, ATP-concentrations were determined for the total suspensions. However, large variations of C:ATP ratios in kelp detritus limit the use of ATP analyses (Stuart 1982). In the case of *H. grandifolius*, this analysis was inapplicable, due to the masking effects with algal ATP. *Leptosomia simplex* debris, on the other hand, did not interfere with ATP measurement. A steep increase of ATP, after the bacterial colonization had already peaked, indicated a vigorous growth of suspended bacteria sustained by dissolved organic substrates (Fig. 7).

3.3 Food Value of Macro-Algal Debris

Despite the low temperature regime and the occurrence of largely endemic macro-algae in Antarctic coastal waters, conditions for the formation of macro-algal detritus are not more adverse than in the seas of certain other parts of the world. Nevertheless, the functional role of macro-algal detritus in Antarctic marine food chains has yet to be determined.

Preliminary tests to determine the nutritive value of macro-algal debris for Antarctic marine amphipods (Pontogeneiidae) indicated that the red alga, with its low C:N-ratio, was consumed more rapidly than the brown alga. Partly decomposed material was preferred over fresh thalli (Table 1, Fig. 8).

3.4 Decomposition Potential of Selected Enzymes

Bacteria involved in the process of detritus maturation are expected to produce exo-enzymes to depolymerize their particulate substrates. For detritus derived from *Spartina*, biphasic decomposition kinetics have been described (Marinucci 1982). Yet, as in most approaches to detritus biochemistry, it is extremely difficult to separate activities of the partly living algal cells from those of epiphytic bacteria. Ultra-violet irradiation showed only limited efficiency in helping to overcome this problem (Fig. 9). Nevertheless, at least at the beginning of an experiment, differences between the untreated and the UV-irradiated suspensions could be attributed to the attached micro-flora. In *H. grandifolius* debris, amylase activities maintained their initial level after detritus formation had entered its stationary phase. Protease activities, on the other hand, showed a considerable decrease toward the stationary phase in the untreated samples. Whether this can be seen as a regulatory phenomenon, due to the increment of nitrogenous compounds in the mature detritus, has yet to be investigated.

One of the most intriguing and partly unresolved questions concerns the intrinsic capacity of algal detritus to decompose efficiently at temperatures of 0°C and below, when amylase and protease have rate optima of around 25°C (Fig. 10). This is common among micro-organisms

Fig. 5A–E. Photomicrographs of epiphytic bacteria on debris of *L. simplex* and *H. grandifolius* at different stages of detritus maturation (epi-fluorescence microscopy). **A** Colonization of epidermis cell-wall junctions at the beginning of experiment with *H. grandifolius*. **B**, **C**, and **D** Colonization of *H. grandifolius* debris after 2, 6, and 28 days. **E** *Leptosomia simplex* debris after 20 days

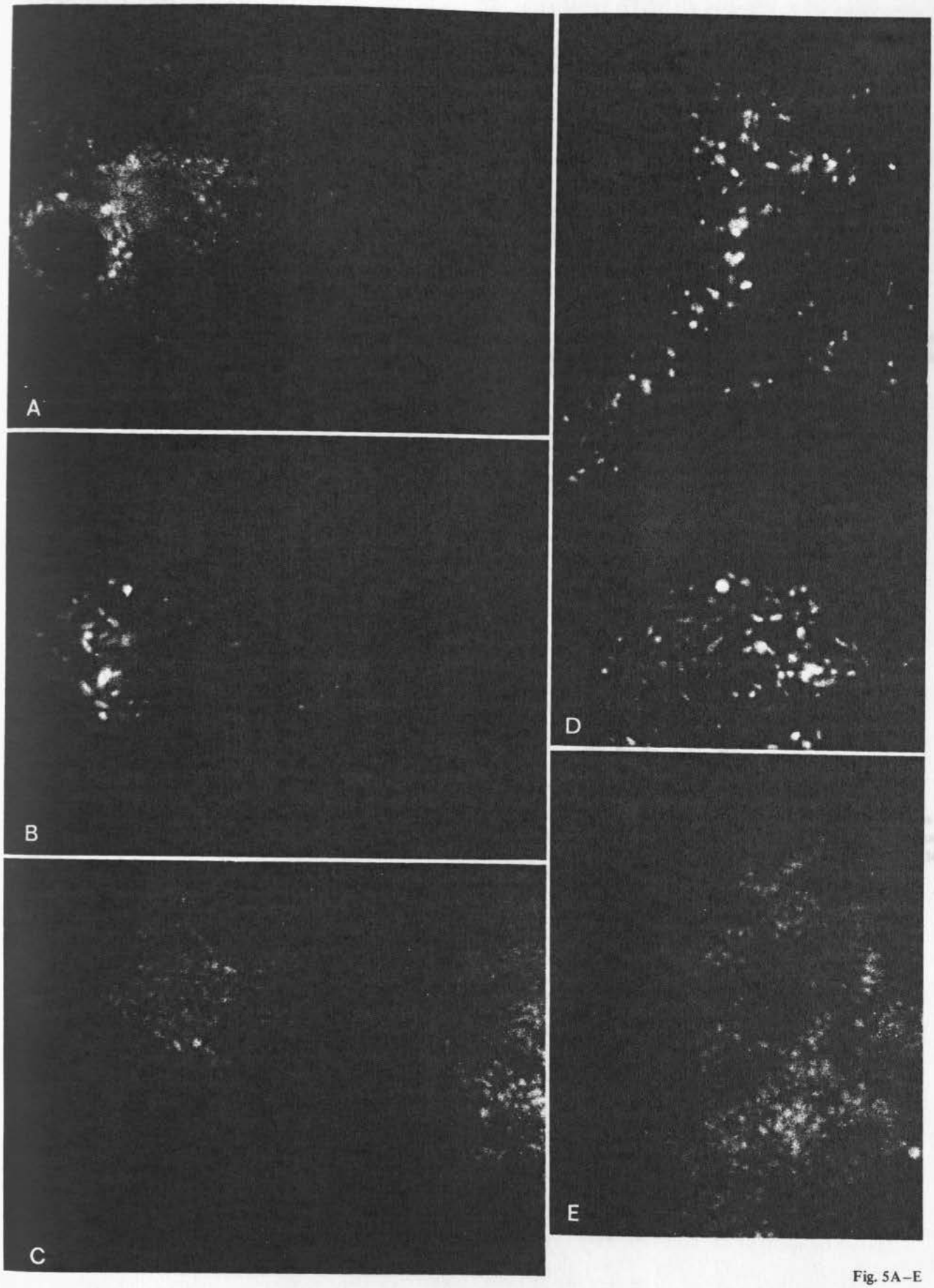

Fig. 5A–E

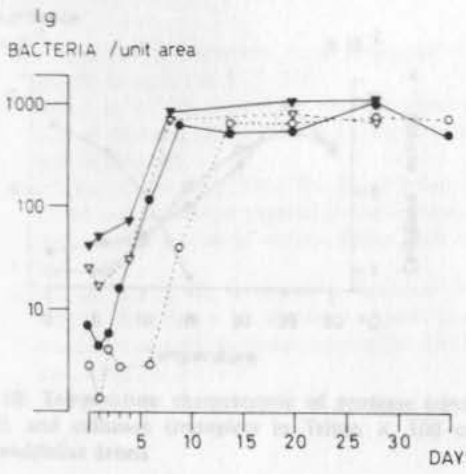

Fig. 6. Time-course of colonization of *H. grandifolius* (*dots*) and *L. simplex* (*triangles*) debris by epiphytic bacteria (log numbers per unit surface area of 2,500 μm^2). *Clear symbols* in all cases indicate UV-irradiation

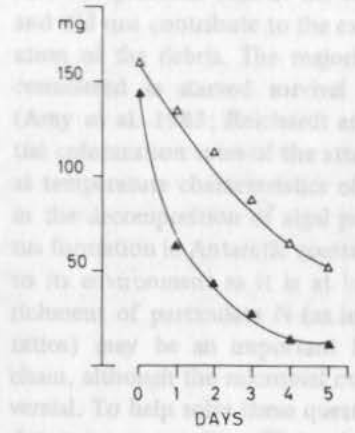

Fig. 8. Loss in fr. wt. of fresh (*clear triangles*) and partly degraded (*shaded triangles*) thallus discs from *L. simplex* due to consumption by intertidal amphipods

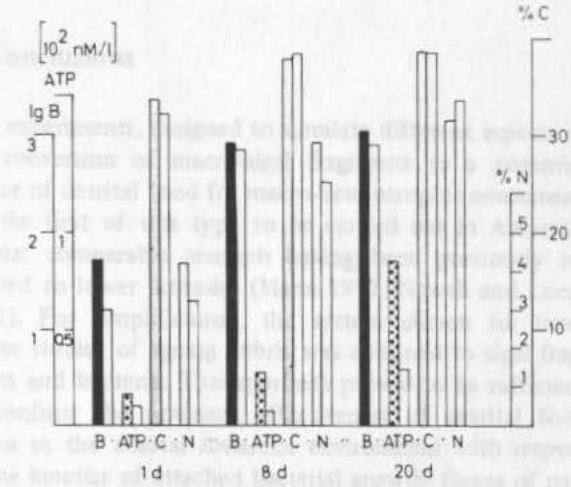

Fig. 7. Chemical and biological parameters for *L. simplex* during different phases of bacterial colonization. *lg B* log plot of bacterial cell densities per 2,500 μm^2 debris surface; nitrogen (*N*), carbon (*C*), and *ATP* concentrations. *Clear columns* in all cases indicate UV-irradiation

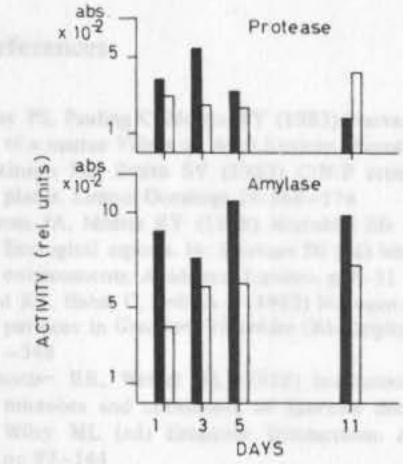

Fig. 9. Activities of protease and amylase in cultured suspensions of *H. grandifolius* debris at 0°C. Relative units based on absorbance (600 nm) of released dye after 18 h of Triton × 100 extract. Clear columns in all cases indicate UV-irradiation

Table 1. Elimination of fresh and partly degraded thallus discs of Antarctic macro-algae consumed by intertidal amphipods. Initial weight losses are after 2 days and 6 days for *L. simplex* and *H. grandifolius*, respectively

	Leptosomia simplex		*Himantothallus grandifolius*	
	fresh	partly degraded	fresh	partly degraded
Initial C:N	8.2	7.7	17.7	15.5
Initial % N	4.3	4.3	1.8	2.0
Initial fr. wt. (mg)	159 ± 10	144 ± 18	578 ± 22	317 ± 3
Weight loss d^{-1} (%)	14.5	30.6	0.9	5.4

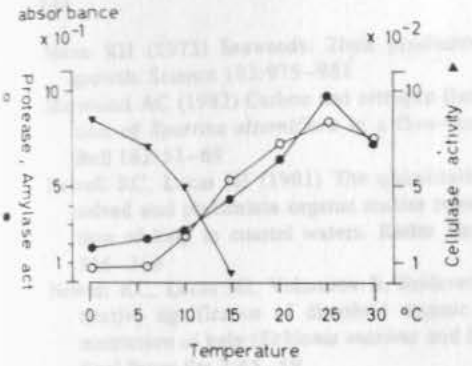

Fig. 10. Temperature characteristic of protease (*circles*), amylase (*dots*), and cellulase (*triangles*) in Triton × 100 extracts from *H. grandifolius* debris

which are adapted to permanently cold environments (Baross and Morita 1978). However, optimum adaptation to the ambient temperature regime of Antarctic waters is shown for cellulase activity from the same source (Fig. 10).

4 Conclusions

Our experiments, designed to simulate different aspects of the conversion of macro-algal fragments to a potential source of detrital food for macro-heterotrophic consumers, are the first of this type to be carried out in Antarctic waters; comparable research having been previously restricted to lower latitudes (Mann 1973; Newell and Lucas 1981). For simplification, the system chosen for time-course studies of ageing debris was confined to algal fragments and bacteria. This approach proved to be sufficient to confirm the potential effectiveness of detrital food chains in the coastal Antarctic environment with respect to the kinetics of attached bacterial growth, fluxes of particulate C and N, activities of selected depolymerizing enzymes and food selection by indigenous macro-heterotrophs.

A mass balance for the conversion of algal to bacterial substance was beyond the scope of this communication. It would have required additional information on the pool of DOM and densities of free supended bacteria, as well as an exact determination of the biomass volume of the total attached bacterio-flora. Since the debris represented living macro-algal fragements, parts of the increments of C% and N% were likely to have been caused by biological processes still associated with them. Physical processes related to mineralization and leaching were covered by the UV-irradiated blanks.

In temperate climates, conversion of kelp C to total bacterial C is strongly influenced by seasonal fluctuations of the predominant bacterial cell forms (i.e. rods or cocci) (Lucas et al. 1981; Newell and Lucas 1981). On the other hand, in our experiments with Antarctic macro-algae at 0°C, coccoid forms were confined exclusively to the surface of epidermal cells at the beginning of the experiment and did not contribute to the exponential phase of colonization of the debris. The majority of these forms may be considered as starved survival stages of rod-shaped cells (Amy et al. 1983; Reichardt and Morita 1982). Exponential colonization rates of the attached bacterio-flora, as well as temperature characteristics of selected enzymes involved in the decomposition of algal polymers, suggest that detritus formation in Antarctic coastal waters is as much adjusted to its environment as it is at lower latitudes. Relative enrichment of particulate N (as indicated by decreasing C:N ratios) may be an important fact for any detrital food chain, although the microbial contribution remains controversial. To help solve these questions, work is in progress to determine conversion efficiencies for attached fractions of detrital bacteria.

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POLYCHAETE TUBE WALLS AS ZONATED MICROHABITATS FOR MARINE BACTERIA

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ABSTRACT - The predominantly anoxic, sulfide-rich sandy sediment of a shallow lagoon at Kiel Fjord was densely inhabited by the polychaete worm *Nereis diversicolor* (280 cm^3 m^{-2} of biomass volume). Burrow walls which contrasted by their brown coloration of at least 1.5 mm thickness with the bulk of the reduced black sediment, made up for 6 % of the total volume of the 10 cm cores investigated. Only the uppermost mm of the internal surface layer contained detectable O_2. As a result of the build up of internal redox gradients, CO_2 dark fixation was activated in the burrow walls (by a factor of approximately 2). Contribution of epi- or endozoic activities associated with *Nereis diversicolor* to this kind of "perizoic" activation in the walls was negligible. Due to the relatively limited internal surface area produced by bioturbation, chemoautotrophic CO_2 fixation in burrow walls may not be very effective on a more global scale. On the other hand, reduced marine sediment devoid of a burrowing infauna showed considerable CO_2 dark fixation only at the oxidized sediment-water interface. Furthermore, assays of ribulose-biphosphate carboxylase activities and viable counts of potentially chemoautotrophic bacteria in different parts of the lagoon sediment suggested that burrow walls as the sites of maximal CO_2 fixation rates would not qualify as microbial barriers. This view of polychaete burrow walls as open enrichment systems for bacteria with key roles in the cycle of carbon was confirmed by additional determinations of heterotrophic (Fe^{3+}, and SO_4^{2-} reducing, proteolytic, chitinolytic, and agarolytic) bacteria.

Key words : CO dark fixation, thiosulfate, detritus, decomposition, sediment, bioturbation.

RÉSUMÉ - Le sédiment sableux riche en sulfures et à dominante anoxique d'une lagune peu profonde du fjord de Kiel, héberge une population dense d'un ver polychète : *Nereis diversicolor* (280 cm^3 m^{-2} en biomasse volume). Les parois des tubes qui contrastent par leur coloration brune d'au moins 1,5 mm d'épaisseur avec la masse de sédiment noir réduit, constituent jusqu'à 6 % du volume total des carottes de 10 cm. La présence d'O_2 n'est décelée que dans les tous premiers millimètres de la couche de surface interne. L'accroissement de la fixation de CO_2 (par un facteur de 2 environ) dans les parois des tubes est le résultat de l'augmentation du gradient rédox interne. La contribution des activités épi ou endozoïques associées à *Nereis diversicolor* dans ce genre d'activation «périzoïque» au niveau des parois est négligeable. La fixation chimioautotrophique de CO_2 dans les parois des tubes n'est pas vraiment importante à l'échelle plus générale, si la surface, limitée, touchée par la bioturbation est prise en référence. D'un autre côté, le sédiment réduit dépourvu d'une endofaune montre une forte fixation de CO_2 à l'obscurité seulement au niveau de l'interface oxydée eau-sédiment. De plus, les dosages de l'activité de la ribulose biphosphate carboxylase et les numérations des bactéries viables et potentiellement chimiotrophes dans les sédiments de la lagune suggèrent que les parois des tubes, comme sites des taux de fixation maximale de CO_2, ne constituent pas une barrière pour les bactéries. Cette hypothèse des parois des tubes de polychètes, comme système d'enrichissement ouvert aux bactéries jouant un rôle clé dans le cycle du carbone, est confirmé par des études supplémentaires sur les bactéries hétérotrophes (réduisant Fe^{3+} et SO_4^{2-}, proteolytiques, chitinolytiques et agarolytiques).

Mots clés : fixation de CO_2 à l'obscurité, thiosulfate, détritus, décomposition, sédiment, bioturbation.

INTRODUCTION

Bacteriological assays aiming at the zonation of different physiological groups in marine

sediments have usually followed the track of vertical gradients that are produced by redox potentials and pO_2 (Novitsky and Kepkay, 1981). The resulting vertical patterns of distinct microhabitats, however, may often be superimposed or even disturbed by burrowing activities of the macrofauna, viz. bioturbation. Beyond that, it has been claimed that bacterial growth rates and metabolic activities are considerably enhanced as a result of infaunal reworking of the sediment (Yingst and Rhoads 1979). Yet, an apparent lack of quantitative data makes it impossible to get an idea of the magnitude and significance of this impact.

In largely anoxic sediments, formation of «internal» oxidized surface areas by the activity of burrowing macrofauna should provide secondary niches for many aerobic or microaerophilic bacteria in an otherwise not inhabitable environment. This may apply particularly to chemoautotrophic bacteria that depend on a constant supply of oxidizable inorganic compounds as supposed by Yingst and Rhoads (1979).

A first approach to understand the impact of burrowing on bacteria with key roles in the carbon cycle focussed primarily at the de novo biosynthesis of organic carbon (CO_2 dark fixation) and secondly at certain aspects of the decomposition of particulate organic matter. Largely anoxic sediment from a shallow lagoon that was intensely bioturbated by the polychaete worm *Nereis diversicolor*, was chosen for a comparative bacteriological study of different areas in 10 cm cores.

Materials and methods

Sediments from Kiel Bay (Boknis Eck) and Limfjorden were sampled by divers in 60 mm wide Plexiglass tubes. Cores of sandy sediment from Stein lagoon (grain sizes : 50.7 %> 500 um, 37.1 %> 250 um) were taken in 7 x 16.5 x 30 cm metal boxes. Redox potentials (measured with an Ingold Pt / Ag-AgCl electrode) as well as coloration differences served to localize selected sampling areas within the box core samples. In addition, pO_2 gradients in the burrow walls were measured using an oxygen microelectrode (Revsbech *et al.*, 1980). Most of the investigations described were carried out with lagoon sediment obtained during a sampling series on 7 days in August 1984.

Assays were carried out with subsamples separated from selected areas in the cores. Subsamples were obtained by breaking the cores into pieces to expose several longitudinal halfs of the burrows. Metal spatulas with U-shaped profiles fitting the open burrow halfs were used to peel off burrow wall layers of roughly one mm thickness from sediment layers showing the same coloration. In certain cases, successive subsamples of approximately 0.5 mm thickness were collected, the uppermost subsample consisting merely of mucous, particle-incrusted pellicles. Subsamples were transferred immediately to sawed-off 1 cm^3 syringes and sealed with Parafilm^r to avoid gross changes of E_h. Cell-free extracts for enzyme assays were prepared at the sampling site. All assays were initiated within 1-2 h after sampling.

Enzyme extraction for assays of RuBPC ase were carried out with 1 cm^3 of sediment per 5 ml of ice-cold extracting buffer containing 12 ‰ of artificial seawater at pH 7.8, 2 % triton X 100, and 2 $g.l^{-1}$ polyvinylpyrrolidone as stabilizing agent. Assays for RuBPC ase followed essentially the technique of Glover and Morris (1979). *In vivo* fixation rates (v) of inorganic carbon (CO_2) were determined by injecting 100 μl (1 μCi) per cm^3 sediment of a stabilized isotonic solution of 1.7 mM Na $H^{14}CO_3$ into either entire 5 cm^3 - subcores or 0.1 -0.5 cm^3 segments of these, (Reichardt, in prep.). rates were calculated using the following formula :

$$v\ (\mu \text{ moles } h^{-1}\ cm^{-3}) = \frac{(12_C) \cdot (14_{C\ ass})}{(14_{C\ add}) \cdot V \cdot t}$$

where (12_C) = μmoles of total carbonate (ΣCO_2) per assay, sum of (ΣCO_2) in the interstitial water of the sample and (ΣCO_2) of the unlabeled fraction of the labeled substrate solution.

$(14_C$ ass) = DPM of assimilated ($\Sigma^{14}CO_2$) ;
$(14_C$ add) = DPM of added ($\Sigma^{14}CO_2$) ;
V = sample volume (cm^3) ; t = incubation time (h).

Concentrations of total carbonate ($\Sigma CO_2 = (CO_2) + (HCO_3^-) + (CO_3^{2-})$) in the interstitial water (sediment wet weight - dry weight) were calculated from carbonate alkalinity data based on measurements of salinity, pH, and temperature according to the equations and tables given by Gargas (1975).

Viable counts were determined at 18°C aerobically and in an anaerobic incubator under Ar gas, using techniques described by Jones (1983) (Fe-reducing bacteria), Krumbein and Altmann (1973) (Mn oxidizing bacteria), Tuttle and Jannasch (1972) (modif. MPN, thiosulfate oxidizing bacteria) and Reichardt (1978) (Cellulose-, chitin-, agar- and gelatine degrading, desulfurizing, and NH_4^+ nitrifying bacteria, MPN).

Results and Discussion

Description of the lagoon sediment

Sediment cores from Stein Lagoon were largely anoxic throughout the upper 10 cm, but heavily bioturbated by *Nereis diversicolor*. This polychaete worm reached a mean standing crop of 4 180 individuals per m^2, equivalent to a biomass volume of 280 cm^3 per m^2 or 50.2 g of dry weight biomass per m^2. Burrows (with a mean diameter of 35 mm) occurred with an average frequency of 6 039 holes per m^2 and represented 6 % of the total sediment volume from 0 to 10 cm depth. Burrow walls of 1.5 mm thickness were characterized by a light brown coloration and redox potentials ranging from + 100 to > + 250 mV. Adjacent sediment areas were predominantly black and sulfide-rich with redox potentials ranging from - 50 to + 50 mV. In the 10 cm-cores studied only the thin top layer of oxidized sediment that accounted for merely 1-2 % of the core volume showed redox potentials equal to or exceeding those of the burrow walls. (Fig. 1).

Microelectrode measurements revealed extremely steep oxygen gradients in the burrow walls. Variations of these pO_2 gradients in three samples are illustrated in Figure 2. Only about one mm below their wall surface burrows were completely deoxygenated, the brown color yet often occurring at much greater depths of penetration. Since the upper half mm layer was characterized by a viscous consistance due to slime excretions, sampling sites of the burrows were occasionally subdivided into 2 or 3 layers of 0.5 mm thickness each (see A1, A2, A3 in Fig. 2).

Dark fixation of CO_2

Whereas primary production by photoautotrophic organisms is routinely determined, assays of the equivalent processes carried out by chemoautotrophs are most difficult to achieve in natural environments, especially, because heterotrophic CO_2 fixation would interfere. Known inhibitors of Calvin cycle enzymes such as iodoacetamide may not be sufficiently specific at those concentrations required for a complete inactivation of ribulose 1.5 biphosphate carboxylase (RUBPCase, -Reichardt, unpubl.). On the other

hand, direct assays of key enzymes of the Calvin cycle such as RUBPCase would overcome heterotrophic interferences, but no longer allow to differentiate between photo- and chemoautotrophic activities.

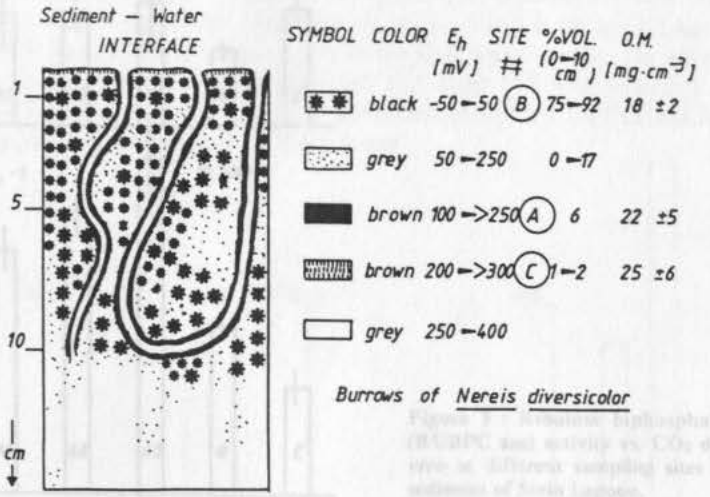

Figure 1 : Stein Lagoon: Schematic illustration of sampling sites A, B and C.
O.M. = organic matter content as ash free dry weight. % of total volume given for 10 cm cores.

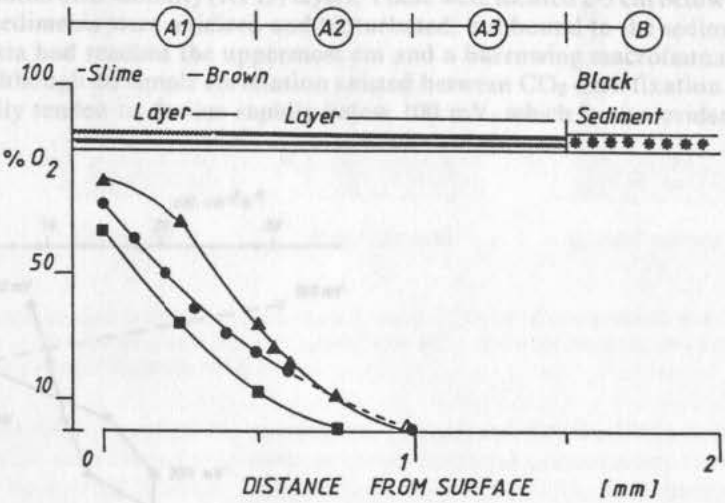

Figure 2 : Oxygen profiles of 3 burrow walls of the polychaete *Nereis diversicolor* from Stein Lagoon sediment. Sampling site A within the brown burrow wall is subdivided into compartments A1, A2 and A3.

In all sediment areas selected for this investigation (Fig. 1) RUBPCase levels showed only minor fluctuations, suggesting an almost evenly distributed capacity to fix carbon dioxide via photo- or chemoautotrophic pathways (Fig.3, upper part). On the other hand, patterns of CO_2 dark fixation *in vivo* were characterized by peaks in the burrow walls and, particularly, in their innermost layer (A3, Fig. 3, lower part).

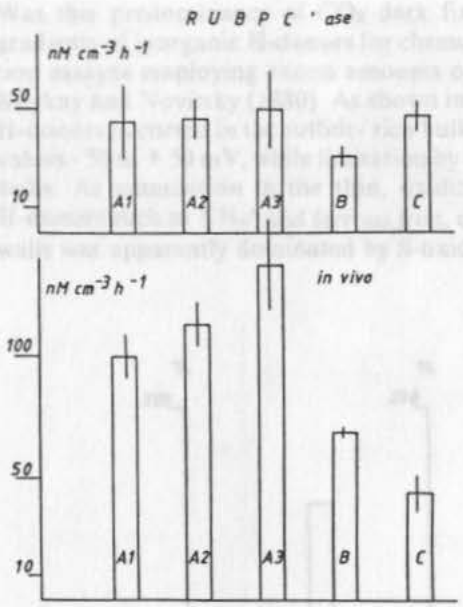

Figure 3 : Ribulose biphosphate carboxylase (RUBPC ase) activity vs. CO_2 dark fixation *in vivo* at different sampling sites in bioturbated sediment of Stein Lagoon.

In vertical profiles from other coastal sediments, CO_2 dark fixation peaked within the redox potential discontinuity (RPD) layers. These were located 2-3 cm below the surface, when the sediments were oxidized and bioturbated, but bound to the sediment surface, when anoxia had reached the uppermost cm and a burrowing macrofauna was absent (Fig. 4). Although no simple correlation existed between CO_2 dark fixation and Eh, the rates usually tended to decline rapidly below 100 mV, which is also evident from this example.

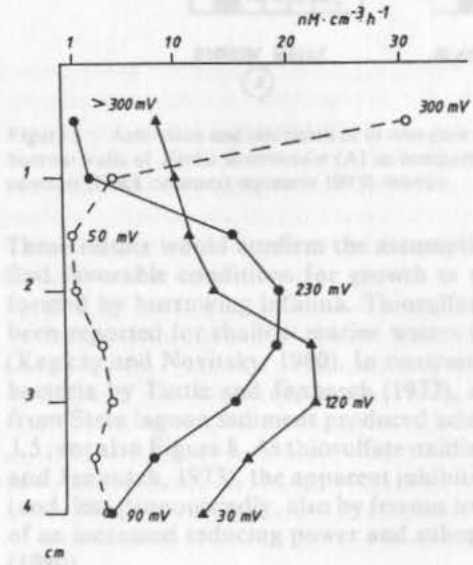

Figure 4 : CO_2 dark fixation in vertical profiles of sediments with and without bioturbation. ● = Kiel Bay at Boknis Eck, 18 m (bioturbated), O = Kiel Bay at Boknis Eck, 28 m (without burrowing infauna), Δ = Limfjorden at Bjornholm Bugt, 1.0 m (bioturbated). E_h values are shown for the depths with maximal and minimal CO_2 fixation rates obtained with core injection technique at *in situ* temperatures in April (O) and August (●, Δ).

Was this predominance of CO_2 dark fixation at intermediate E_h values caused by gradients of inorganic H-donors for chemoautotrophic bacteria ? To check this, «activation assays» employing excess amounts of potential H-donors have first been used by Kepkay and Novitsky (1980). As shown in Figure 5, no stimulation by 8 mM of various H-donors occurred in the sulfide- rich bulk of the sediment from Stein Lagoon (B) at E_h values - 50 to + 50 mV, while limitation by thiosulfate was noted for the adjacent burrow walls. As stimulation in the thin, oxidized top layer (C) was achieved by different H-donors such as NH_4^+ and ferrous iron, chemoautotrophic CO_2 fixation in the burrow walls was apparently dominated by S-oxidizers.

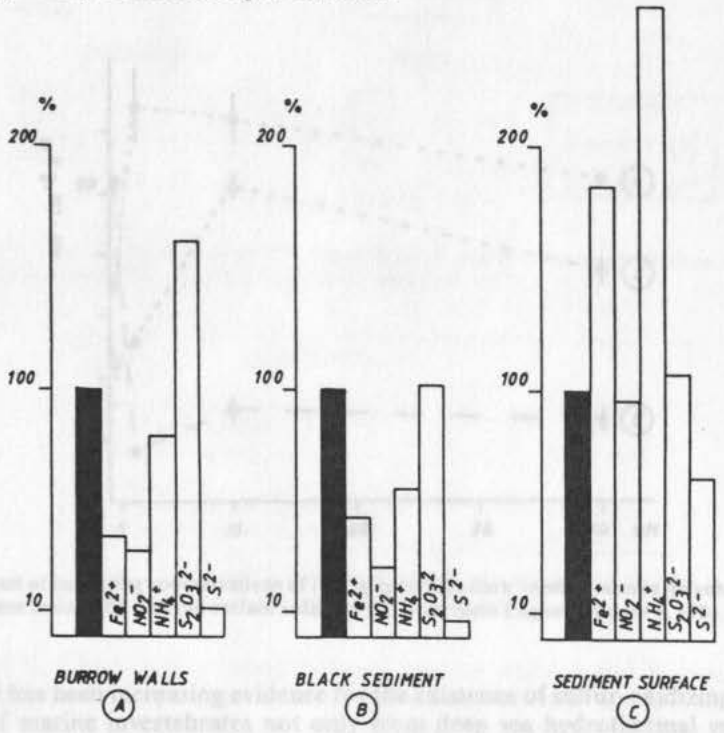

Figure 5 : Activation and inhibition of *in vivo* dark fixation of CO_2 by various potential H-donors (8mM) in burrow walls of *Nereis diversicolor* (A) as compared with adjacent anoxic parts (B) and top sediment (C) controls (Black columns) represent 100 % activity.

These results would confirm the assumption (Yingst and Roads, 1980) that thiobacilli find favorable conditions for growth at redox potential discontinuity layers that are formed by burrowing infauna. Thiosulfate-stimulation of CO_2 dark fixation has also been reported for shallow marine waters (Tuttle and Jannasch, 1977) and marine mud (Kepkay and Novitsky, 1980). In contrast to isolations of thiosulfate-oxidizing marine bacteria by Tuttle and Jannasch (1972), all the isolates obtained by MPN enrichment from Stein lagoon sediment produced acid as indicated by pH values decreasing to 2.6 - 3.5 ; see also Figure 8. As thiosulfate oxidizers may also utilize sulfide as H-donor (Tuttle and Jannasch, 1973), the apparent inhibition of CO_2 dark fixation by additional sulfide (and, less pronouncedly, also by ferrous iron and nitrite) may be interpreted as the result of an increased reducing power and suboptimal E_h according to Kepkay and Novitsky (1980).

Ammonia-stimulation of dark fixation in the oxidized top layer did not necessarily indicate a peak of bacterial nitrification ; for NH_4^+ ions are also known to increase dark CO_2 fixation by marine diatoms (Goldman and Dennett, 1983).

It should be stressed that stimulation of chemoautotrophic CO_2 fixation by potential H-donors provides a useful tool only, if concentrations of the compound added are limiting in the sample. In sediment samples with a low E_h even minor amendments of thiosulfate (1 mM) may cause the reverse effect, as demonstrated by thiosulfate inhibition of CO_2 fixation in sampling site B (Fig.6).

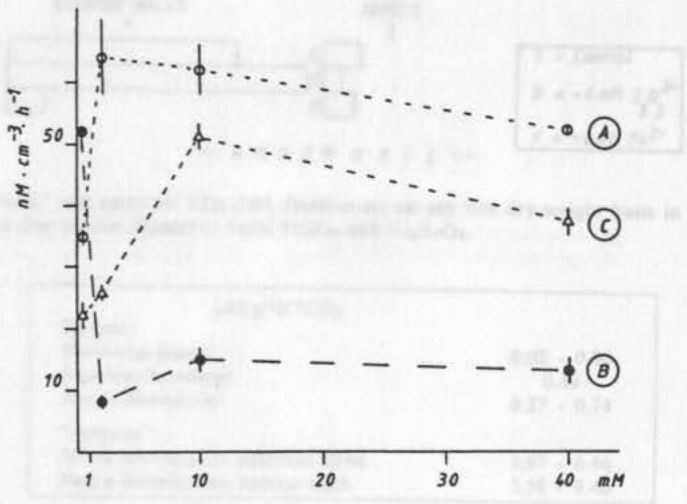

Figure 6 : Impact of increasing concentrations of $Na_2S_2O_2$ on CO_2 dark fixation rates in polychaete burrow walls (A), adjacent anoxic (B), and top surface sediment (C) from Stein Lagoon, August 6, 1984.

Lately, there has been increasing evidence for the existence of sulfur-oxidizing bacterial symbionts of marine invertebrates not only from deep sea hydrothermal vents (f.e. : Cavanaugh *et al.*, 1981 ; Ruby *et al.*, 1981), but also from coastal marine sediments (Cavanaugh, 1983 ; Giere *et al.*, 1982 ; Ott *et al.*, 1982). As yet, it would be premature to interprete the stimulation and enrichment of sulfur-oxidizing bacteria in polychaete burrow walls as evidence for a continuum of sulfur-based energy conservation extending from thiosulfate-oxidizing chemoautotrophs in the burrow walls to epi- and endozoic symbionts of a similar type.

There was no indication that the producer of the burrows, *Nereis diversicolor*, was carrying a substantial amount of epi-or endozoic chemoautotrophs (Fig.7). On an ash-free dry weight basis, CO_2 fixation by this polychaete worm amounted to less than 5 % of the activity detected in the burrow walls. It may be speculated that most of the CO_2 fixation by Nereis was channeled through heterotrophic pathways via anaplerotic enzymes (Hammen and Lum, 1964) and, in particular, via phosphoenolpyruvate carboxykinase (Schöttler and Wienhausen, 1981). Fixation rates obtained from «perizoic» microbiota embedded in slime excretions or in the burrow walls were considerably greater than rates determined in isolated worms including their epi- and endozoic microfloras. (Tab. 1).

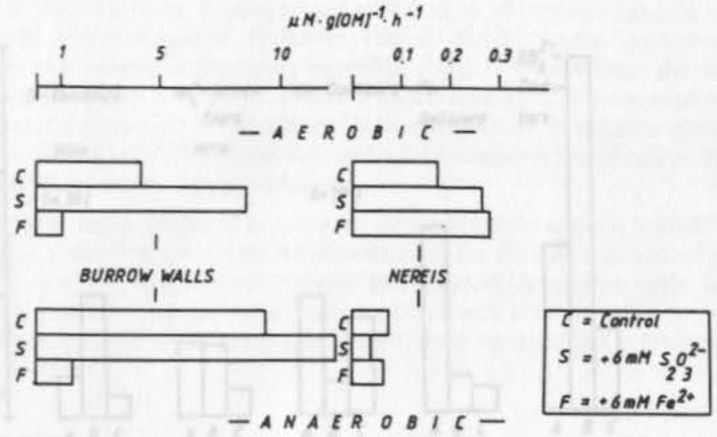

Figure 7 : "Perizoic" and epic-zoic CO_2 dark fixation on an ash free dry weight basis in burrows of the polychaete *Nereis diversicolor*. Impact of 6mM $FeSO_4$ and $Na_2S_2O_3$.

	$\mu M\ g^{-1}h^{-1}CO_2$
Epizoic:	
Pectinaria koreni	0.02 - 0.04
Nepthtys hombergi	0.49
Nereis diversicolor	0.37 - 0.74
"Perizoic":	
Nereis diversicolor, adherent slime	3.97 - 6.46
Nereis diversicolor, burrow walls	3.58 - 9.40

Table 1 : CO_2 dark fixation by various epicoic and pericoic microbiota. Specific rates based on g of ash free dry weight.

Enhanced CO_2 fixation in oxidized infaunal burrow walls should be recognized as a rather widespread phenomenon. F.e., fixation rates in deep sea sediment from the Antarctic Ocean doubled in the area of polychaete burrow walls as compared with the reduced surroundings. (Reichardt, in prep.). However, instantaneous rates of CO_2 fixation along polychaete burrows would have to be much greater than twofold to merely equal CO_2 fixation in the reduced surroundings on a global, volumetric scale. (Just to cause a doubling of CO_2 fixation rates per unit of sediment volume, burrow walls from Stein lagoon representing 6 % of 10 cm cores would have to be about 15 times more active than the surrounding sediment). Despite of these limitations on a larger scale, polychaete burrows in sulfide-rich sediments may still be regarded as the center and starting point of various microbial activities that may easily spread out into surrounding areas. This assumption was supported by supplementary data on viable counts of certain bacteria and *in vitro* enzyme assays.

Viable counts

While boundary layers are expected to constitute an ecological niche for bacteria that depend on the supply of either reduced or oxidized inorganic ions, thiosulfate-oxidizers, ammonia - nitrifyers and manganese-oxidizers were not less abundant in other parts of Stein lagoon sediment than in the burrow walls (Fig. 8). Although these viable counts were no proof of activities *in situ*, their pattern of distribution may still indicate rapid

spreading (by meiofaunal carriers ?) or at least a long continued survival under adverse conditions.

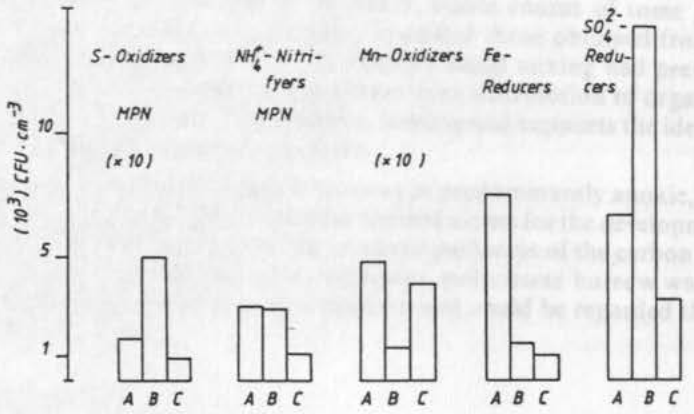

Figure 8 : Viable counts and MPN of anaerobic bacteria (Fe^{3+} and SO_4^{2-} - reducers) and aerobic bacteria ($S_2O_3^{2-}$-oxidizers, NH_4^+-nitrifyers, and Mn-oxidizers) plotted as log of CFU in burrow walls (A) and other parts of bioturbated sediment from Stein Lagoon. Examples from August 15, 1984.

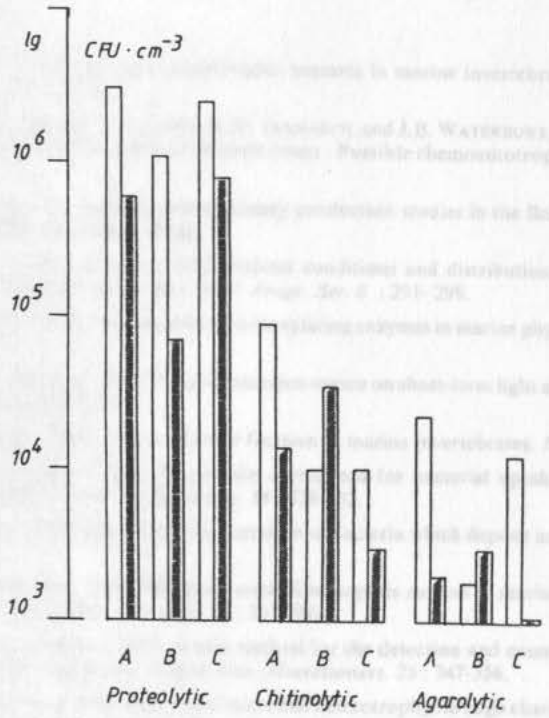

Figure 9 : Abundance (plate counts) of selected groups of bacteria depending on reduced or oxidized inorganic ions in burrow walls and other parts of bioturbated sediment from Stein Lagoon. Example from August 6, 1984.

Due to the feeding habit of *Nereis diversicolor,* particulate organic matter (POM) accumulate in their burrows. Again, strong enrichment of bacteria capable of decomposing this POM was anticipated. However, viable counts of some pertinent groups of bacteria from the burrow walls rarely exceeded those obtained from the reduced bulk sediment (sampling site B. Fig. 9). Possibly rapid mixing had prevented an excessive accumulation of decomposers. An almost even distribution of organic matter (ash-free dry weight) in all areas of the sediment investigated supports the idea that burrow walls were poor barriers for microorganisms.

It was concluded that polychaete burrows in predominantly anoxic, sulfide-rich marine sediments were able to create essential zonated niches for the development of bacteria that play key roles in either anabolic or catabolic pathways of the carbon cycle. In the case of Stein lagoon, a sulfide-rich sandy sediment, polychaete burrow walls turned out to be rather poor barriers for sediment bacteria and could be regarded therefore as an open enrichment system.

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ENZYMATIC POTENTIAL FOR DECOMPOSITION OF DETRITAL BIOPOLYMERS IN SEDIMENTS FROM KIEL BAY

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ABSTRACT

Enzymatic activities involved in the solubilization of particulate organic matter (POM) were determined in the surface samples from coastal sediments of Kiel Bay (Baltic Sea). For this purpose dye release assays were developed and standardized to measure the enzymatic conversion of dye-labelled particulate protein, cellulose, chitin, agar, and algal cell walls to soluble fragments.

On the average, about half of the total POM-solubilizing activities were proteolytic. These were reduced when oxygen availability or Eh became limiting. Seasonal and spatial distribution patterns showed opposite trends for protein- and polysaccharide-solubilizing enzymes (most clearly for cellulase). Among the polysaccharases investigated, agarase activities were consistently predominant. During periods of intensive advection of macroalgae in winter they reached even the level of proteolytic activities. Intermediate levels of activity were noted for stained cell walls as a mixed natural substrate.

Seasonal changes of the enzyme activities during winter and spring were dominated by the impact of a settling phytoplankton bloom at 18 m water depth, whereas samples from the maximal depth (28 m) with similar ATP levels showed extremely low activities. Concluding from the temporal patterns, the half-lives of the enzymes were a few weeks or less. During the main sedimentation event, agar- and cellulose-solubilizing enzymes peaked earlier than the proteolytic enzymes. Time-lags between the maxima of ATP-related and volume-based protease activities suggested particularly strong regulatory impacts on the proteases. The analytical potential of dye-release assays to elucidate rate-controlling factors and kinetic details of POM degradation in marine sediments is discussed.

INTRODUCTION

The energy flow in marine benthic environments is fuelled by the input and degradation of organic matter. These processes have been evaluated in order to describe the cycling of organic carbon in coastal sediments of the Baltic Sea (Smetacek *et al.* 1978, Pollehne 1980, Balzer *et al.* 1985). Biodegradation rates for organic matter were based on concentration changes of dissolved oxygen and nutrient ions in bell-jar experiments and were deduced from flux models (Balzer 1984). These methods were designed to describe only the overall processes of decomposition. Accordingly, they could not provide any background information needed to understand the

kinetics of rate control along the individual biogeochemical pathways. Due to their biocatalytic nature, however, basic kinetic properties should be detectable through enzymatic activities.

Conversion of particulate organic matter (POM) to dissolved organic matter (DOM) comprises the first major change of the spectrum of organic compounds during decomposition. This process is catalyzed by extracellular endo-enzymes and exo-enzymes. With their random cleavage of internal bonds of their particulate substrates, endo-enzymes produce soluble fragments which may subsequently serve as substrates for exo-enzymes. Hence, the potential for controlling the overall rates of POM-decomposition may be attributed to these endo-enzymes (Linkins *et al.* 1984).

Routine assays for individual polysaccharide- or protein-degrading enzymes fail to distinguish unequivocally between random (= endo-enzymatic) and terminal (= exo-enzymatic) bond cleavages. As a first approximation, however, dye release from dye-labelled particulate substrates (such as cellulose) may largely reflect endo-enzymatic activities (or endo-cellulases; Leisola & Linko 1976). In addition to this catalytic specificity, solubilization of particulate biopolymers may increase decomposition rates by several orders of magnitude (Reese 1977).

It has to be found out yet, if the enzymes responsible for such rate-changing steps can be adequately determined in marine sediments by using dye-labelled substrates as substitutes for either individual constituents or the total of detrital POM. In contrast to terrestrial soils (Burns 1978) levels of POM-solubilizing enzymes have scarcely been investigated in marine sediments (Kim & ZoBell 1974, Lackland *et al.* 1982, Vance *et al.* 1982). To develop a methodological basis for dye-release assays in sediments, sorption of enzymes and their products as well as dosage and grain size of the substrate particles should be addressed in the first place. Since decomposition of POM depends on the presence of depolymerizing enzymes for a variety of chemically defined compounds, differentiation by sub-

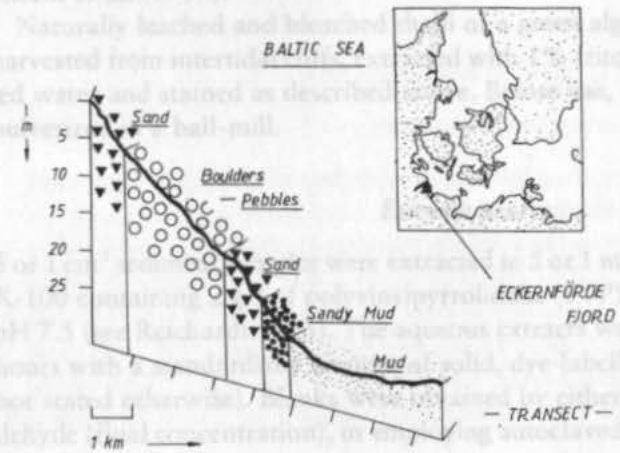

Fig. 1. Schematic diagram of sampling sites in Kiel Bay and transect at Eckernförde Fjord.

strate-specific solubilization rates was desirable. On the other hand, solubilization rates for natural mixtures of detrital biopolymers promised more realistic results. Therefore stained algal cell walls were included as a model substrate for natural detritus particles.

A series of enzyme analyses in the topmost sediment layers was designed to reveal potential enzymatic responses during periods with extremely changing input of POM (Smetacek 1980). To compare temporal or spatial patterns of activities with pertinent geochemical parameters, an intensively investigated research area of Kiel Bay was chosen as sampling site (Schulz 1983, Meyer-Reil 1983, Balzer 1984).

MATERIALS AND METHODS

Sampling sites

Box-core samples (Reineck grab) were obtained from sediments of several near-shore stations of Kiel Bay (Baltic Sea). A transect close to the inlet of Eckernförde Fjord was chosen to study horizontal distribution patterns in different types of sediment (Fig. 1). Two stations (at 18 and 28 m water depth) located nearby in an area restricted for research of the University of Kiel (Wefer 1974) served to investigate seasonal changes.

Substrates for dye-release assays

Dye-labelled particulate protease substrate (commercial 'Hide Powder Azure') was obtained from Sigma Chemicals. Substrates for cellulase, chitinase, and agarase were prepared by staining 'Avicel'-cellulose, highly purified chitin (Hercules Inc. Res. Center, Wilmington, Delaware), and agar (Merck), respectively, with remazolbrilliantblue R (Höchst), using the procedure of Stamm (1963) and Rinderknecht *et al.* (1967).

Naturally leached and bleached thalli of a green alga, *Enteromorpha* sp., were harvested from intertidal cliffs, extracted with 1% triton X-100 plus boiling distilled water and stained as described above. Before use, all of these substrates were pulverized in a ball-mill.

Enzyme assays

5 or 1 cm^3 sediment samples were extracted in 5 or 1 ml, respectively, of 2% triton X-100 containing 2 g/l of polyvinylpyrrolidone (PVP) in 0.01 M tris-HCl buffer, pH 7.5 (see Reichardt 1973). The aqueous extracts were incubated for usually 20 hours with a standardized amount of solid, dye-labelled biopolymer (7 mg/ml, if not stated otherwise). Blanks were obtained by either addition of 5% of formaldehyde (final concentration), or employing autoclaved aliquots of the sample. The

incubated extracts were centrifuged (15 min at 6000 g) and the absorbance of the soluble stained hydrolyzate was determined at 600 nm wavelength (Zeiss PMQ-spectrophotometer).

Activity units of the solubilizing enzymes were expressed as $\mu g \cdot h^{-1} \cdot cm^{-3}$ and were calculated according to the following formula.

$$\text{Activity unit } (\mu g \cdot h^{-1} \cdot cm^{-3}) = (10^3 \cdot \text{absorbance} \cdot F \cdot V_a)/(V_s \cdot t)$$

with

F = substrate-specific conversion factor ($\mu g \cdot l^{-1}$)
V_a = total assay volume (l)
V_s = volume of sediment sample used for assay (cm^3)
t = incubation time (h).

To convert the absorbance reading into quantities of solubilized POM, the dye labels of the stained particulate substrates were determined photometrically after complete enzymatic or acid (6 N HCl) hydrolysis. Absorbance readings (600 nm) of the completely solubilized substrates led to the following conversion factors:

Protease substrate:	4557 $\mu g \cdot l^{-1}$
Chitinase substrate:	844 $\mu g \cdot l^{-1}$
Cellulase substrate:	1789 $\mu g \cdot l^{-1}$
Agarase substrate:	4650 $\mu g \cdot l^{-1}$
Enteromorpha substrate:	1714 $\mu g \cdot l^{-1}$

Triplicate assays gave average coefficients of variation ($S \times 100/x$) of 5.6%.

ATP concentrations in the sediments were obtained from Schulz (1983). As an estimate of the organic matter content (OM), ash-free dry weight determinations were carried out after combustion of the dried sediment at 550°C. Viable plate counts of proteolytic and chitinolytic bacteria were determined according to Reichardt 1978.

RESULTS AND DISCUSSION

Methodological aspects

Staining of insoluble biopolymers or detrital particles with reactive dyes has opened up an almost unlimited source of potential substrates for enzymes involved in the solubilization of both chemically simple and complex POM (Hagen *et al.* 1966, Rinderknecht *et al.* 1968, Leisola & Linko 1976). A disadvantage of dye-labelled biopolymers is their reduced accessibility to enzymatic degradation in comparison with their unstained counterparts. On the other hand, their contribution to a wide spectrum of rapid and simple enzyme assays remains unmatched by alternative substrates which are used for viscosimetric or radiotracer techniques (Hagen *et al.*

1966, Vance *et al.* 1982, Reichardt in prep.). Currently, examples for their use in aquatic environments are rather scarce and largely lacking an adequate critical evaluation (Kim & ZoBell 1974, Little *et al.* 1979, Meyer-Reil 1983).

In sediments, assay efficiencies can be strongly reduced by adsorption to particles of a) the enzymes and b) their solubilized products. In 'one-step' assays (without removal of the sediment after the extraction was completed) adsorption effects could be partly reversed by detergents such as triton X-100. Homogenizing the sediment (with an agate mortar, 'Pulverisette') did not always increase the efficiency of extraction. Rather the opposite effect was observed in protease assays (Fig. 2), where homogenized samples produced lower activities, irrespective of the addition of triton X-100. This could possibly be explained by re-adsorption or masking of the extracted enzyme molecules in the presence of the pulverized sediment particles.

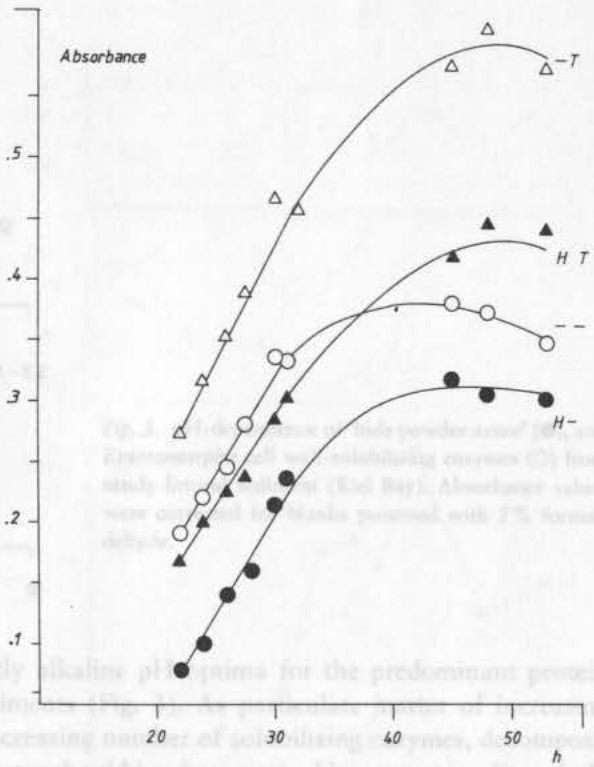

Fig. 2. Time course of formation of dissolved stained products (absorbance) from protease substrate 'hide powder azure' (Sigma) in sandy beach sediments of Kiel Bay. Diluted 1:3 in artificial sea water; with homogenization in agate mortar (H) and/or extraction with 2% triton X-100 (T), at 20°C.

Dyed products of proteolysis were subject to strong adsorption in native or autoclaved sediments, whereas adsorption became negligible in the presence of combusted sediment stripped of its organic coating (Reichardt, in prep.). It seems that humic substances which may account for the bulk of organic coating of the sediment particles, have a strong impact on sorption of both enzymes and their products (Ladd & Butler 1975, Linkins *et al.* 1984, Martin *et al.* 1978).

In an effort to standardize the assays, saturation of protease-solubilizing enzymes was not achieved within a manageable range of substrate concentrations (100 g/l). To allow comparisons among different substrates, standardized quantities (usually 7 mg/ml) were employed. This permitted absorbance readings of the soluble products within 20 h of incubation at 20 °C.

pH-dependence of POM-Solubilizing Enzymes

Individual enzymes involved in the decomposition of polysaccharides and proteins are characterized by a wide range of pH optima (Barman 1969, Boyer 1971). Therefore the average pH of the sediments was taken as an arbitrary assay standard.

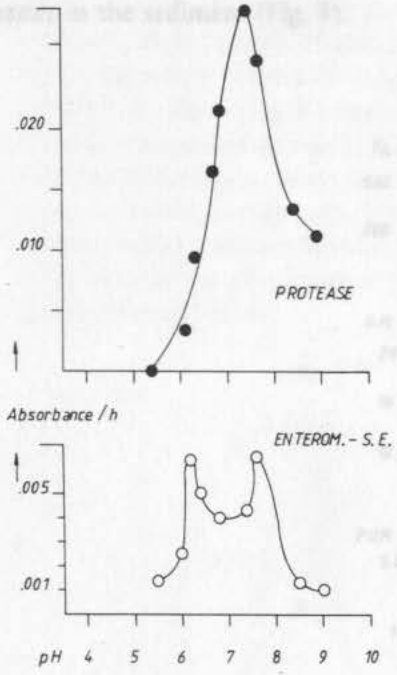

Fig. 3. pH-dependence of 'hide powder azure' (●), and *Enteromorpha* cell wall-solubilizing enzymes (○) from sandy littoral sediment (Kiel Bay). Absorbance values were corrected for blanks poisoned with 5% formaldehyde.

pH spectra showed slightly alkaline pH optima for the predominant protein solubilizing enzymes in sediments (Fig. 3). As particulate matter of increasing heterogeneity required an increasing number of solubilizing enzymes, decomposition of more complex substrates should be characterized by more complicated pH spectra (see above). This was confirmed for the chemically complex substrate prepared from green algal cell walls. The pH spectrum for sediment enzymes which solubilized *Enteromorpha* cell walls was characterized by two distinct peaks, one presumably due to protease (in the alkaline range) and one at pH 6, which corresponded to that of cellulase from salt marsh sediments (Lackland *et al.* 1982).

Activity patterns during a spring bloom of phytoplankton

Activities of different POM-solubilizing enzymes (POM-SE) in the surface layer (0-1 cm) of nearshore Kiel Bay sediments at 18 m water depth showed seasonal fluctuations similar to the concentrations of total organic matter (OM). In particular, sedimentation of a phytoplankton bloom between 10 and 17 March 1982 (with an ATP-based biomass of 4 $\mu g \cdot l^{-1}$ in the upper 7.5 m of the water column according to Schulz 1983) was reflected by POM-SE in the sediment. An instantaneous response to the bulk input of POM was noted for agarase and cellulase, whereas protease, chitinase, and *Enteromorpha* cell wall-solubilizing enzymes (ECW-SE) peaked somewhat later, i.e. simultaneously with the peak of the organic matter in the sediment (Fig. 4).

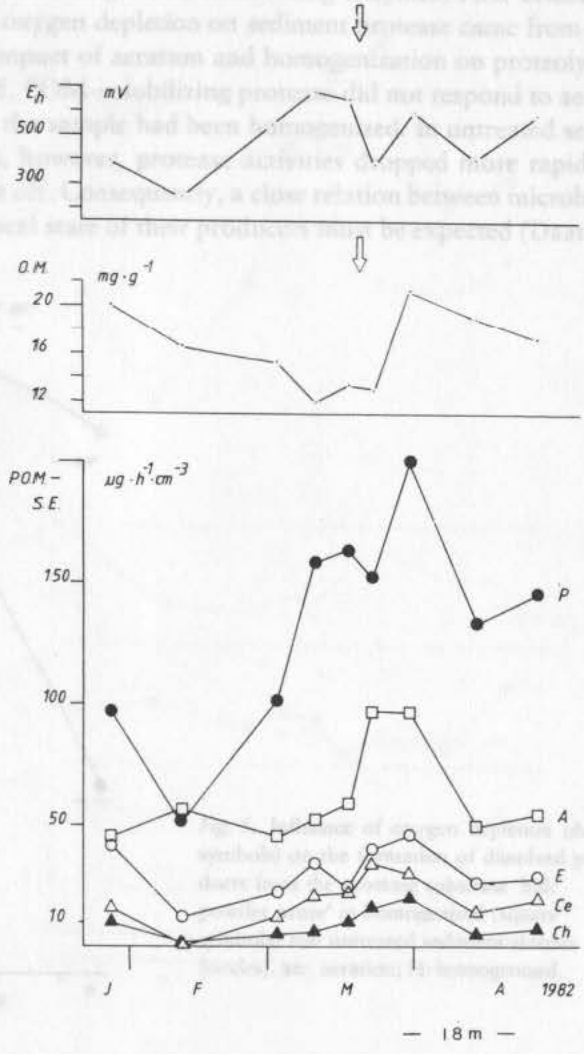

Fig. 4. Temporal fluctuations of redox potential (Eh), total organic matter content (OM), and POM-solubilizing enzymes at the sediment surface (0-1 cm) at 18 m water depth in Eckernförde Fjord during winter and spring. P: protease; A: agarase; E: ECW-SE; Ce: cellulase; Ch: chitinase.

Temporal fluctuations during winter and spring suggested a relatively short half-life (of less than two weeks), especially for the polysaccharases. After the main sedimentation event, these enzyme activities decreased at a quicker rate than the pool of total organic matter.

Maximal activities were noted for protease. As compared with the polysaccharases, enzymes capable of solubilizing detrital particulate protein were most closely correlated with the redox potentials. A close correlation with Eh was also noted for enzyme activities involved in the solubilization of algal cell walls.

Sensitivity to oxygen depletion

These correlations led to the question how far physico-chemical conditions in the sediment were capable of controlling POM-solubilizing enzymes. First evidence for a detrimental impact of oxygen depletion on sediment protease came from an experiment addressing the impact of aeration and homogenization on proteolytic activities. As shown in Fig. 5, POM-solubilizing protease did not respond to aeration or O_2-depletion, when the sample had been homogenized. In untreated sediment containing living cells, however, protease activities dropped more rapidly, when aeration had been shut off. Consequently, a close relation between microbial proteases and the physiological state of their producers must be expected (Daatselaar & Harder 1974).

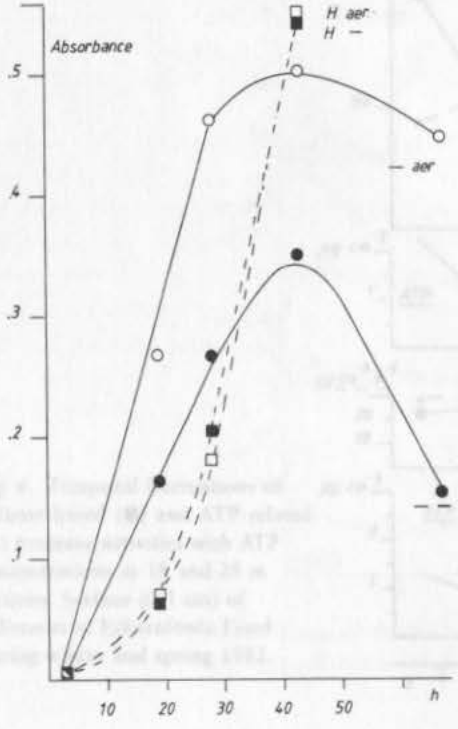

Fig. 5. Influence of oxygen depletion (dark symbols) on the formation of dissolved products from the protease substrate 'hide powder azure' in homogenized (square symbols) and untreated sediment slurries (circles). aer: aeration; H: homogenized.

Relationship to biomass parameters

POM-solubilizing enzymes are extracellular. Hence, a positive correlation with biomass data would disappear, when the enzymes 'outlive' their producers.

Temporal (weekly) changes of enzyme activities at two stations (sandy sediment at 18 m, muddy sediment at 28 m) were compared with those of ATP biomass equivalents. At both stations (volume-based) protease activities and ATP biomass equivalents coincided over a considerable stretch of time at both stations (Fig. 6). This would support the view that proteins are degraded by organism- (bacteria-) associated enzymes (Hollibaugh & Azam 1983). On the other hand, protease activities at 18 and 28 m differed by a factor of about 10, whereas ATP concentrations were in the same range.

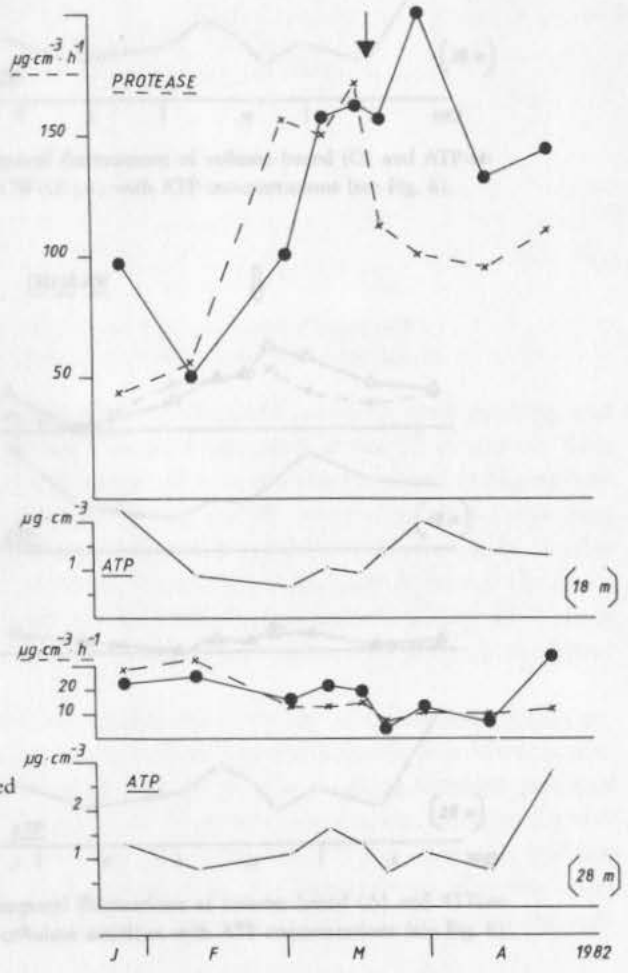

Fig. 6. Temporal fluctuations of volume-based (●) and ATP-related (×) protease activities with ATP concentrations at 18 and 28 m stations. Surface (0-1 cm) of sediments in Eckernförde Fjord during winter and spring 1982.

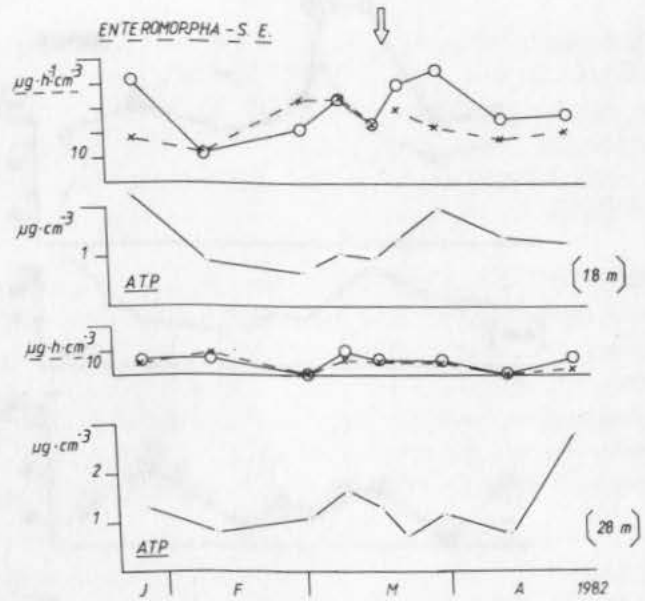

Fig. 7. Temporal fluctuations of volume-based (○) and ATP-related ECW-SE (×) with ATP concentrations (see Fig. 6).

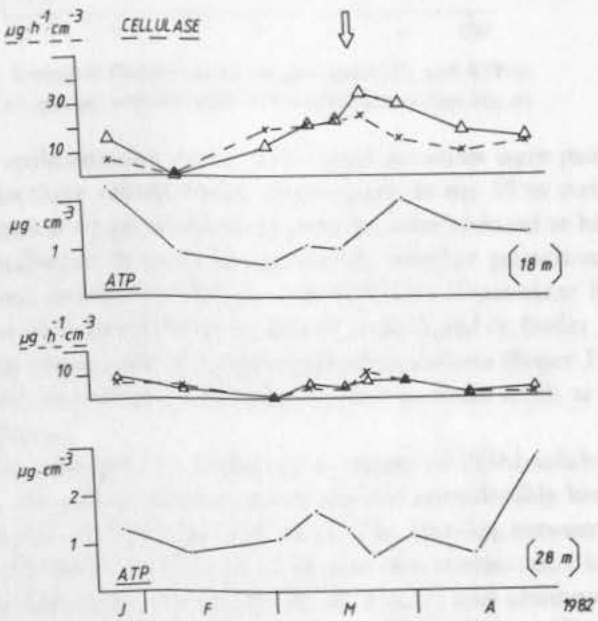

Fig. 8. Temporal fluctuations of volume-based (△) and ATP-related (×) cellulase activities with ATP concentrations (see Fig. 6).

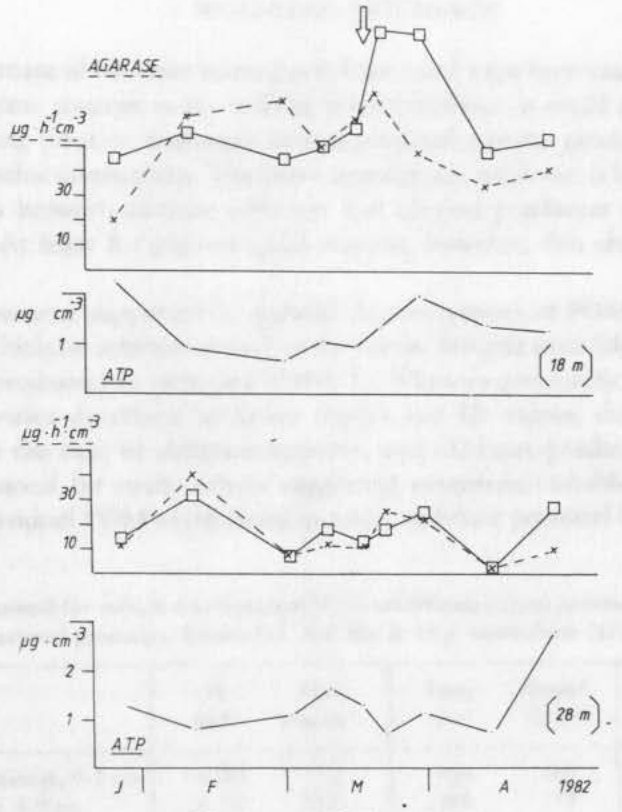

Fig. 9. Temporal fluctuations of volume-based (□) and ATP-related (×) agarase activities with ATP concentrations (see Fig. 6).

During the main sedimentation event, ATP-based activities were peaking and declining earlier than their volume-based counterparts at the 18 m station. This indicated that production or preservation of protease were reduced at higher biomass levels in the sediment. It could be speculated, whether growth-supporting compounds had acted as inhibitors of protease synthesis (Daatselaar & Harder 1974). Other factors such as inhibition by humic acids (Ladd & Butler 1975), or removal by chelating compounds of activating divalent cations (Boyer 1971) may be considered as well, to explain the exorbitantly low protease levels at the lower end of the slope (28 m).

Could protease be regarded as a model for a variety of POM-solubilizing enzymes? In fact, also the polysaccharases tested showed considerably lower activities at the 28 m station as compared with 18 m. The time-lag between peaks of biomass- and volume-based activities at 18 m was also conspicuous for *Enteromorpha* cell wall-solubilizing enzymes (ECW-SE, Fig. 7) and chitinase, but less evident for cellulase (Fig. 8) and agarase (Fig. 9). Nevertheless, also the latter enzymes were characterized by decreasing biomass-related activities after the sedimentation event.

This decrease of biomass-related activities could have been caused by exhaustion of the enzyme sources in the settling phytoplankton. It could also have resulted from missing positive responses among potential enzyme producers in the endogenous benthic community. The latter assumption, however, is based on a positive correlation between enzyme activities and enzyme producers (such as sediment bacteria). At least for physiological reasons, however, this should rather be an exception.

This view was supported by parallel determinations of POM-solubilizing protease and chitinase activities as well as the colony-forming units (cfu) of their potential bacterial producers in sediment (Table 1). Whereas proteolytic bacteria and protease activities decreased at lower depths and Eh values, this correlation was missing in the case of chitinase activities and chitinase-producing bacteria. This example stood for many others suggesting extremely variable relationships between individual POM-solubilizing enzymes and their potential bacterial producers *in situ*.

Table 1. Example for vertical distribution of POM-solubilizing enzyme activities and CFU of pertinent bacterial producers, Boknis Eck, Kiel Bay at 18 m water depth (13 April 1985).

	Eh (mV)	OM (mg/cm^3)	Protease*	Proteol. CFU**	Chitinase*	Chitinolyt. CFU**
Surface sediment, 0-2 cm	+280	35.2	429	100	137	20
RPD-layer, 4-9 cm	+ 90	41.0	269	77	114	4
Reduced zone, >9 cm	0	45.9	91	20	139	2

*$\mu g \cdot h^{-1} \cdot cm^{-3}$. **CFU $\times 10^3 \cdot cm^{-3}$.

Impact of water depth

Besides the described temporal fluctuations, basic differences were noted between the enzyme activities in the middle (18 m) and the lower end of the slope (28 m). Were these differences reflecting a continuous decrease of enzymatic decomposition of POM down the slope? How were POM-SE related to accumulation rates for organic matter? For a transect orthogonal to the shoreline at the south-western margin of the Kiel Bay and extending to the maximal depth of 29 m, accumulation rates for organic carbon were characterized roughly by a 20-fold increase from 20 to 28 m (Balzer *et al.* 1985).

Along a parallel transect (Fig. 1), selected activities of POM-SE in the top sediment layer (0-1 cm) were determined in June 1985. In contrast to the activity distribution described for winter and spring, cellulase peaks occurred at 28 m rather than at 18 m (Fig. 10). The enzymatic potential to solubilize cellulose particles increased down the slope of the transect and matched also with the organic matter content. On the other hand, the enzymatic potential to solubilize

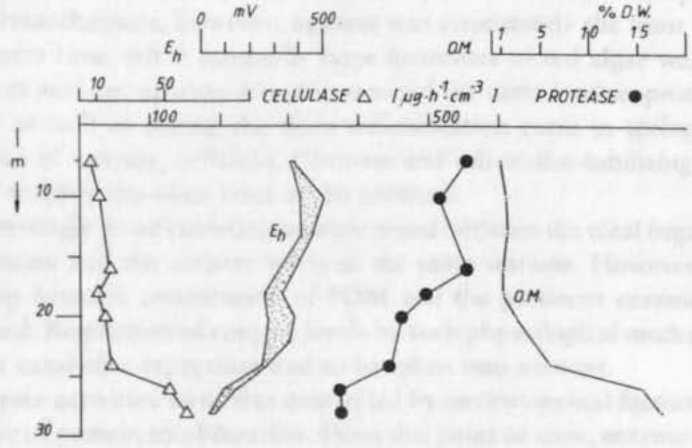

Fig. 10. Spatial distribution of POM-SE activities of cellulase (△) and protease (●) in sediments (0-1 cm) along transect in Eckernförde Fjord (Kiel Bay), with redox potentials (Eh) and concentrations of organic matter (OM).

protein particles declined towards the maximal depth and corresponded with the decreasing tendency of the redox potential. This decrease of protease activities towards the maximal depth was consistent during all seasons (1981-82; Reichardt, unpublished). In conclusion, the most efficient activities of POM-solubilization and the accumulation rates for organic carbon followed opposite trends.

Conclusions

When determined under reproducible, standardized conditions, enzyme activities can also be regarded as a measure of enzyme concentrations. The described assays for POM-solubilizing sediment enzymes reflected degradation capacities. By using sediment extracts, physico-chemical control mechanisms which may have been effective *in situ*, were excluded. The specificity of the assays depended on the purity of the substrates employed. Staining with covalently bound dyes lowered the solubilization rates, but was not expected to change the specificity (Hagen *et al.* 1966).

Due to the heterogeneity of marine POM, the process of POM-solubilization should be resolved into single reactions. Since roughly 50% of autotrophically produced organic matter constitute proteinaceous compounds (Little *et al.* 1979), the high degradation rates obtained for the particulate scleroprotein substrate (hide powder) were to be expected. The derivative of a natural mixed substrate representing structural green algal polymers, brought about the most realistic simulation of POM-degradation *in situ*. Its pH spectra and its negative correlation with Eh confirmed that it contained also proteinaceous constituents. Enzymes solubilizing

single polysaccharides showed considerably lower activities than protease. Among the polysaccharases, however, agarase was consistently the most pre-eminent. In the winter time, when unusually large quantities of red algae were found on the sediment surface, agarase activities reached the same level as protease (Fig. 4). In winter as well as during the main sedimentation event in spring, the combined activities of agarase, cellulase, chitinase and cell-wall-solubilizing enzymes maintained roughly the same level as the protease.

Surprisingly good correlations were noted between the total organic matter concentrations and the activity levels at the same stations. However, a simple relationship between constituents of POM and the pertinent enzymes could not be expected. Regulation of enzyme levels by such physiological mechanisms as induction or catabolite repression had to be taken into account.

Enzyme activities were also controlled by environmental factors such as pO_2 in the case of protein solubilization. From this point of view, enzyme activities would gain indicator properties for the degradative response of the enzyme producing biota. Accumulation of organic matter at the lower end of the transect (Balzer *et al.* 1985) may, at least partially, be explained by the drastically reduced protease levels.

The production of inducible enzymes degrading relatively recalcitrant POM is usually stimulated, when easily degradable organic matter becomes less available (Reese 1977). Therefore, increased levels of cellulase have been suggested as a quality indicator for the decomposition of organic matter in salt marshes (Lackland *et al.* 1982). In Kiel Bay sediment, high cellulase activities occurred together with high levels of total organic matter, but low protease activities (Fig. 10). In terms of enzymatic degradation capacities, this indicated a reversal of usual sequences of organic matter decomposition (i.e. protein is degraded more rapidly than cellulose). Other inducible polysaccharidases may be useful indicators for changing inputs from defined sources of organic matter as e.g. advected macroalgae. In this context, the coincidence of high agarase levels and accumulation of red algae deserves further investigations.

The data referred to samples from the sediment surface (0-1 cm). This choice appeared justified, because the major processes of organic matter decomposition were assumed to proceed near the sediment surface (Müller & Suess 1979, Balzer 1984). It must be emphasized, however, that vertical activity gradients may not develop because of an intensive shifting of sediment material in the tidal zone, or due to bioturbation by the burrowing macrofauna (Reichardt, in prep.).

If solubilization of POM is a rate-limiting step in the decomposition of organic matter, major terminal processes of mineralization should follow the same trend. In fact, the *in situ* O_2-consumption rates reported from bell-jar experiments located near the described sampling sites followed largely the same seasonal trends as the enzyme activities (Balzer *et al.* 1985).

Dye-release assays for POM-solubilizing enzymes are particularly attractive

because of their simplicity and wide range of potential substrates. For a final evaluation of their significance in marine biology, however, further calibration with more established techniques is needed (Reichardt, in prep.). Also, more extensive efforts to standardize the techniques will be necessary in order to compare enzyme activities from different types of sediments.

The sedimentation regime of Kiel Bay offered almost ideal conditions to investigate the ecological significance of the described enzyme assays. These cannot replace *in situ* rate measurements of POM-decomposition, but they provide useful information about the enzyme degradation potential, its quality and its control by environmental parameters.

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Measurement of enzymatic solubilization of P.O.M. in marine sediments by using dye release - techniques

by

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Abstract

Dye-labeling of structural biopolymers with "reactive" covalently bound dyes is a simple method to modify substrates for measuring the enzymatic solubilization of P.O.M.. It is applicable to pure polysaccharides and proteins as well as mixed detrital compounds. Increases of absorbance resulting from the release of soluble stained hydrolyzates served as a measure of the largely endoenzymatic P.O.M.-solubilizing activities which are likely to play a key role in benthic carbon re-cycling.

Dye release from a commercial scleroprotease substrate was characterized by the absence of substrate saturation and a strong inhibition at larger particle sizes. Autoclaved sediment particles (in contrast to ashed sediment) were effective adsorbents for both enzyme and product molecules. Minimal grain sizes (silt) caused maximal inhibition of the enzymatic dye release. An extraction - desorption procedure based on tritonx100 played a crucial role in standardizing activity measurements of marine sediments.

Activity peaks during the sedimentation of a plankton bloom raised the question where these benthic POM-solubilizing enzymes originated from. Sedimentation experiments with an input of enzyme-free P.O.M. suggested the absence of short-term benthic responses.

Key words: Marine sediment; particulate organic matter; decomposition; sorption; enzyme activity; methodology

Scope of the investigation

Where marine benthic ecosystems depend largely on the input of organic detritus sedimenting from the water column, conversion of particulate into dissolved organic matter comprises the primary steps in benthic carbon and energy flows. To find out to what extent these are rate-limiting steps in benthic carbon cycling, it is necessary to measure either the solubilization rates for P.O.M., or enzymatic activities that are responsible for these processes (Fig. 1, Linkins et al., 1984; Meyer-Reil; Reichardt, in prep).

Techniques which may be used to determine P.O.M.-solubilizing enzyme activites in sediments are summarized in Table 1. Besides direct detection and quantification of the dissolved decomposition products, there are a few, less specific techniques such as measuring weight losses of the substrate, viscosimetry, or use of substrates that are labeled with either radiosotopes or reactive dyes.

Since endo-enzymes degrade solid biopolymers into relatively large dissolved molecules, their relative importance as P.O.M.-solubilizing catalysts is most likely to exceed considerably the importance of exo-enzymes (which, by definition, attack the polymers at their ends, (Fig. 1). Yet, there is no rapid method to measure exclusively endoenzymatic polysaccharases or proteases in sediments. Nevertheless, among the few non-specific assay procedures dye-release techniques measure largely endo-enzymatic solubilization of biopolymers (Tab. 1).

The current number of commercially available structural biopolymers labeled with covalently bound dyes may be considerably enlarged. Chemically less defined mixed substrates representing detrital biopolymers may be prepared by using the basic procedure for reactive dye-staining (Stamm 1963; Rinderknecht et al., 1968). First experiences with new labeled substrates for POM-solubilizing enzymes will be described. Finally, dye release techniques were tested as a tool to understand the mechanisms which control the breakdown of detritus particles following major sedimentation events.

Due to the odd size relationship between POM-solubilizing enzymes and their solid substrates, it is the smaller enzyme molecule which is mobilized and

adsorbed. In the presence of inorganic sediment particles, sorption processes are likely to become more complicated and may strongly affect the reaction kinetics (Fig. 2). Due to their usually high sorption capacities, sediments are likely to present most unfavourable conditions for measuring the conversion of particulate to dissolved organic matter, (f.e. Burns, 1980).To get an idea of the impact of sorption in sediment samples, selected aspects were investigated by using either dye-labeled scleroprotein or algal polymers as substrates for sediment-extracted or commercial protease.

Methods

Dye-labeled scleroprotein substrate was obtained from Sigma ("hide powder azure"). Dye-labeled "Avicel"-cellulose, highly purified chitin (Hercules Inc. Res. Center, Wilmington, Delaware), and agar (Merck) were stained with remazol brilliant blue R. (Höchst) according to Stamm, 1963, and Rinderknecht et al., 1967. Naturally leached and bleached thalli of the green alga Enteromorpha sp. were harvested from intertidal rocks, extracted with 1 % triton X-100 (Serva) in boiling distilled water and subsequently stained as described above. This mixed substrate will be referred to as "Enteromorpha substrate". It contained 60 % carbohydrates and reflected both cellulase and protease activities. All substrates were ground in a ball mill and sieved through a set of steel sieves with mesh sizes ranging from 1 000 to 63 µm. If not stated otherwise, the fraction smaller than 63 µm was used.

Bacillus subtilis - protease was obtained from Sigma (P5380) and used in 100 µl aliquots of 2 mg/ml solutions in distilled water. POM-solubilizing enzymes were extracted from 100 cm^3 of Kiel Bay sediments with 200 ml ice cold 15 o/oo arteficial seawater containing (usually) 2 % tritonx100 and 2 g/l polyvinylpyrrolidone in 200 mM tris-HCl buffer, pH 7.5. If not stated otherwise, the suspensions were shaken in the cold for 30' and subsequently centrifuged (6000 g). 2 ml aliquots of the supernatants were used per assay in 10 ml polystyrene centrifuge tubes containing weighed amounts (0.5 - 100 mg/ml) of a defined size fraction of the substrate. Blanks received 5 % formaldehyde (final concentration) prior to incubation at 20 ° C for 2-20 h on a shaking machine. After terminating the

incubation with 5 % formaldehyde (final concentration) and centrifugation (6000 g) the absorbance in the 3 ml-supernatants was measured at 600 nm wavelenght using a Zeiss PMQ spectrophotometer (1 cm).
Activity units of the solubilizing enzymes were expressed as $\mu g\ h^{-1}\ cm^{-3}$ and were calculated according to the following formula:

$$\text{Activity unit } (\mu g\ h^{-1}\ cm^{-3}) = \frac{10^{-3} \cdot \text{absorbance} \cdot F \cdot V_a}{V_s \cdot t}$$

with F = substrate specific conversion factor ($\mu g\ l^{-1}$)
V = total assay volume (l)
V = Volume of sediment sample used for assay (cm^3)
t = incubation time (h).

To convert the absorbance readings into quantities of solubilized POM, the dye labels of the stained particulate substrates were determined photometrically after complete enzymatic or acid (6 N HCL) hydrolysis. Absorbance readings (600 nm) of the completely solubilized substrates led to the following conversion factors: protease substrate: 4557 $\mu g\ l^{-1}$; chitinase substrate: 844 $\mu g\ l^{-1}$; cellulase substrate: 1789 $\mu g\ l^{-1}$; agarase substrate: 4650 $\mu g\ l^{-1}$; Enteromorpha substrate: 1714 $\mu g\ l^{-1}$.
Soluble degradation products for adsorption tests were prepared enzymatically and subsequently autoclaved (30', 120 °C).

Sediment to be used in the sorption studies was from different locations in Kiel Bay. It was pooled, autoclaved (120 °C for 30 '), dried at 70 °C and sieved through a steel sieve (1000 - 63 µm mesh sizes). A fraction of it was ashed (550 °C for 24 h) prior to sieving.

Total dissolved carbohydrates were determined using the phenol-H_2SO_4 - method (Herbert et al., 1971).

Results and Discussion

Limitation by substrate concentrations and grain sizes

Enzyme levels as reflected by their activities are usually measured under optimal or standardized conditions that ensure saturation with the substrate. This prerequisite, however, can hardly be reached with certain

substrates, as for example the commercially available (Sigma) "hide powder azure" which is used to measure enzymatic solubilization of scleroprotein particles. Up to a concentration of 100 g l^{-1} - beyond which the assay was no longer feasible for technical reasons - activities were limited by the concentration of the substrate (Fig. 3).

This phenomenon may be explained by the reduced molecular activity of the enzymes (bonds hydrolyzed per enzyme molecule per min), when particulate instead of dissolved substrates are attacked. Corresponding with an increased surface: volume ratio for smaller substrate particles, enzymatic solubilization of particles was a function of particle sizes. It declined by a factor of 4 over a range of particle sizes from 63 µm to 1000 µm (Fig. 4). The relative increase of measured activities after treatment with 1.5 % triton x-100 indicated a certain degree of (re-) adsorption of the soluble degradation products.

This limitation of proteolytic activities by the size of substrate particles was also evident from the apparent k_M - values. Substrate affinities (plotted as $1/k_M$) decreased exponentially by a factor of 8.3 (Fig. 5), when the size of the substrate particles was shifted from 63 to 5000 µm. Hence, a strong reduction of attachment sites for the solubilizing enzyme must be anticipated, when particle sizes reach a diameter of 1 mm.

Interactions of enzymes and sediment particles

Activity measurements in sediments are further complicated by kinetic changes as a result of largely non-competitive inhibition by inorganic sediment particles. For example, 1 g (dry weight) of autoclaved sediment per 2 ml assay solution caused marked changes of the activity of Bacillus subtilis protease as a function of substrate concentrations (Fig. 6). In order to obtain the same activity in a silty sediment as in the water column, enzyme levels would have to be higher by more than one order of magnitude. This methodological obstacle may be overcome using only sediment-free extracts for the assay.
A sufficient knowlegde of enzyme sorption to particles is required, when enzyme activities from different types of sediment are to be compared. Small silt particles may reduce proteolytic activities to less than 10 %

sediment-free controls (Fig. 7). Water-sediment interfaces are layers with high biochemical activities. Here is the site of resuspension of sediment and detritus particles. As can be inferred from the inhibition constants (k_i) in Table 2, inhibition by sediment particles applied also to particle concentrations being considerably lower than in the densely packed sediment.

Fig. 8 illustrates that an addition of the detergent triton x 100 (1 %) could only partly eliminate the inhibitory effects of small sediment particles on Bacillus subtilis protease activities. It is noteworthy that adding 2 g/l of polyvinylpyrrolidone (PVP) as a protective polymer may further reduce the inhibition by sediment particles. In assays without particles, however, PVP additions did not result in higher activities (Table 3).

Sorption of the decomposition products

Extraction of sediments with triton x 100 may be applied as a standardized assay procedure. It overcomes also the problem of re-adsorption of the soluble products. In nature, however, the largest portion of soluble degradation products is likely to be adsorbed to sediment particles. This is demonstrated for solubilized scleroprotein compounds in the presence of different concentrations of autoclaved muddy sediment from Kiel Bay (Fig. 9).
Similar to the adsorption of enzymes by sediments, the adsorption of soluble degradation products increased towards small grain sizes. Scleroprotein hydrolyzate showed 100 % adsorption in autoclaved fine sand and silt. (Fig. 10).

The adsorption of degradation products was largely caused by the organic coating of the sediment particles. This became evident from a comparison of autoclaved and ashed sediment particles which served as adsorbents (Fig. 11). Humic substances that are part of the organic coating, have occasionally been made responsible for inhibitory influences on enzyme reactions in sediments (Martin et al., 1978). In the case of proteolytic solubilization products obtained from stained Enteromorpha-substrate, however, enzymatic activities were rather stimulated by adding humic

substances that had been extracted from Baltic Sea sediment (Fig. 12).

Applications

In Kiel Bay sediments the main peaks of enzyme activities involved in the solubilization of scleroprotein, cellulose, chitin, agar and Enteromorpha substrate coincided with the sedimentation of a major phytoplankton bloom during spring (Reichardt, in prep.). Simultaneous shifts of different biological and biochemical parameters during this sedimentation event have been interpreted as "benthic response" (Smetacek et al. 1978; Graf et al. 1982, Meyer.Reil 1983). On the other hand, the origin of the increased enzyme activities in the sediment remained largely unknown.

A box core sample experiment was designed to describe the impact of enzyme-free POM on solubilizing activities in bioturbated sediment from 17 m water depth in Kiel Bay. An arteficial input of 4.8 g m^{-2} consisted of equal amounts of fine powdered cellulose, chitin, agar, and scleroprotein (Fig. 13). As compared with the control boxes, minor responses were limited to protease activity already 3 h after the input. Far more significant, however, was the impact of perturbation after each sampling which resulted in similar increases of enzyme activities in both the control and the enriched sediment. Increases similar to those observed during the field investigations could not be triggered. So it became most likely that the increased enzyme levels noted during the sedimentation event in situ, were not (predominantly) produced by the benthic microorganisms, but had originated from the sedimenting bloom itself.

Conclusion

A great advantage of dye release assays for POM-solubilizing enzymes is the broad spectrum of chemically defined or complex potential substrates which can be stained with reactive dyes. Photometric determination of the soluble stained reaction products is the most simple way of analysis. As shown in table 4, the sensitivity of direct absorbance measurements of the stained reaction products is even slightly better than for chemical analyses of the total dissolved carbohydrates in the same sample.

Although the dyed particulate enzyme substrates have been available for some time (Hagen et al., 1966), they have only infrequently been used to measure enzyme activities in ecological samples (Little et al., 1979; Leisola & Linko 1976; Kim & ZoBell 1974). In contrast to enzyme assays in solutions, quantitative activity measurements with particulate substrates are complicated by different kinetics with grain size effects that have to be controlled in order to obtain reprducible results. This cannot be achieved with using arbitrary quantities of commercial preparations of undefined grain size (Little et al., 1979).

Major problems arise when activities are to be measured in sediments. Severely standardized techniques are required to perform a reproducible extraction of the enzymes. Due to sorptive processes, concentration and grain size of the substrate play an even more crucial role than in water samples. Sorption of the dissolved reaction products to sediment particles is largely overcome by use of buffered tritonx100.

The strong impact of small sediment particle sizes on the activities may partly serve as a mechanistic explanation for differences noted between different types of sediment along a slope at Kiel Bay (Reichardt, in prep.). Adsorption of POM-solubilizing enzymes to the organic coating (i.e. humic compounds) of sediment particles could be made responsible for decreased activities. On the other hand, protease activity was considerably enhanced in the presence of humic compounds, when the test system did not contain any sediment particles.

Dye release assays with a variety of chemically defined substrates were successfully applied to measure solubilizing activities in sediment samples that had received a high load of enzyme-free P.O.M.. It was concluded that activity peaks of P.O.M.-solubilizing enzymes which had been observed during the sedimentation of phytoplankton blooms in Kiel Bay (Reichardt, in prep.), may have been introduced with the sedimenting bloom rather than constituting a part of the benthic response. It is concluded that dye release assays for P.O.M.-solubilizing enzymes in marine sediments are meaningful only, if enzyme extracts obtained from sediments using standardized procedures such as described under 'Methods' are subject to assays with defined (standardized) concentrations and grain sizes of the particulate substrate. For comparing activities in different types of

sediments, corrections for the inhibitory impact of different grain sizes are strongly recommended.

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Figure Legends

Fig. 1 Role of solubilizing enzymes in the decomposition of organic matter.

Fig. 2 Illustration of sorptive processes between solubilizing enzyme (E) its particulate substrate (P.O.M.), its product (P) and inorganic sediment particles (inorg. P.M.).

Fig. 3 Influence of concentration of the particulate protease substrate "hide powder azure" (Sigma) on protease activity in sandy littoral sediment from Kiel Bay. Double log plot.

Fig. 4 Influence of grain size of substrate particles on the activity (adsorbance units per cm^3 . h) of scleroprotease in Baltic Sea sediment at 17 m water depth at 20 °C. Substrate concentration : 2.5 g/1. The difference between separating the tritonx100 extract before(o)or after(●)the incubation is also illustrated.

Fig. 5 Dependence of apparent substrate affinities($1/k_M$) of scleroprotease in Baltic sea sediment (17 m) on grain sizes of the particulate substrate (2.5 g l^{-1}, 20 °C).

Fig. 6 Influence of substrate concentration (125-250 µm hide powder azure particles) on the scleroprotein solubilizing activity (in triton x100 containing assay medium) of Bacillus subtilis protease (absorbance per h at 20 °C in the absence and presence of sediment particles, 1 g dry weight/2 ml).

Fig. 7 Influence of particle size of autoclaved sediment (1 g dry weight per 2 ml) on scleroprotein solubilizing activities as % of sediment-free controls for S = 2 (●) and 4 g l^{-1} (▲) (125-250 µm) at 20 °C.
Source of the enzyme: Bacillus subtilis (Sigma). Abscissa shows upper units of grain size.

Fig. 8 Influence of sorption by autoclaved sediment particles of different size on the activity (absorbance readings) of scleroprotein solubilizing enzyme from Bac. subtilis, using 2,5 gl of hide powder azure particles (63-125 µm) as substrate. Black columns indicate increase of the foregoing activity readings in the presence of 1.6 % tritonx100 (or 1.6 % tritonx100 + 2gl^{-1} of polyvinylpyrrolidone, last column. Controls were run without sediment particles.

Fig. 9 Sorption (decreasing adsorbance values in the supernatant) of solubilized scleroprotein substrate to different concentrations of autoclaved muddy sediment from Kiel Bay at 28 m. Liquid assay volume : 2,5 ml.

Fig.10 Adsorption of stained proteolytic products to autoclaved sediment (1 g/2 ml) as function of substrate concentration and grain size: Assay with Bac. subtilis protease using hide powder azure particles (125-250 µm) as substrate at 20 °C.
% increase of absorbance after addition of tritonx100 (final concentration: 1.5 %).

Fig. 11 Adsorption (decreasing adsorbance at 600 nm) of the soluble degradation products of hide powder azure to sediment fractions of different grain sizes (upper limits). Comparison between autoclaved (□) and ashed (■) sediment.

Fig. 12 Influence of humic compounds (sodium pyrophosphate extracts from Baltic Sea sediment, as % of their natural extractable concentration) on scleroprotease activity (abs./h) on particulate Enteromorpha-substrate.

Fig. 13 Sedimentation experiment in box core samples at 5°C containing Baltic Sea sediment from 17 m water depth. Input of fine powdered POM (4.8 g m^{-2}) consisting of equal amounts of Avicel-cellulose, chitin, agar and scleroprotein (hide powder, Sigma). Controls without addition of POM. Dye release assays of the upper 1.5 cm for protease, chitinase, agarase, and cellulase; standard deviations as bars (n=3).

Fig.2

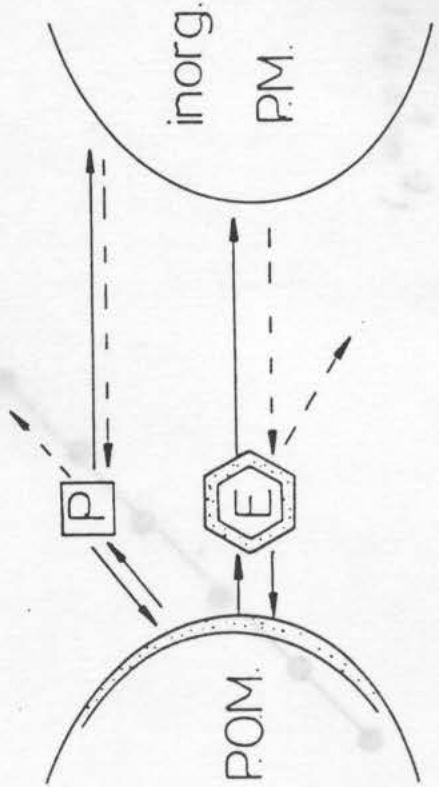
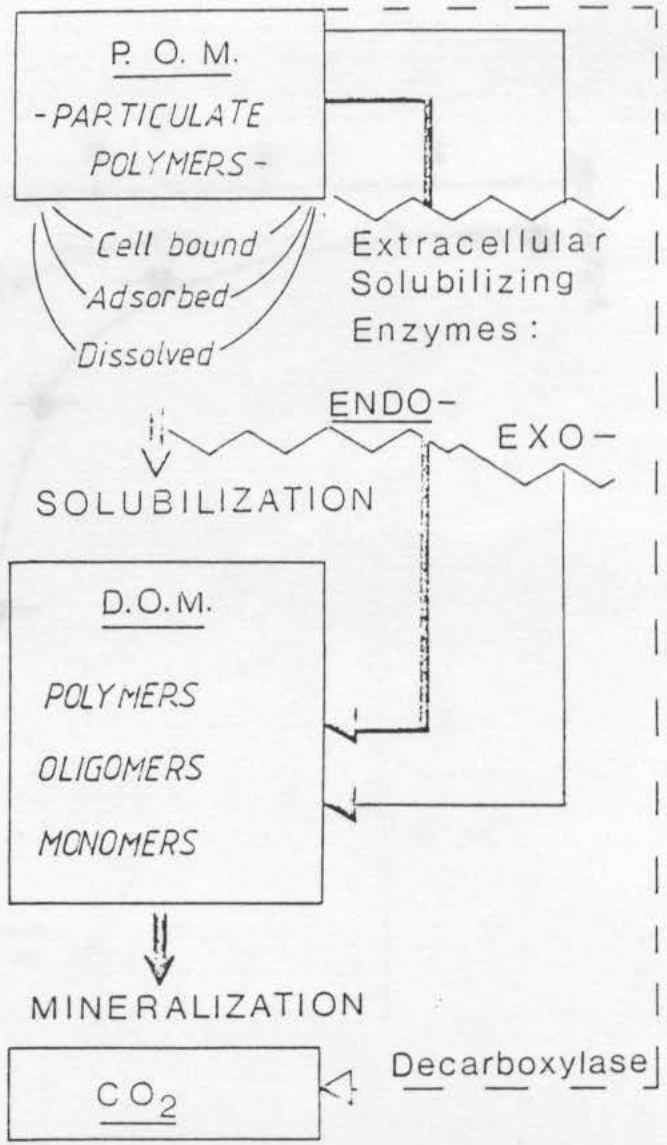

inorg.
P.M.
P
E
POM.

Fig.1


P. O. M.
-PARTICULATE
POLYMERS-
Cell bound
Adsorbed
Dissolved
Extracellular
Solubilizing
Enzymes:
ENDO-
EXO-
SOLUBILIZATION
D.O.M.
POLYMERS
OLIGOMERS
MONOMERS
MINERALIZATION
CO_2
Decarboxylase

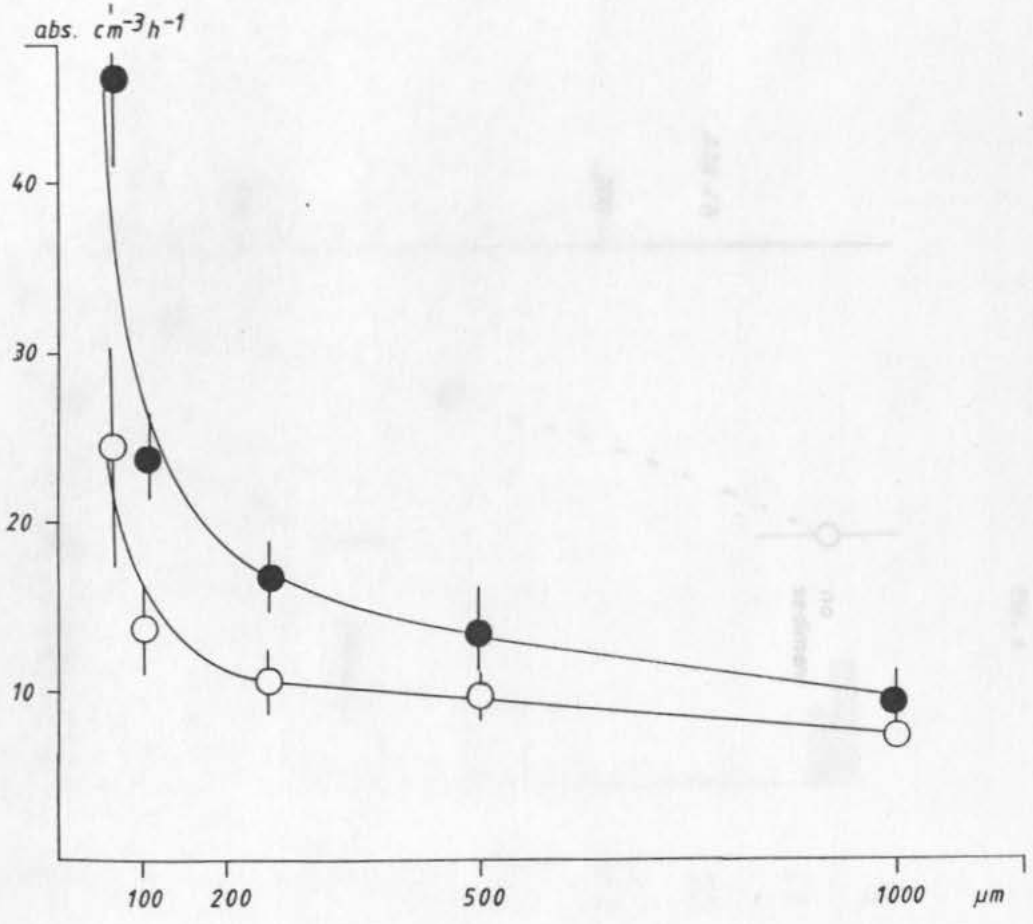

abs. cm⁻³h⁻¹
40
30
20
10
100
200
500
1000
µm

Fig.4

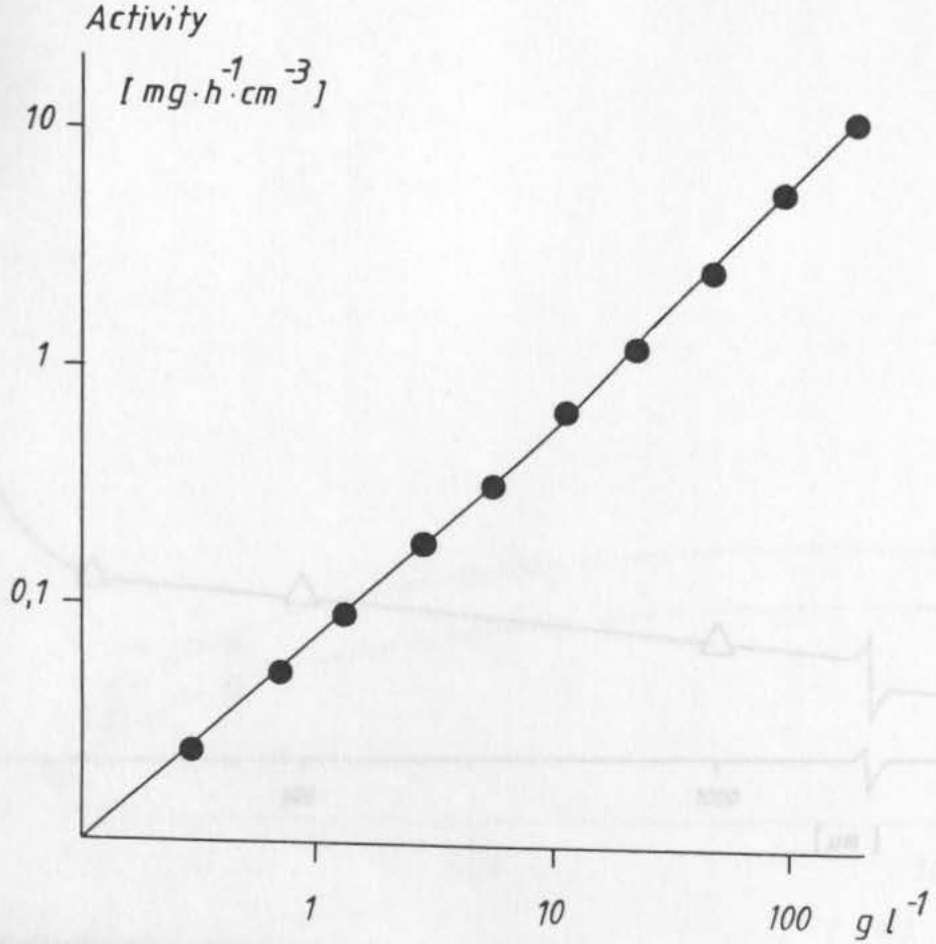

Activity
[mg·h⁻¹·cm⁻³]
10
1
0,1
1
10
100
g l⁻¹

Fig.3

Fig. 6

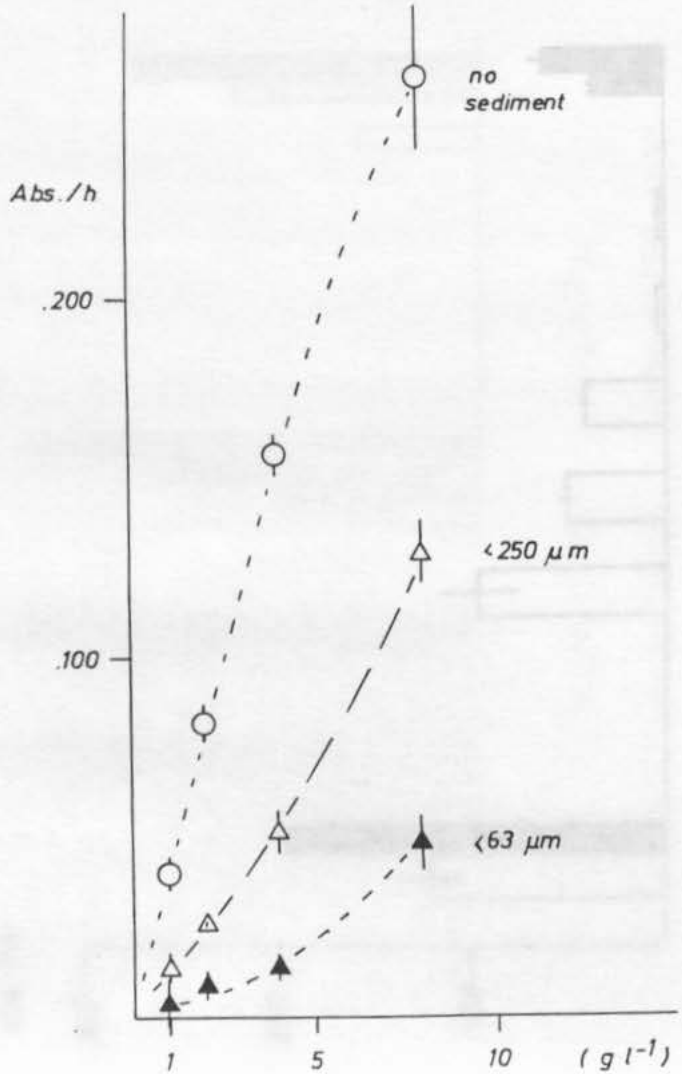
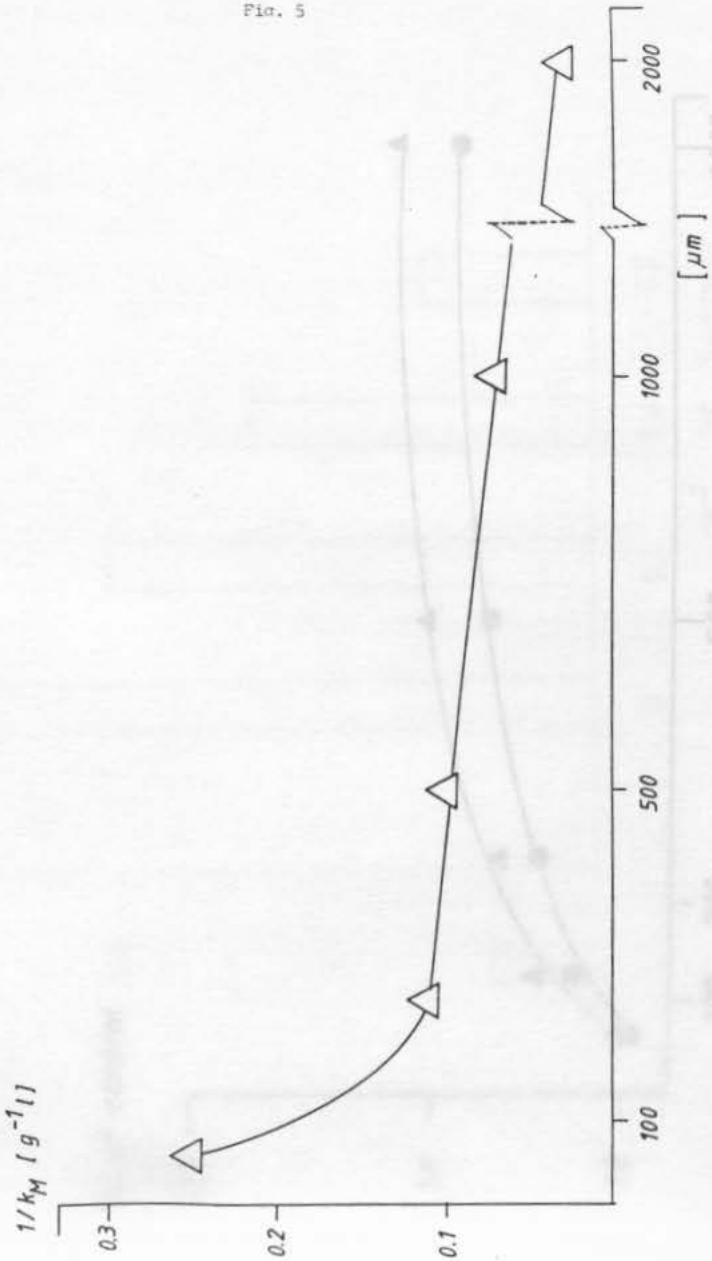

Abs./h
.200
.100
no sediment
<250 µm
<63 µm
1
5
10
(g l⁻¹)

Fig. 5


1/K_M [g⁻¹ l]
0.3
0.2
0.1
2000
1000
500
100
[µm]

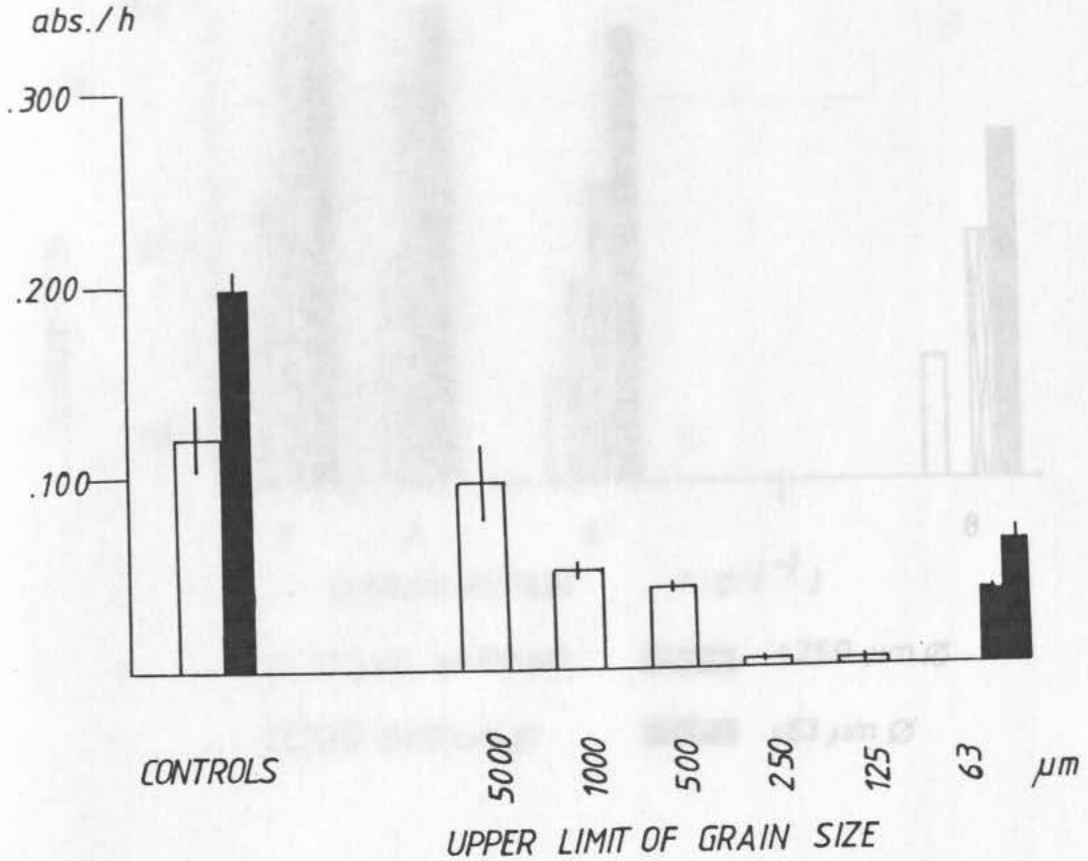

abs./h
.300
.200
.100
CONTROLS
5000
1000
500
250
125
63
µm
UPPER LIMIT OF GRAIN SIZE

Fig. 8

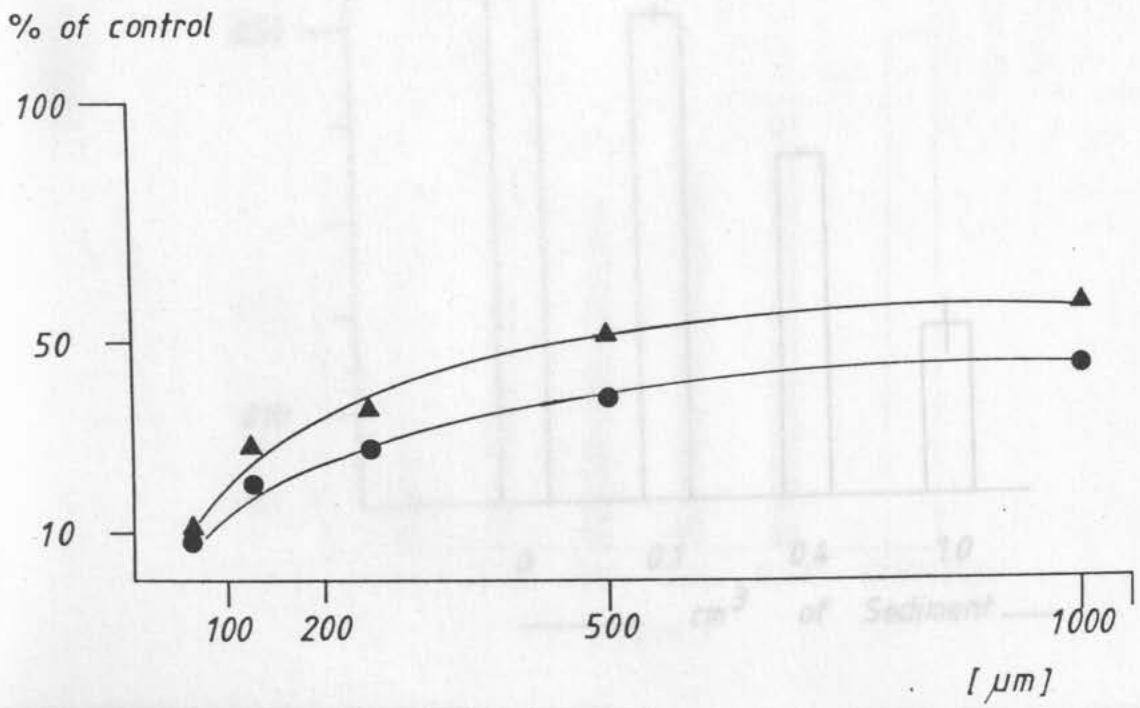
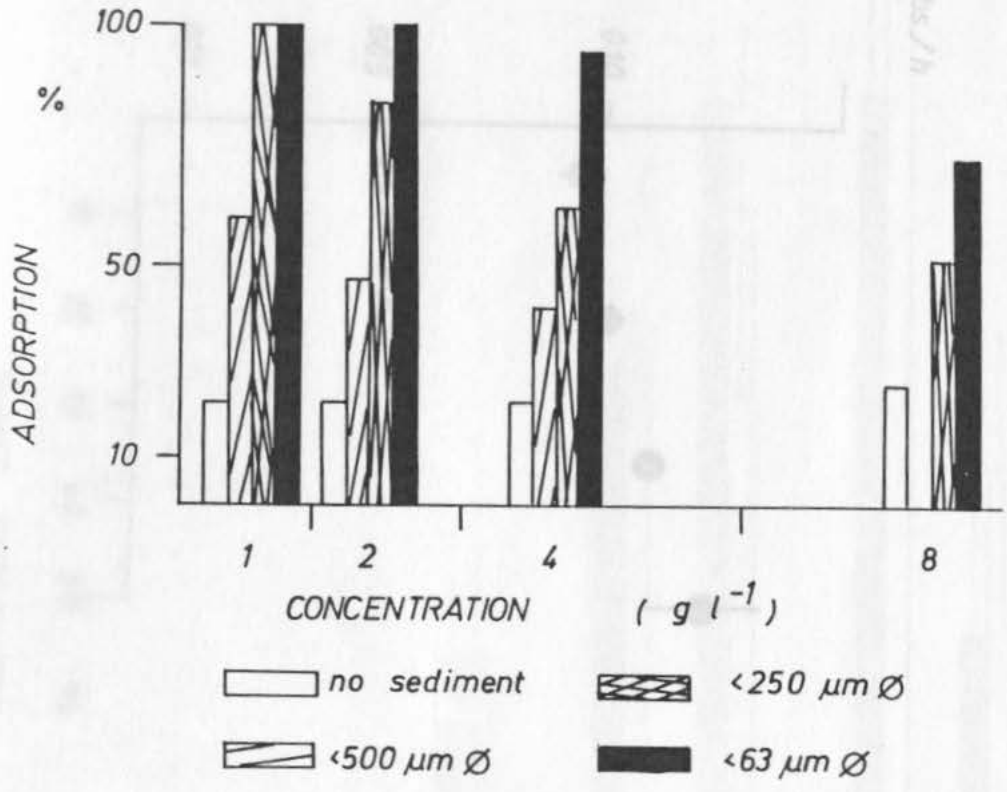

% of control
100
50
10
100
200
500
1000
[µm]

Fig. 7


100
%
50
10
ADSORPTION
1
2
4
8
CONCENTRATION (g l -1)
no sediment
<250 µm Ø
<500 µm Ø
<63 µm Ø

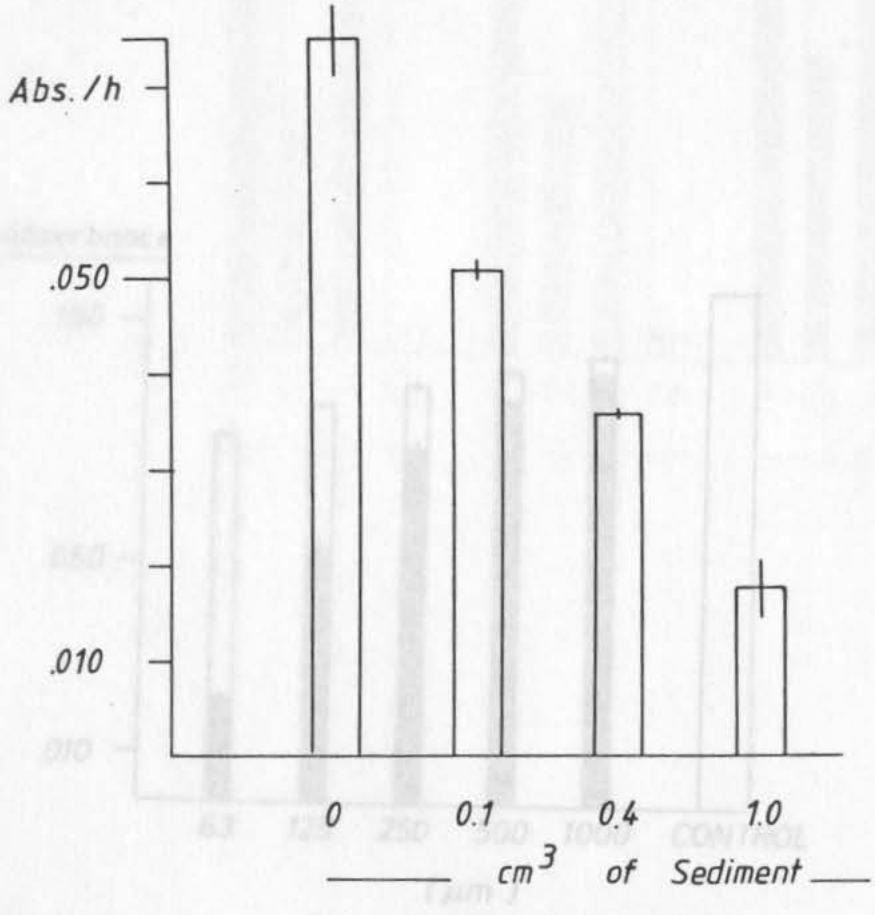

Abs./h
.050
.010
0
0.1
0.4
1.0
cm 3 of Sediment

Fig. 12

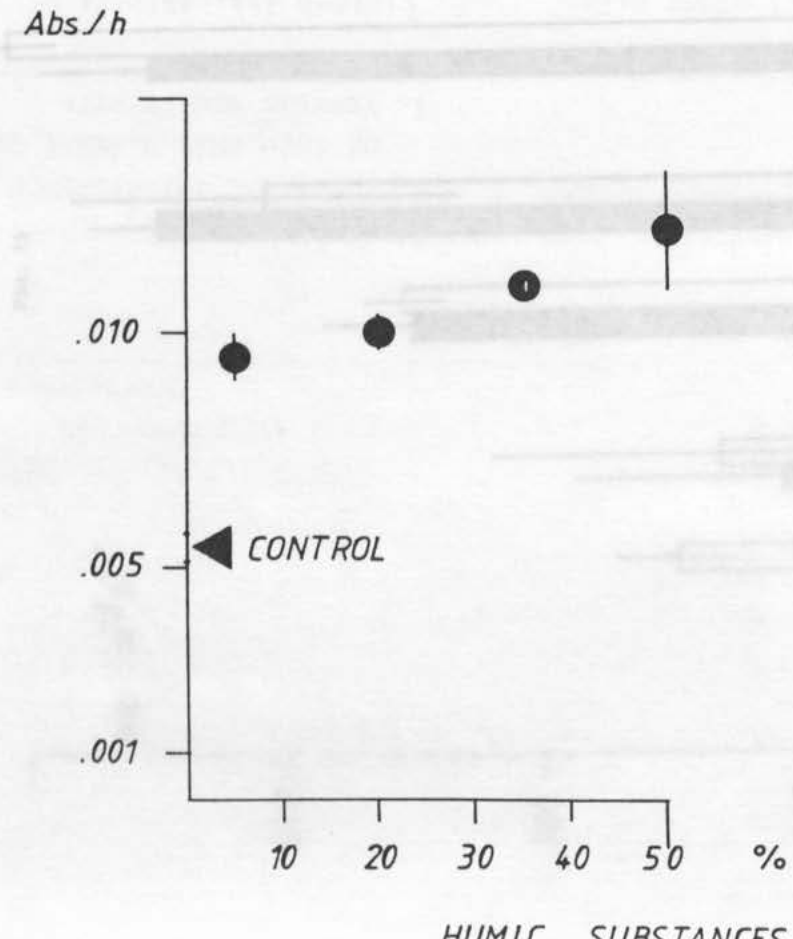

Abs / h
.010
.005
.001
CONTROL
10
20
30
40
50
%
HUMIC SUBSTANCES

Fig. 11

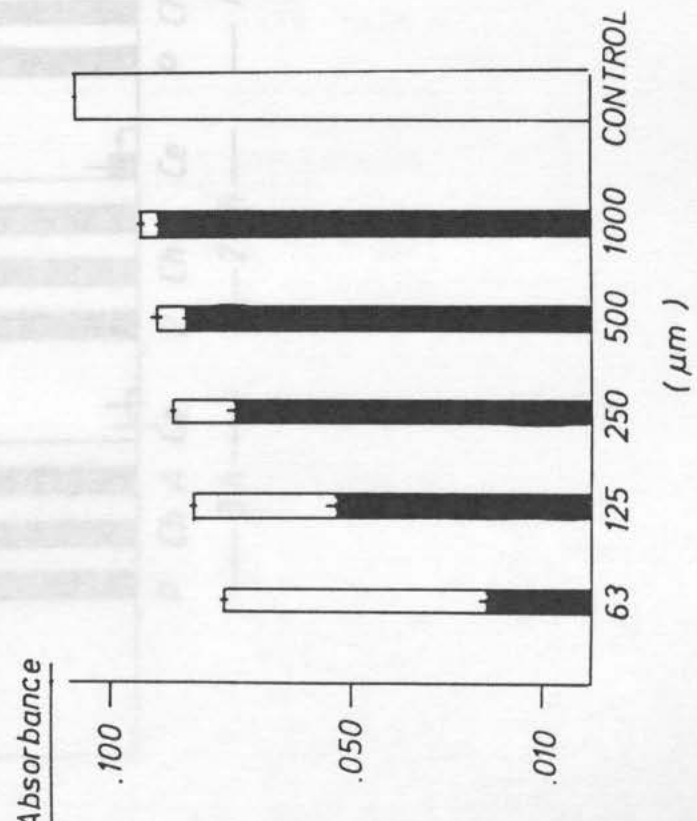

CONTROL
1000
500
250
125
63
(µm)
Absorbance
.100
.050
.010

Fig. 13

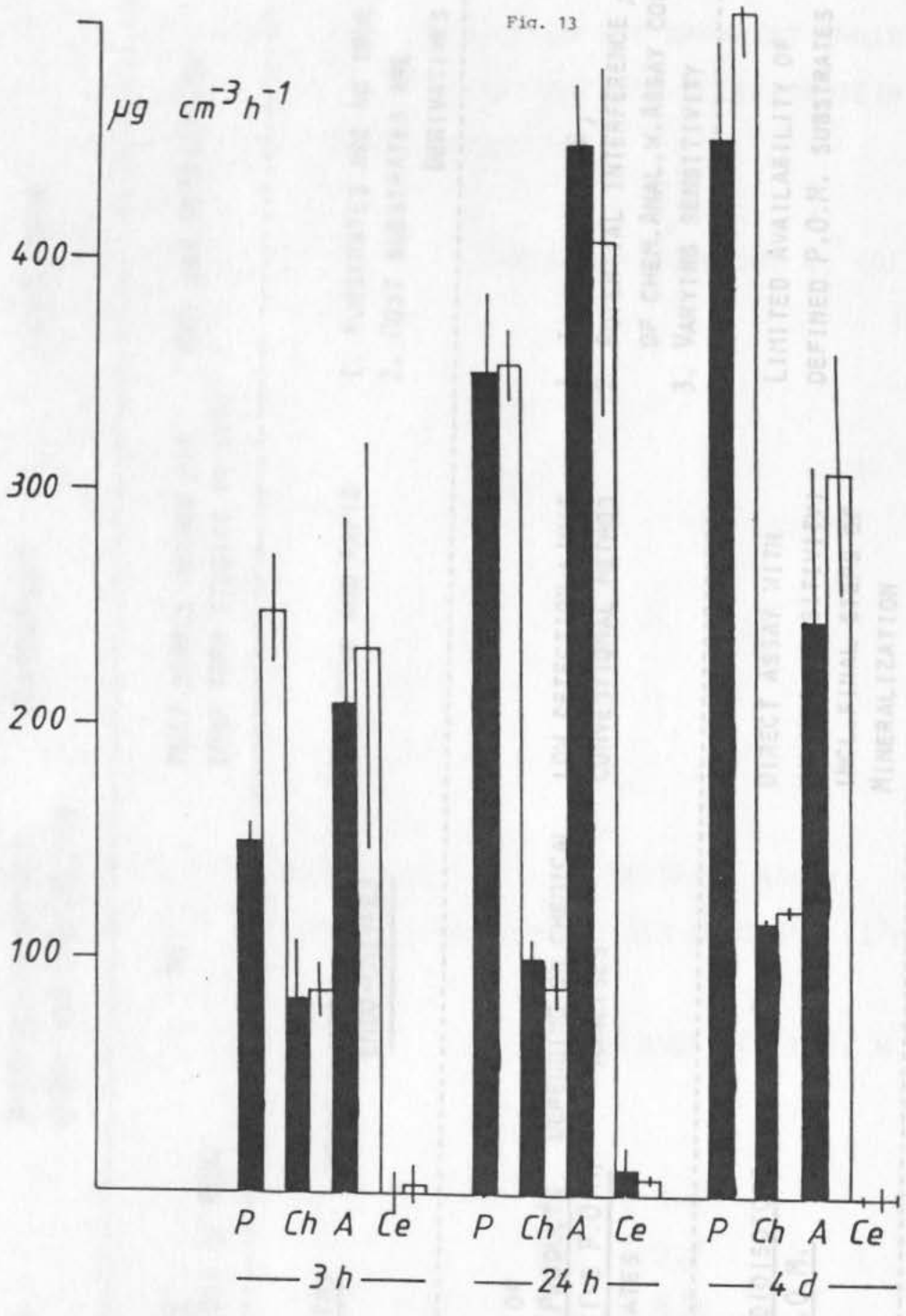


TABLE 1. ASSAY TECHNIQUES FOR P.O.M. - SOLUBILIZING ENZYMES

TECHNIQUE	DISTINCTION BETWEEN ENDO- AND EXO-ENZYMES	PREFERENCES	LIMITATIONS
GRAVIMETRY (WEIGHT LOSS OF P.O.M.)	NO	MOST SIMPLE METHOD FOR LONG TERM STUDIES IN SITU	VERY LOW SENSITIVITY
VISCOSIMETRY	PREDOMINANTLY ENDO-ENZYMES	SENSITIVE AND RAPID	1. SUBSTRATES ARE NO TRUE P.O.M.; 2. MOST SUBSTRATES ARE DERIVATIVES
DETECTION OF DISSOLVED PRODUCTS - WITH NATIVE P.O.M. AS SUBSTRATES	DEPENDING ON CHEMICAL ANALYSES	LOW DETECTION LIMIT; CONVENTIONAL METHOD	1. TIME-CONSUMING; 2. POTENTIAL INTERFERENCE; OF CHEM. ANAL. W. ASSAY COND.; 3. VARYING SENSITIVITY
- WITH RADIOISOTOPE-LABELLED P.O.M.	NO	DIRECT ASSAY WITH MAXIMAL SENSITIVITY; INCL. FINAL STEPS OF MINERALIZATION	LIMITED AVAILABILITY OF DEFINED P.O.M. SUBSTRATES
- WITH DYE-LABELLED P.O.M. AS SUBSTRATES	PREDOMINANTLY ENDO-ENZYMES	RAPID; WIDE RANGE OF SUBSTRATES	1. DYE COUPLING CAUSES UNDER-ESTIMATES; 2. MODERATE SENSITIVITY OF COLORIMETRIC ANALYSIS

TABLE 2

INFLUENCE OF SEDIMENT PARTICLE SIZE ON THE APPARENT INHIBITOR CONSTANTS (K_I) OF (AUTOCLAVED) SEDIMENT FOR SCLEROPROTEIN SOLUBILIZATION BY BAC.SUBTILIS PROTEASE.

SIZE RANGE OF SEDIMENT PARTICLES (/UM)	INHIBITOR CONSTANT K_I (G/L)
< 63	530
500 - 1000	1395

TABLE 3

IMPACT OF POLYVINYLPYRROLIDONE (PVP) ON PROTEASE ASSAY WITH TRITON X100 - EXTRACTS FROM BALTIC SEA SEDIMENT (17M)

CONCENTRATION OF PVP (MG L^{-1})	ABSORBANCE(600 NM)/ H
0	9.2 $\pm$ 1.2
100	9.1 $\pm$ 0.7
1000	8.7 $\pm$ 0.8
2000	8.9 $\pm$ 0.2
10000	6.8 $\pm$ 0.9

TABLE 4

COMPARISON OF PHOTOMETRIC READINGS FOR STAINED SOLUBLE DECOMPOSITION PRODUCTS OF CHITINASE SUBSTRATE. DIRECT READINGS AT 600 NM VS. TOTAL CARBOHYDRATE ASSAY (PHENOL METHOD) AT 488 NM

CONCENTRATION (MG L^{-1})	ABSORBANCE AT 600 NM	TOTAL CARBOHYDRATE ABSORBANCE (488 NM)
16.4	.250	.152
9.4	.158	.087
1.9	.036	.018
0.2	.002	.000

Carbon dioxide dark fixation in marine sediments and its implications for benthic energy flow concepts

by

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Abstract

Dark fixation of carbon dioxide in marine sediments occured largely through the autotrophic pathways of the Calvin cycle as indicated by iodoacetamide inhibition of the key enzyme ribulose-1-5 diphosphate carboxylase. Chemoautotrophic CO_2 -fixation rates obtained from dark fixation by using iodoacetamide-treated assays as blanks are summarized for different marine sediments ranging from shallow littoral (Western Baltic) to the deep sea (Norvegian Sea, Antarctic Ocean). Their relation to current concepts of benthic carbon and energy flow is discussed.

The significance of the de novo synthesis of benthic biomass from CO_2 for the carbon flow in shallow sediments of the phototrophic zone is emphasized with particular reference to the small food webs existing in macrofauna burrow walls. Enhanced rates of both dark fixation and evolution of CO_2 (from 14-C-Glucose) in the surface layer of polychaete burrow walls suggested a very rapid turnover of inorganic carbon.

When considering the role of Carbon cycle - mediated CO_2 -fixation, (i.e., photo- and chemoautotrophic processes) in deep sea sediments, chemo-autotrophic primary production accounted for a major fraction of primary synthesis of benthic biomass in the Antarctic. In the Bransfield Strait this process even matched the yearly energy input via sedimentation.

Carbon flow in marine sediments is often extremely dependent on sedimentation processes (Smetacek, 1980). Detrital POC fluxes as measured in sediment traps are usually taken as a measure of total energy input into aphotic marine sediments. For methodological reasons lateral advection of POC is scarcely quantified and therefore thought to represent a missing link in conceptual models of energy and carbon flow. Another largely unknown potential source of energy is chemoautotrophic primary production which is measurable as part of the CO_2 dark fixation. Likewise, primarily methodological reasons may account also for the omission of this parameter from carbon flow models. In summarizing CO_2 dark fixation measurements in the western Baltic, the Norvegian Sea, and the Antarctic Ocean, the role of both total CO_2 dark assimilation and chemoautotrophic primary production in benthic food webs will be addressed.

Material and methods

Sediment box core samples were obtained from:

1) Kiel Bay (Eckernförde Fjord and Stein Lagoon) at 18 - 28 m and 0.5 m, respectively during 1981 through 1985.
2) the Norvegian Sea (Vöring Plateau, 600 - 1900 m water depth) on a cruise with R/V "Poseidon" in July 1985
3) the Bransfield Street and NW Weddell Sea Antarctica, (110 - 4500 m water depth) on cruises of R/V "Polarstern", ANT II-3 and ANT II-2, in 1983 and 1984, respectively.

In vivo CO_2-fixation rates were determined in triplicate subsamples taken from Reineck box cores with 5 cm^3 sawed off syringes. Into these subcores 100 µl ($3.7 \cdot 10^4$ Bq) per cm^3 of isotonic solutions of NaH $^{14}CO_3$ were injected using a 1cm^3 syringe driven by means of a microscope micrometer pinion. Blanks were obtained from samples into which 100µl/cm^3 of 18 % formaldehyde had been injected. After dark incubation for 1-4 h at in situ temperatures the subcores were sectioned at 1 cm-intervalls and immediately acidified. After two rinses with 10 ml of sterile sea water and subsequent centrifugation the samples were dried at 60°C and combusted with an automatic Packard ^{14}C- sample oxidizer for liquid scintillation counting. Radioactivity of the assimilated carbon was estimated using a Beckman LS 100 liquid scintillation system with quench corrections based on external standards and channels ratios. Concentrations of total carbonate in the pore water were calculated from carbonate alkalinity according to Gargas (1975). Anaerobic incubations were carried out under Ar atmosphere. Further details are described elsewhere (Reichardt (1986).

Mineralization rates of ^{14}C (U)- glucose were determined using serum flasks with a bucket assembly attached to the serum caps (Reichardt & Morita,1982). 0.2 cm^3 aliquots of the sediment sample (triplicate) were incubated in 100 ml- serum flasks with 2 ml of isotonic sterile sea water and 200 µl ^{14}C (U)-glucose ($5.5.10^3$ Bq; 163 . 10^3 Bq/µmol). Samples treated with 4 % of formaldehyde served as blanks. After incubation for 30-60 min at in situ temperatures the assay was terminated by acidification (1 N H_2SO_4; final pH2.0). At the same time 0.15 ml of the CO_2-absorbent phenetylamine was injected onto a piece of fluted filter paper held by the bucket assembly. After 1 h of shaking the filter paper was removed and placed in a toluene based scintillation fluid. Its radioactivity was measured as described above.

Results and Discussion

Conceptual models

A simplefied illustration of benthic carbon and energy flow based on the major processes of energy transfer rather than trophic levels is shown in Fig. 1. This functional scheme appeared particularly adequate for a discussion of CO_2 fixation, which is more directly a process-oriented parameter than are production measurements at higher trophic levels. The question to be addressed in this paper is whether a better knowledge of CO_2-fixation processes would finally help to shape better conceptual models of carbon- and energy flow.

More recent models of benthic food webs stress the importance of the so-called "small food web" which consists largely of microheterotrophs and meiofauna (Kuipers et al., 1981). In Fig. 2 the uptake of dissolved organic nutrients (termed "adsorption" according to Whittaker, 1969) is a predominant process. As a result of sediment diffusion barriers, part of the incorporated carbon which is respired as CO_2 may subsequently be re-assimilated. This can be accomplished by photosynthetic, but also chemoautotrophic primary producers. Finally, variable amounts of CO_2 may be incorporated through heterotrophic pathways (Li, 1982, Overbeck, 1979). Therefore the "small food web" has the potential for a rapid turnover of carbon dioxide even in the aphotic benthos through dark fixation via chemoautotrophic or heterotrophic pathways.

Inhibition by iodocetamide (IAM) of the Calvin cycle key enzyme was tested in lagoon sediment with blue-green algae as predominant photoautotrophs. Assuming that the enzyme is inhibited to the same extent in photo- and chemoautotrophs, exclusively heterotrophic processes may be measured at IAM concentrations sufficient to produce 100% inhibition of the photosynthetic reaction (i.e., light reaction minus dark reaction). This inhibition occured at approximately 15 mM IAM (i.e., a concentration which was about 30 times greater than that being effective in cell-free systems (Argyroudi-Akoyunoglou & Akoyunoglou, 1967). The resulting rate for heterotrophic dark fixation was within the expected range of 0.5 to 12 % of the total (Overbeck & Daley, 1973). Hence, chemoautotrophy plays by far the predominant role among dark fixation processes.

Activation patterns as indicators of chemoautotrophy

In vertical sediment profiles dark fixation may often peak at discontinuity layers of the redox potential indicating a limitation by the supply of electron donors for the chemoautotrophs. This was demonstrated for sediment cores from the western Baltic Sea. Most often, chemoautotrophic CO_2-fixation is limited by the supply of thiosulfate. Addition of 8 mM caused a considerable stimulation, in contrast to ferrous iron as an alternative electron donor. Strongly reduced sediments from maximal depths in Kiel Bay could no longer be activated by thiosulfate. On the contrary, a concentration of 1 mM thiosulfate caused here already strong inhibitory effects (Reichardt, in press).

Besides thiosulfate, also ammonia was able to cause a slight stimulation of CO_2 dark fixation in Kiel Bay sediments (Fig. 4). This occured also under anaerobic incubation (Ar-atmosphere). According to the described activation patterns, thiosulfate oxidizers and ammonia nitrifyers turned out to be major constituents of the chemoautotrophic microflora.

Impacts of burrowing macrofauna

Discontinuities of redox profiles that give rise to an enrichment of the chemoautotrophs are most abundantly found at inner sediment surfaces which are the result of macrofaunal burrowing. In an intensely bioturbated lagoon sediment of Kiel Bay f.e., CO_2 dark fixation was activated along the polychaete burrow walls where pO_2 and reduced inorganic ions reached most favorable

conditions for chemoautotrophic CO_2-fixation. Similar observations were made in Antarctic deep sea sediments. At stations with intense bioturbation by burrowing polychaetes, high CO_2-dark fixation rates penetrated to considerable depths. On the other hand, this process was limited to the surface when burrowing macrofauna was largely absent. Subsamples taken from oxidized burrow walls were characterized by twofold higher CO_2-fixation rates than in the adjacent sediment (Reichardt, in prep.).

In the more oxidized sediments from the Vöring Plateau in the Norvegian Sea the relative contribution of ammonia -nitrification and thiosulfate-oxidation to the chemoautotrophic pathways showed a considerable degree of patchiness. Also in vertical profiles the contribution of either nitrification or thiosulfate oxidation changed from one horizon to the other, even, if the overall redox profile did not indicate any discontinuities (Reichardt, in prep.). An explanation for such patterns may become likely by considering also the distribution and metabolic activity patterns of the burrowing infauna present. When macrofauna body surface or burrow material was used to differentiate between the two main processes, nitrification prevailed usually. So vertical CO_2-fixation profiles could largely be attributed to macrofauna-dependent nitrification. With regard to the mechanisms resonsible for this kind of macrofauna-favored CO_2-fixation, it must be considered that in reduced sediments the supply of potential electron donors to the largely aerobic chemoautotrophic organisms is likely to occur as a result of biopumping and aeration of inner boundary layers. In less reduced sediments the excretion of potential electron donors such as ammonia may be even more important, as indicated by the CO_2-fixation data from the Vöring Plateau.

CO_2-turnover

Furthermore it appears most likely that due to the inevitable diffusion barriers in benthic microhabitats, carbon dioxide is turned over in closed circuits and exchanged fairly rapidly between the members of the so-called "small food web". At least measurements of CO_2 evolution from ^{14}C-labeled glucose in Kiel Bay lagoon sediment showed in increase in subsamples from the burrow walls of Nereis diversicolor (Fig. 5). It would not be surprizing, if the most active sites for CO_2-evolution are at the same time most active in CO_2-assimilation.

Significance for energy flow

Finally the crucial question has to be answered, if CO_2 dark fixation in marine sediments is really important enough to be treated as a kind of missing link in conceptual models. A preliminary answer at least may be attempted for aphotic sediments of the Antarctic Ocean and with particular reference to deep-sea benthos. Unfortunately there are no reliable production estimates available for the macrofauna which is present at very high population densities. Nevertheless it seems to be legitimate to compare CO_2 dark fixation with calculated fluxes of P.O.C. (Table 1).

The fluxes are based on two data sets, i.e., maximal primary production estimates which had been obtained for the very productive Bransfield Strait (von Bodungen et al., 1986) and average estimates for the Antarctic Ocean (Clarke, 1985). They were calculated according to the empirical formula of Suess (1980). It turned out that CO_2-dark fixation per m^2 and year was of the same order of magnitude as the P.O.C-flux, or even higher, when the P.O.C. flux was based on average primary production. At one of the three stations considered, dark fixation matched even the P.O.C. flux calculated for maximal primary production.

Conclusions

It can be concluded that dark fixation of CO_2 in deep aphotic sediments is significant enough to be included in conceptual models of benthic carbon and energy flow. In different marine sediments this predominantly chemoautotrophic de novo synthesis of benthic biomass was most significant in macrofaunal burrows that function as niches for "small food webs" (Reise & Ax, 1979) which consist of microorganisms and meiofauna. In shallow littoral sediments with higher rates of photosynthetic primary production, CO_2 dark fixation would primarily accelerate the carbon cycling within the "small food webs".

In energy flow models which are currently conceived, chemoautotrophic primary production is entirely neglected. On the other hand, CO_2 dark fixation in Antarctic deep-sea sediments reached the order of the P.O.C. flux to the sediment, which is currently used as the only applicable measure of energy supply in aphotic sediments. It is suggested to make at least more adequate use of what is usually taken merely as a correcting parameter for photosynthetic primary production measurements.

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TABLE 1

COMPARISON OF POC -FLUX TO THE SEDIMENT SURFACE AND CO_2-FIXATION IN ANTARCTIC SEDIMENTS

- BRANSFIELD-STRAIT-

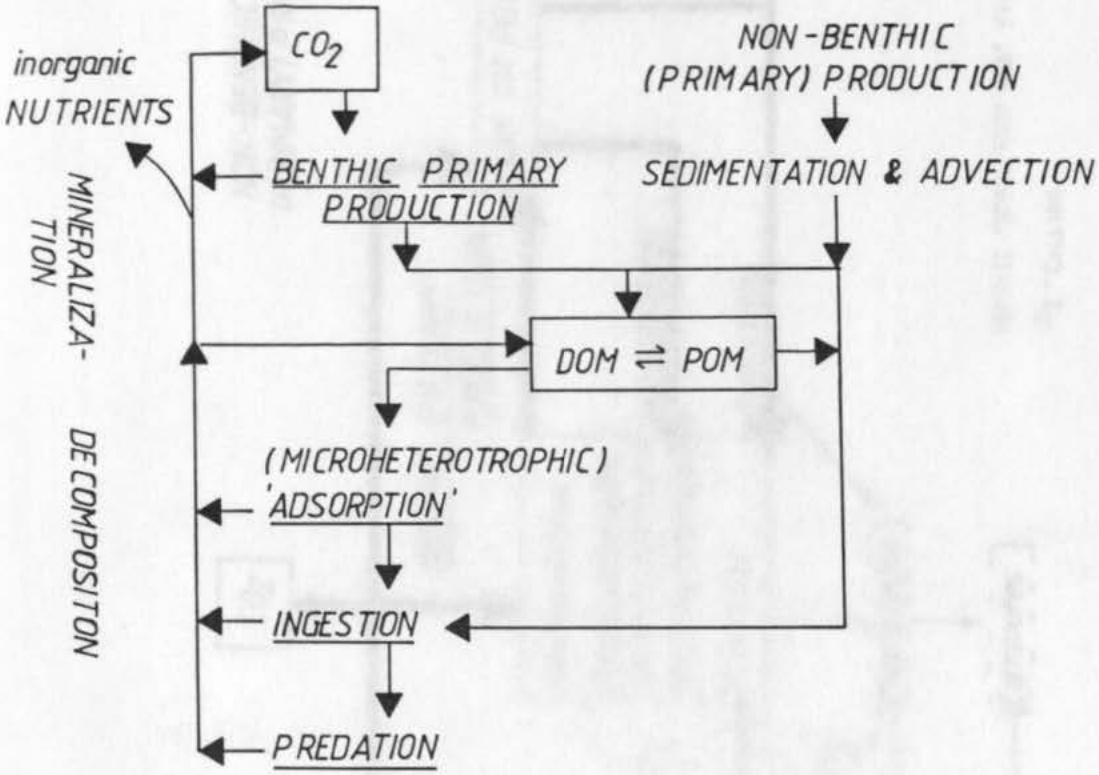
LOCATION	WATER DEPTH (M)	PRIMARY PRODUCTION (G C M^{-2} A^{-1}) (A)	PRIMARY PRODUCTION (G C M^{-2} A^{-1}) (B)	POC-FLUX[C] (G C M^{-2} A^{-1}) (A)	POC-FLUX[C] (G C M^{-2} A^{-1}) (B)	CO_2-DARK FIXATION (G C M^{-2} A^{-1})	CO_2-DARK FIXATION (KJ)
$62^{o}14.2$ S $58^{o}17.9$ W	500	250	20	20.4	1.60	0.93	≙ 38.9
$62^{o}20.3$ S $57^{o}49.9$ W	1970	250	20	5.3	0.42	4.98	≙ 208.5
$61^{o}38.1$ S $54^{o}46.8$ W	2280	121	20	2.2	0.36	0.46	≙ 19.3

(A) MAXIMAL ESTIMATES (V.BODUNGEN ET AL.1986)

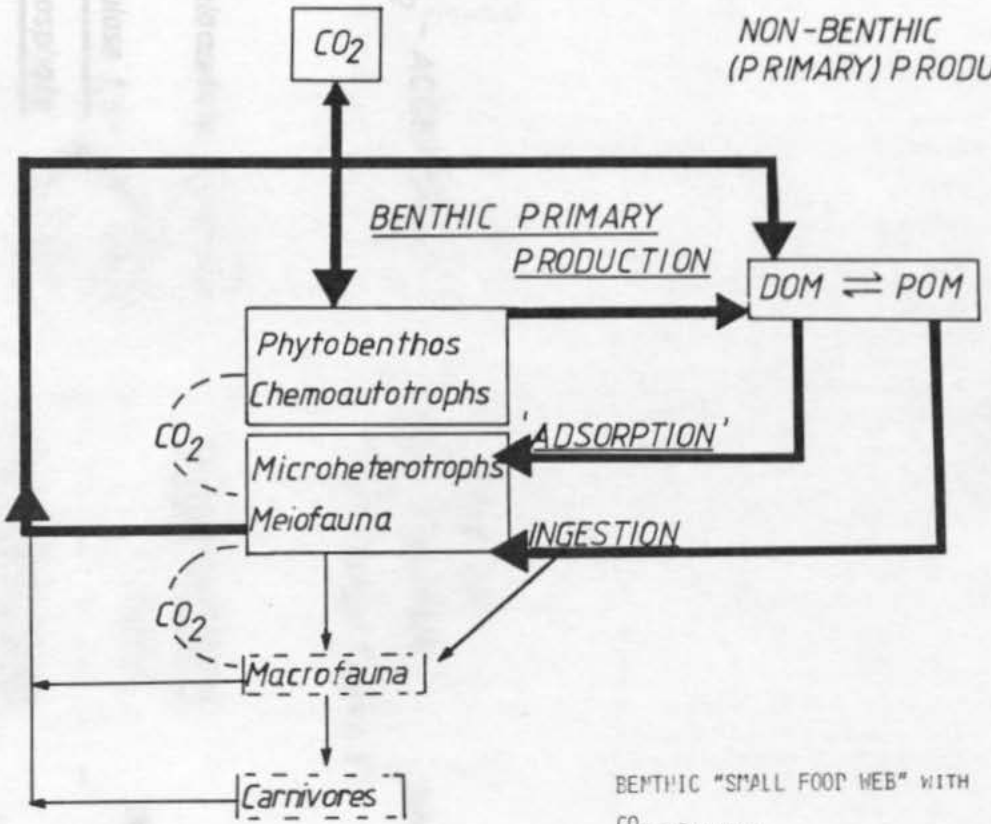
(B) AVERAGE ESTIMATES FOR ANTARCTIC OCEAN (CLARKE, 1984)

C) CALCULATED FROM (A) AND (B) ACCORDING TO SUESS, 1980

Fig.1



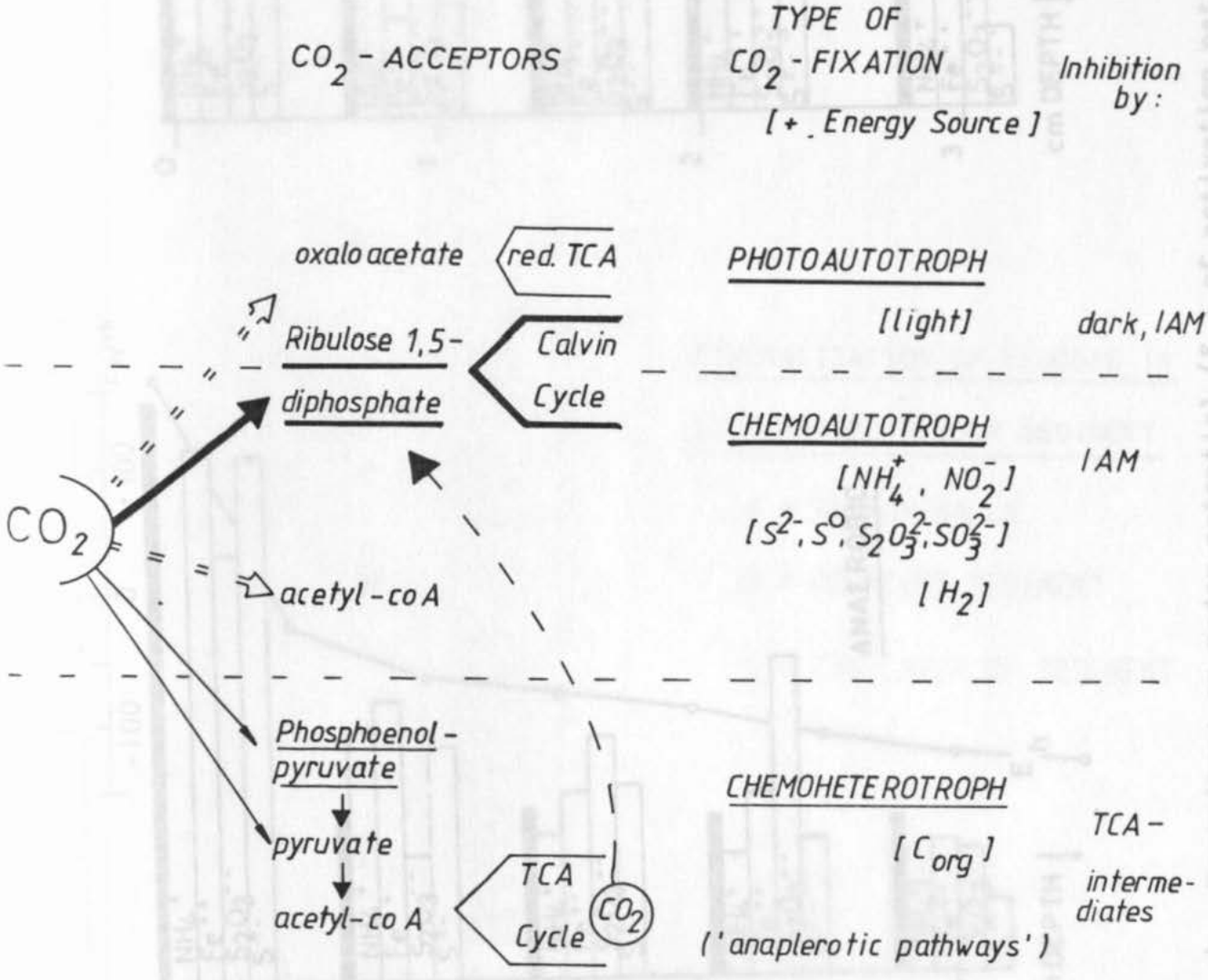
SEQUENCE OF MAJOR PROCESSES INVOLVED IN BENTHIC FLOW OF CARBON (BOXES) AND ENERGY (UNDERLINED)



BENTHIC "SMALL FOOD WEB" WITH
CO_2 - CYCLING

Fig.2

Fig. 3



DE NOVO SYNTHESIS OF BIOMASS VIA MAJOR PATHWAYS OF CARBON DIOXIDE FIXATION (SIMPLIFIED)

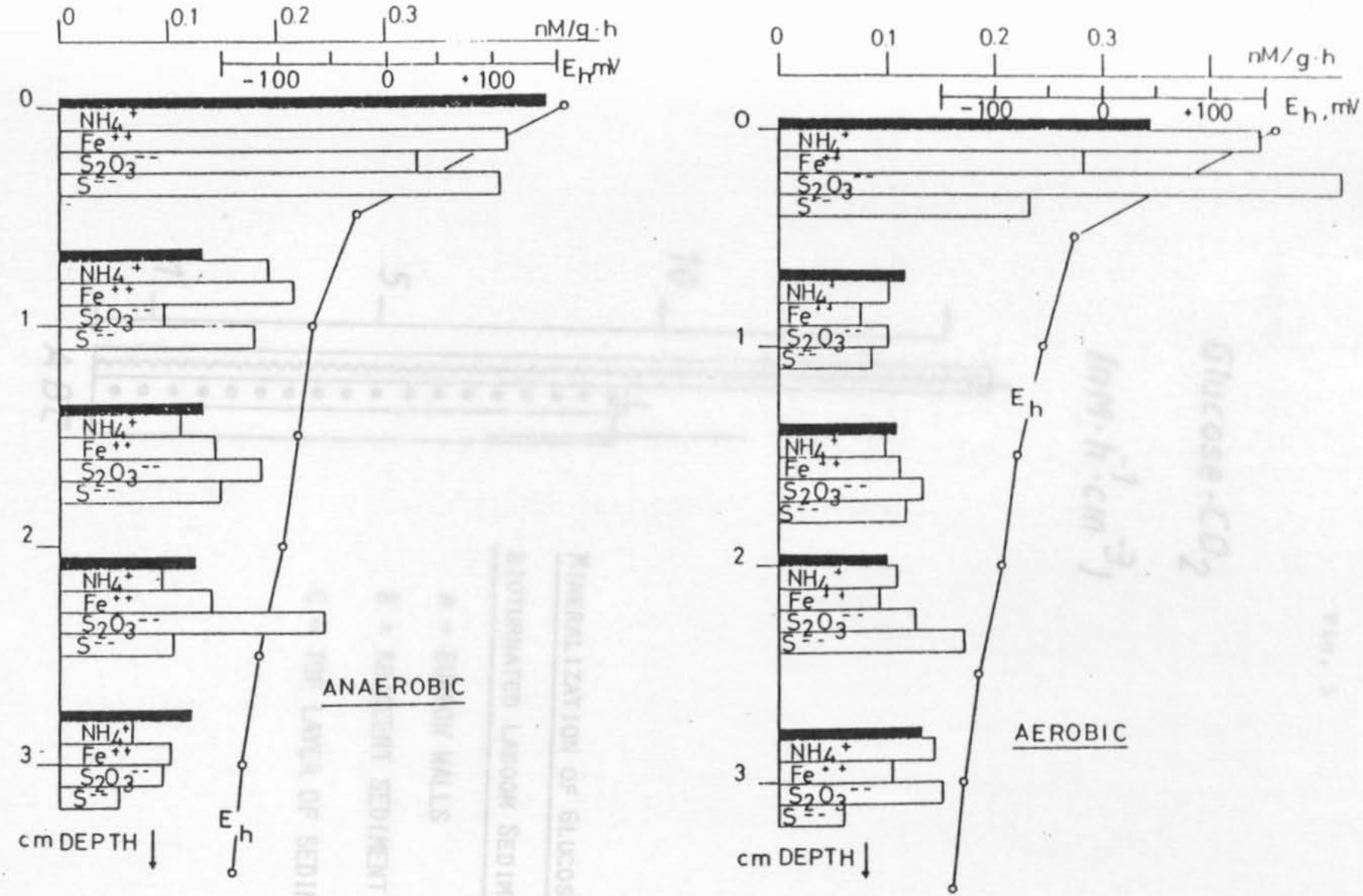
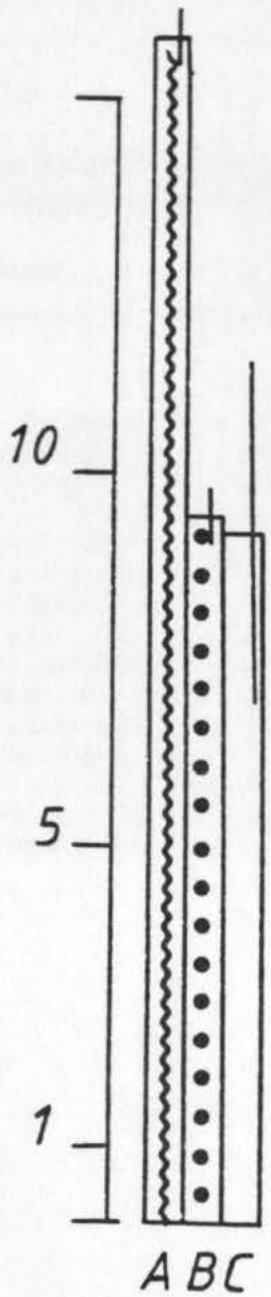


Fig.4. Dependence on redox potential (E_h) of activation patterns for aerobic and anaerobic $^{14}CO_2$-dark fixation at different depths of littoral sediment cores from Kiel Bay. Impact of 10 mM $(NH_4)_2SO_4$, $FeSO_4$, $Na_2S_2O_3$, and Na_2S.

Fig. 5



MINERALIZATION OF GLUCOSE IN BIOTURBATED LAGOON SEDIMENT

A = BURROW WALLS

B = ADJACENT SEDIMENT

C = TOP LAYER OF SEDIMENT

II

LRH: W. Reichardt
Microb Ecol (1987) 15:000–000

MICROBIAL ECOLOGY

Impact of the Antarctic Benthic Fauna on the Enrichment of Biopolymer Degrading Psychrophilic Bacteria

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Abstract. Stenothermic cold adaptation was a predominant growth characteristic among biopolymer degrading bacteria from Antarctic shelf sediments. Psychrophilic decomposers of protein (gelatin), chitin, and cellulose accounted for up to 84, 93, and 68%, respectively, of 0°C-isolates from selected compartments of the sediments. Macroinvertebrates were recognized as a selective pressure on these fast-growing (zymogenous) psychrophiles. Psychrophilic properties of growth and biopolymer degradation coincided most in the case of proteolytic isolates. On the other hand, the majority of psychrophilic chitin- and cellulose-decomposers showed less efficient biopolymer degradation at environmental temperatures (0°C). Temperature optima of the activities of pertinent depolymerizing enzymes (e.g., scleroprotease) exceeded by far the temperature optima for growth (between 4 and 12°C). Therefore, it appears likely that enhanced rates of enzyme synthesis at low temperatures play a crucial role for the degradation of detrital organic matter in this permanently cold environment.

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Abstract

Stenothermic cold adaptation of growth predominated among biopolymer degrading bacteria from Antarctic shelf sediments. Psychrophilic decomposers of protein (gelatin), chitin, and cellulose accounted for up to 84, 93, and 68 % , respectively, of 0 °C-isolates from selected compartments of the bioturbated sediments. Macroinvertebrates were recognized as a selective force for these fast-growing (zymogenous) psychrophiles. Psychrophilic properties of growth and biopolymer degradation coincided most in the case of proteolytic isolates. On the other hand, the majority of psychrophilic chitin- and cellulose-decomposers showed less efficient biopolymer degradation at environmental temperatures (0 °C). Temperature optima of the activities of pertinent depolymerizing enzymes (e.g.scleroprotease) exceeded by far the temperature optima for growth (between 4 and 12 °C). Therefore it appears likely that enhanced rates of enzyme synthesis at low temperatures play a crucial role for the degradation of detrital organic matter in this permanently cold environment.

Introduction

Adaptation to the permanently cold Antarctic marine environment is largely characterized by "k-selection" of slow growth rates of the benthic macrofauna (3). As a result of permanently low temperatures ($\leq$ 0 °C) population dynamics are controlled by low basic metabolic rates, reduced individual energy requirements and greatly increased standing crops. Similar strategies have been anticipated for the vast majority of cold-stenothermic ("psychrophilic") bacteria (with growth from 0 °C or less to 20 °C and temperature optima at or below 15 °C; (22). It has been speculated that this group of psychrophilic bacteria is dominated by autochthonous populations, whereas the fast-growing opportunists would occur mainly as psychrotrophs which grow at 0°C , but also far beyond 20 °C; (2).

On the other hand, (presumably) zymogenous psychrophiles have been isolated in great numbers from Arctic marine sediments (25). Nevertheless, the majority of the biopolymer degraders among these isolates were rather psychrotrophic than psychrophilic. This could indicate a low degree of cold adaptation with respect to bacterial biopolymer degradation.

As seasonally changing energy flows in polar benthic communities are initiated by decomposition of detrital biopolymers, cold adaptation of these processes would affect rate-limiting biogeochemical transformations. To evaluate the role of temperature control, different aspects must be taken into account: 1) Although fast-growing psychrophiles have been isolated from the Antarctic (16, 22, 34) nothing is known about the fraction of biopolymer decomposers that are psychrophilic. 2) Considering the patchy distribution of nutrients in sediments, these zymogenous psychrophiles should prevail in high-nutrient environments like the "zoosphere", viz. the immediate surroundings of mechanically and metabolically active benthic invertebrates (1, 35). 3) Psychrophilic growth characteristics of biopolymer decomposing bacteria do not necessarily imply that biopolymer decomposition is also cold-adapted (22).

In this paper a first attempt is described to evaluate the significance of fast-growing (zymogenous) psychrophiles as geochemical agents of biopolymer degradation in Antarctic marine sediments. The "zoosphere", comprising microhabitats for epi- and perizoic bacteria on the body surface of burrowing macrofauna and at the walls of burrows and tubes, was chosen to isolate aerobic protein- and polysaccharide degrading psychrophiles. Surrounding sediment material served as a "control" for the assessment of enrichment effects in the "zoosphere".It could be shown that zymogenous psychrophiles were the predominant group of biopolymer degrading bacteria in the"zoosphere". Cold adaptation of decomposition processes corresponding to psychrophilic growth prevailed only among proteolytic isolates.

Materials and Methods

Sampling sites

Sediment samples were obtained during a cruise of the ice breaker R/V "Polarstern" (ANTIII-2) from 5 Antarctic shelf stations south of the South Shetland Islands archipelago from November 20 to December 3, 1984 (Table 1). Subsamples from sediment and "zoosphere", viz., macroinvertebrate infauna and their burrows or tubes, were taken aseptically from Reineck box cores (50 x 50 cm). The macroinvertebrate infauna was dominated by maldanid, terebellid, and sabellid polychaetes as well as echiurid worms. Most of the bacterial isolates originated from fine grained sediments (Table 1).

Treatment of samples

All laboratory work was carried out in a cooled (0°C) lab container aboard the ship. Epizoic bacteria were isolated by means of agar plate impressions (using contact times of 1-3 days) from the body surface of pretreated polychaete and echiurid worms or pieces of their epidermis. The moribund worms used in this procedure had been rinsed several times and kept for up to 24 h in repeatedly changed in filter-sterilized Antarctic seawater to exclude contaminations from the sediment or from the gut contents as far as possible. Surfaces of inhabited worm tubes and burrows were usually coated

with mucoid layers or membranes. To enumerate and isolate "perizoic" bacteria from these habitats, the surface films together with adhering sediment particles were transferred into sawed off 1 ccm syringes for suspension of 0.1 cm aliquots in sterile sea water. Dilution series from different sediment compartments were all treated in the same way: The first dilutions were treated two times for 15 sec with a high-speed blendor (ULTRATURRAX, Janke &Kunkel, Staufen, W.Germany) in an ice bath. 0.1 ml aliquots of serial dilutions (1:10) were plated on precooled agar plates using ice-cold glas spatulas and a cold tray (34).

Enrichment media

For enumeration and isolation of aerobic cold-adapted bacteria (sum of psychrophiles and psychrotrophs) the agar plates were incubated for 14 d at 0 °C. Proteolytic bacteria were detected by gelatin liquefaction on nutrient gelatin (Merck) containing: gelatin (120 g/1), peptone (5 g/1), meat extract (3 g/1), and agar (3g/1) in 3/4 strength Antarctic sea water. Chitinolytic colonies were detected by clearing zones on chitin agar containing 20 ml of Bacto peptone (5g/1) and agar (12 g/1) as bottom layer and 5 ml of nutrient-free agar (12 g/1) with reprecipitated chitin (5 g/1) as top layer, in 3/4 strength Antarctic sea water. For detection of cellulolytic bacteria the same medium with cellulose (Avicel) instead of chitin was employed.

Isolation and thermal classification

Single colonies with positive degradation responses for gelatin, chitin and cellulose were quantitatively isolated from the impression plates as well as from the spread plates derived from the highest dilutions showing positive responses. The isolates were transferred in duplicate onto the same agar media on which they had developed. Growth and hydrolytic action of the colonies were compared after 5 d at 20 °C and 0 °C. Visible preferences of either parameter for the lower temperature were recorded as psychrophilic growth or decomposition.

Exponential growth rates were determined in liquid batch cultures (1 % Bacto peptone in Antarctic sea water) using 5 ml of the culture medium in 1.3 x 16 cm test tubes immersed in a set of temperature controlled water

baths. Absorbance readings at 550 nm (1 cm, Zeiss PMQ3 spectrophotometer) served as biomass equivalents.

Activity measurements

Protease activity was determined using a stained scleroprotein (Hide Powder Azure, Sigma) as substrate (21, 28). Cell suspensions were extracted in tris-HCl (0.2 M)- buffered (pH 7.8 triton X 100, final conc.: 2%) and incubated for up to 20 h with 5 mg/ml of the solid substrate. Absorbance readings (600 nm, 1 cm) of the solubilized dyed product of proteolysis served as activity units. Corresponding dye release assays were carried out for cellulase, chtinase, and agarase (28).

Results

Viable counts at 0 °C of biopolymer decomposing bacteria reflected patchy distribution patterns. Surface layers of inhabited macrofaunal burrows, in particular, tended to be preferred enrichment sites for cold-adapted primary decomposers. Examples from two sampling stations are shown in table 2. It was also noted that the surface of an abandoned burrow contained significantly less chitin decomposers than the surrounding sediment.

605 proteolytic, chitinolytic and cellulolytic strains were isolated at 0°C from sediment and "zoosphere", viz."perizoic" microflora from burrow surfaces as well as "epizoic" microflora from invertebrate body surfaces (table 1). In this mixture of psychrotrophs and psychrophiles the latter group was identified by their failure to grow at 20 °C. About the same percentage (> 80%) of the proteolytic isolates from each of the three ecological niches considered were psychrophiles. Polysaccharide decomposing psychrophiles, however, were more abundant among the perizoic and epizoic isolates (fig.1). 93 % and 68 % of the perizoic chitin degraders and cellulose degraders, respectively, turned out to be psychrophiles, as compared

with 35 % and 13 % , respectively, of the isolates from the plain surrounding sediment. Epizoic isolates showed a similar predominance of psychrophiles.

Cold adaptation of biopolymer decomposition was different from that of growth. Nearly all of the proteolytic psychrophiles from the "zoosphere" (burrow walls and invertebrate tissue surfaces) showed enhanced proteolysis at 0 °C as compared with 20 °C. On the other hand, only 63 % of the isolates from plain sediment were psychrophilic in terms of proteolysis. This discrepancy between psychrophilic growth and psychrophilic biopolymer decomposition became most striking among the chitin- and cellulose degraders (fig.2). With respect to the decomposition of these polysaccharides, a very limited number of the pertinent isolates preferred the low temperature. An exception were cellulose degrading isolates from plain sediment. Whereas protein degradation by isolates from the "zoosphere" revealed an extreme degree of cold adaptation, this did not apply to the decomposition of chitin and cellulose.

Attempts to detect protein- , chitin-, or cellulose- depolymerizing enzymes with temperature optima in the psychrophilic growth range (fig.4) up to 20 °C) have failed so far. Temperature optima around 40 °C and higher were typical for proteases of the psychrophilic isolates (fig.3).

Exponential growth rates that were determined in liquid cultures of 20 randomly selected psychrophilic protein degraders indicated temperature optima for their exponential growth between 4 and 12 °C. Two examples are illustrated in fig 4.

Discussion

Not before Morita's (22) terminological clarification has it been possible to estimate the ecological importance of psychrophilic bacteria. Ecological

surveys have shown that psychrophilic populations persist only in stable cold environments (2). Yet, the significance of certain physiologically defined groups of psychrophiles in nature has rarely been investigated (25). Furthermore, to distinguish between psychrotrophic and psychrophilic populations simply on the basis of conventional plate counts (e.g. 4 °C vs.20 °C) may become problematic in certain cases (12).

The psychrotrophic populations considered in this investigation had to be able to grow and compete with psychrophilic populations at 0 °C and below. Therefore a temperature of 0 °C was chosen for enumeration and isolation of both groups. To distinguish between the two thermal groups, temperature responses of isolated strains were assessed instead of using estimates merely based on plate counts at critical temperatures (8, 12, 19). This more direct procedure was limited by the number of isolates screened. On the other hand, it offered greater precision and the advantage of using isolated group members for further characterizations (16, 17).

Predominance of psychrophilic heterotrophic bacteria has been reported for polar marine environments (25, 34) and even for non-polar regions (30). Yet, unequivocal assessments of zymogenous psychrophiles are still missing. Differential incubation periods such as 3 months at 2 °C vs. 1 month at 20 °C (30) do not appear as a reliable basis to distinguish between defined thermal and nutritional groups. Extremely extended incubation periods of more than 2 weeks are likely to favor the enrichment of oligotrophic populations.

In contrast to the current lack of ecological data on fast growing psychrophiles there is at least physiological evidence for their competitiveness. Psychrophilic bacteria have been shown to outcompete psychrotrophic bacteria also at high nutrient concentrations (13, 23).

The predominance in Antarctic shelf sediments of fast growing biopolymer degrading 0 °C-isolates with growth temperature optima below 15 °C suggests that these psychrophiles are primarily responsible for a fast turnover of organic matter in these fauna-rich habitats. The combination of fast growth and stenothermic cold adaptation seems not to apply to the majority of Antarctic macrobenthos which is predominantly k-selected (slow growing) - (3).

Epizoic bacteria attached to the external surfaces of invertebrates and "perizoic" bacteria colonizing infauna-made microhabitats comprise what may be termed "zoosphere" (analogous to:phytosphere). Although no quantitative estimates are available, it seems plausible that sediment bacteria rely in part on benthic invertebrates for their organic substrates (9). External surfaces of invertebrates and, particularly, infauna-made internal sediment interfaces such as burrow walls are characterized by extremely high concentrations of dissolved and particulate organic matter (9, 29).

In isolated extreme environments the "zoosphere" seems to be particularly selective for fast growing bacteria with properties being characteristic of the environment, as e.g. barophily (6). A similar selection among the marine microflora is likely to occur also on the Antarctic sea floor. The largely endemic benthic infauna of the Antarctic ocean (5) would ensure over long periods of time both permanently cold and nutrient-rich attachment sites for zymogenous psychrophiles. This would explain the described predominance of obligate psychrophiles among the epizoic and "perizoic" isolates. In addition to increased levels of nutrients in the zoosphere, a general stimulation of surface-associated growth rates (7) may strengthen the selective forces acting on bacterial epibionts.

To minimize interferences from specific binding forces on the animal tissue (18), epizoic bacteria were isolated using a combined impression-incubation technique. Although great care was taken to remove loosely attached cells before each attempt to isolate truly epizoic bacteria, their psychrophilic share differed only slightly from that of perizoic psychrophiles. The plain sediment should have contained the microflora of both the diet and the feces of the deposit feeding infauna. It is unknown, to what extent the composition of the sediment microflora is influenced by fecal material as a potential source of mesophilic and psychrotrophic bacteria derived from warm-blooded animals such as marine mammals and penguins.

It may be speculated whether similiarities and differences between epizoic, perizoic, and plain sediment bacteria reflect also taxonomic relationships. However, taxonomic aspects were beyond the scope of this investigation and pertinent published data are lacking. A few investigations suggest that differences between epizoic bacteria and the microflora of surrounding

habitats may be of minor importance, at least on the generic level (4, 33).

This investigation aimed at a group of aerobic, rapidly isolated bacteria which were characterized by their degradation of biopolymers. Psychrophilic adaptation to their permanently cold habitat was correlated with maximal abundance in the "zoosphere". This result supports the idea that bacterial biodeterioration in the deep sea is largely mediated by scavenging animals (32).

It has also been noted that conversion of particulate organic matter to dissolved organic matter in an aquatic environment was primarily temperature-controlled, whereas subsequent mineralization processes depended mainly on the availability of oxygen (11). When applied to the extreme conditions on the Antarctic sea floor, this would imply an optimal cold adaptation of pertinent polysaccharide - and protein- depolymerizing enzymes. However , psychrophilic adaptations on the enzyme level were not evident from temperature spectra of protease (fig.3) as well as chitinase and cellulase activities (Reichardt, in prep.). These enzymes possessed temperature optima far beyond the growth range of the isolates (fig.4). Therefore enhanced biopolymer degradation by certain isolates at 0 °C had to be attributed to an increased production of the enzymes.

On principle, synthesis of enzyme proteins can increase at low temperatures (0 °C) in both psychrophilic and psychrotrophic bacteria (31). In Arctic marine sediments, proteases, amylases and chitinases were mainly produced by psychrotrophs, while cellulolytic or agarolytic activities were not detected at all (25). Cold adapted freshwater bacteria possessed mainly gelatinases and lipases, whereas chitinases were less common and cellulases were absent (20). These findings indicated that cold adaptation of biopolymer degrading bacteria may be primarily associated with protein degradation. In general, this view was supported by the isolates from the Antarctic benthos.

Yet, stenothermic cold adaptation among the Antarctic strains was considerably more pronounced: Almost 100 % of the cold-adapted proteolytic strains (84% of these being psychrophiles) from the "zoosphere" liquefied gelatin more effectively at 0 °C than at 20 °C. Furthermore, the Antarctic benthos contained also, though in low densities, zymogenous psychrophiles

that were capable of degrading agar (Reichardt,unpubl.) and cellulose most effectively at 0 °C. Also macroorganisms tend to exhibit a higher degree of metabolic cold adaptation in the Antarctic than in the Arctic ocean (10).

How efficient bacterial biopolymer degradation can be, depends on the thermal characteristic of both enzyme production and activity. As mentioned before, pertinent enzyme activities showed temperature optima far beyond the growth temperature range of their producers. This contrasts with other metabolic parameters such as the assimilation of organic substrates (14, 15, 24). There is, however, as yet indirect, evidence that the higher temperature optima of the biopolymer degrading enzymes are efficiently compensated for by increased production rates. Culture experiments to quantify this production are in progress.

In conclusion, the Antarctic benthos fauna favors the enrichment of fast growing (zymogenous) biopolymer degrading bacteria that are predominantly psychrophilic. Therefore the hypothesis has to be rejected that in these permanently cold marine environments psychrotrophic bacteria represented the zymogenous "population" (2). In contrast to freshwater environments where high levels of nutrients can select for populations with higher growth temperature maxima (8, 26, 27), this mode of selection seems not to apply to the "zoosphere" of the investigated Antarctic sediments. Whereas the uptake processes for dissolved organic matter in the Antarctic marine environment possess similar temperature optima as the growth rates, the efficiency of biopolymer degradation by extracellular enzymes may largely depend on enhanced enzyme production rates at the in situ temperature.

Acknowledgements

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Legends

Fig.1 Number of bacterial isolates degrading protein (prot.), chitin (chi.), and cellulose (cell.) at 0 °C, classified according to their sources: Isolates from plain sediment (S), perizoic isolates from infaunal tube and burrow walls (P), epizoic isolates from burrowing macroinvertebrates (E). Hatched parts indicate portion of psychrophiles (no growth at 20 °C).

Fig.2 Number of psychrophilic isolates from fig.1. Hatched parts indicate here the portion of isolates with stronger degradation responses at 0 °C than 20 °C .

Fig.3 Temperature dependence of scleroprotease activity from psychrophilic isolate.

Fig.4 Temperature dependence of exponential growth rates (ln μ) in batch cultures of two proteolytic isolates.

Table 1. Origin of protein- and polysaccharide degrading bacteria isolated at 0°C.

Station	I	II	III	IV	V
Coordinates	62°14 S 58°18 W	61°15 S 55°00 W	63°30 S 54°15 W	62°43 S 55°56 W	62°09 S 58°24 W
Water Depth (m)	504	125	120	105	484
Type of Sediment:					
% Silt & Clay	30	48	5	5	29
% veryfine/fine sand	56	48	55	18	49
% medium/coarse sand	14	5	21	33	18
% Very coarse sand/ pebbles	-	-	19	44	4
isolates from plain sediment	131	33	-	4	-
isolates from "zoosphere" (total)	286	85	38	26	2
from body surfaces	111	33	38	21	2
from burrows/tubes	118	52	-	5	-

(Revised June 23,1987)

Table 2 Biopolymer degrading bacteria (cfu /cm^3 x 10^3, after 14 d at 0 °C)

Station	Group	Sediment	Surfaces of tubes or burrows void		inhabited	
I	Proteolytic bacteria	123 ±15		(M)	197 ±38	*
				(S)	480 ±180	*
II	-"-	170 ±80	0.5 ±0.7 *	(M)	130 ±44	
I	Chitinolytic bacteria	33 ±4		(S)	8000 ±1400	*
II	-"-	122 ±35	4.2 ±3.5 *	(M)	150 ±60	
I	Cellulolytic bacteria	< 0.1				
II	-"-	< 0.1	5 ±8 *	(M)	5 ±0	*

Station I: 62°14'S, 58°18'W, (504 m); Station II: 61°15'S, 55°00'W, (125 m)

(M) Mucous membrane forming surface layer; (S) Burrow wall without mucous membrane

*) Significantly different from surrounding sediment (P= 99%) . Mean values are given with standard deviations (n= 3).

Fig. 1

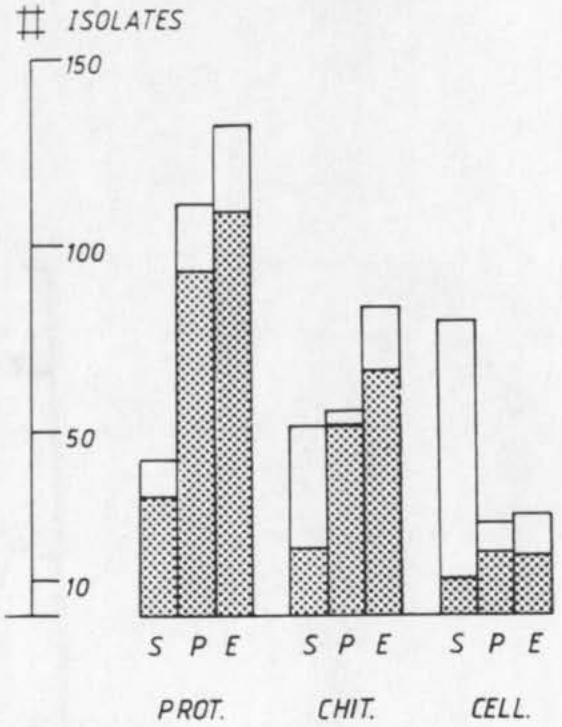
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Fig. 2

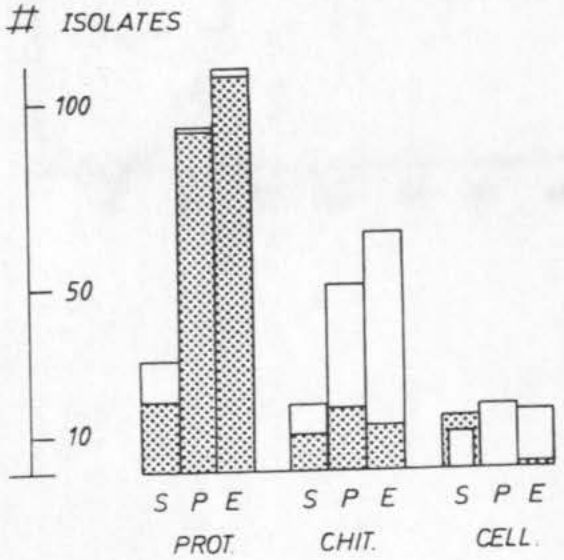
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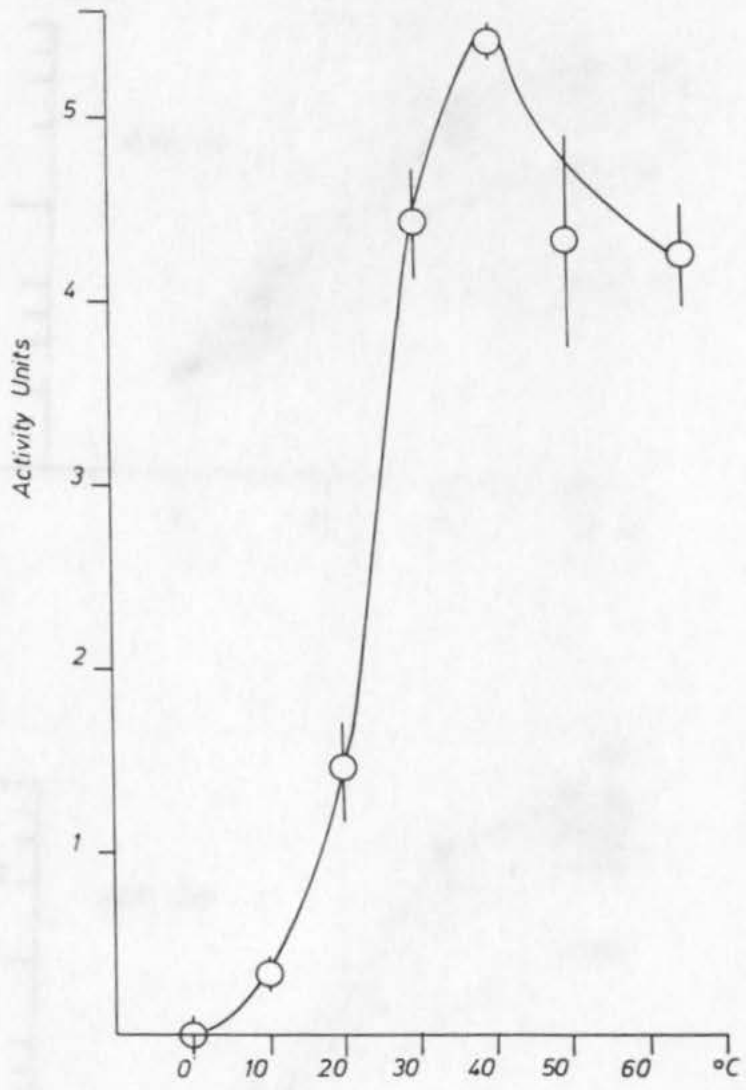
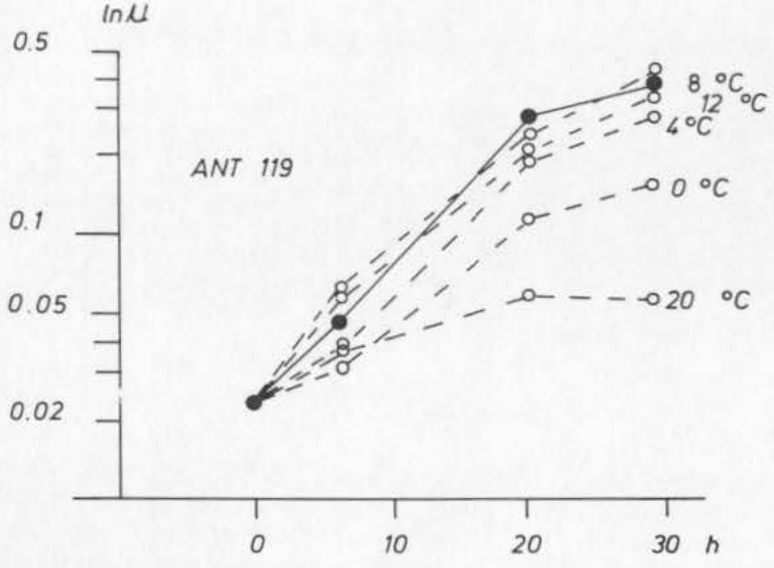
Fig 3




ln μ
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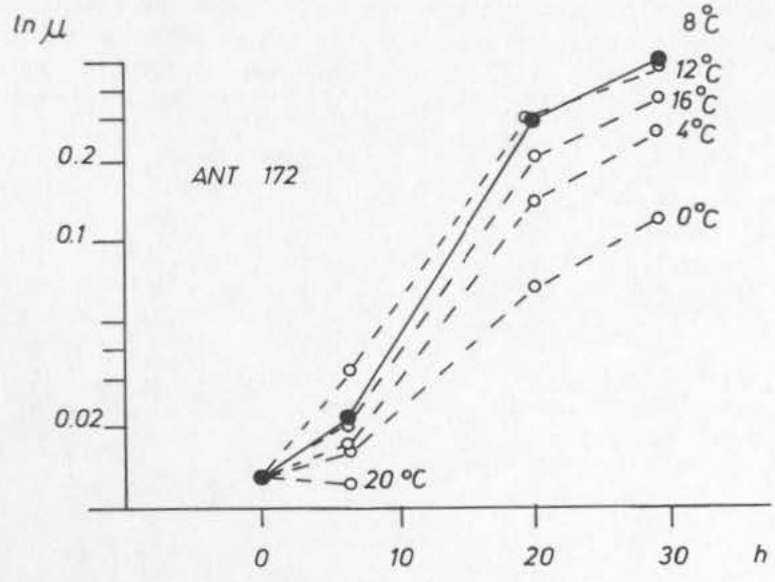
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NOTE

Burial of Antarctic macroalgal debris in bioturbated deep-sea sediments

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(*Received* 2 *March* 1987; *in revised form* 30 *April* 1987; *accepted* 8 *May* 1987)

Abstract—In two sediment samples from the Antarctic Ocean (Weddell Sea) at 2280 and 1570 m water depth the upper 8–10 cm contained considerable amounts of macroalgal fragments larger than 1 mm (22.9 and 20.8 g m^{-2} of ash-free dry weight), derived from brown and red macroalgae. With C : N ratios (>17) being rather typical for initial stages of bacterial colonization and transformation to detritus, the thallus fragments served as a matrix for epiphytic bacteria. The occurrence of low densities of bacteria on the algal surfaces therefore could be interpreted as the result of grazing by a polychaete-dominated infauna. The input of large macroalgal fragments locally into deep-sea sediments could help modulate the extreme seasonality of benthic energy flow which is thought to prevail at high latitudes.

INTRODUCTION

PRIMARY production of planktonic algae is usually considered to be the ultimate energy source for benthic life in the deep sea (HONJO, 1978; SPENCER *et al.*, 1978; DEUSER and ROSS, 1980; DEUSER *et al.*, 1981; HONJO *et al.*, 1982; BILLETT *et al.*, 1983; BETZER *et al.*, 1984; SMITH and BALDWIN, 1984; VON BODUNGEN *et al.*, 1986). In coastal areas, however, primary production of benthic macroalgae and other macrophytes often becomes a predominant energy source for benthos that depends on detrital food chains (MANN, 1973; WOODWELL *et al.*, 1977; NEWELL *et al.*, 1980; STEPHENSON *et al.*, 1986). A contribution of macroalgae to the energy flow of deep-sea benthos has been considered rather infrequently (MENZIES *et al.*, 1967; MENZIES and ROWE, 1969; SCHOENER and ROWE, 1970). To determine if detrital food chains based on macroalgae play a role in deep-sea sediments, evidence for both advection of macroalgal particles and utilization of these by benthic consumers is required.

Macrophytic fragments, including macroalgal debris, have been encountered down to the hadal region in deep-sea traps (WIEBE *et al.*, 1976; HONJO, 1978; HINGA *et al.*, 1979) as well as on the sea floor (HEEZEN *et al.*, 1955; MENZIES *et al.*, 1967; MENZIES and ROWE, 1969; SCHOENER and ROWE, 1970; GEORGE and HIGGINS, 1979). A few were recognized as fragments of *Halimeda* (ERICSON *et al.*, 1952) or red algae (GEORGE and HIGGINS, 1979). With few exceptions (MOORE, 1963; MENZIES *et al.*, 1967; WIEBE *et al.*, 1976), the importance of macrophytic POM as an energy source for the deep-sea benthos has been discounted (JOHNSON, 1974; HONJO, 1978; HINGA *et al.*, 1979; HONJO *et al.*, 1982).

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Macrophyte fragments may be moved downwards by submarine landslides (slumping) and turbidity currents (HEEZEN *et al.*, 1955; ANIKOUCHINE and LING, 1967; WOLFF, 1979), but also by vertical migration of pelagic organisms (HINGA *et al.*, 1979; ROWE and GARDNER, 1979). Horizontal transport over long distances may occur by surface currents (MENZIES *et al.*, 1967) or in nepheloid layers (MCCAVE, 1984). Macrophytic detritus is known as a source of food for deep-sea benthic echinoderms such as echinoids (HEEZEN, 1955), Ophiuroidea (SCHOENER and ROWE, 1970) and sea urchins (SUCHANEK *et al.*, 1985), and also for herbivorous nereide polychaetes (WOLFF, 1979). But it has been impossible to assess the actual significance of such observations for benthic energy budgets (STOCKTON and DELACA, 1982).

Recent observations from deep-sea sediments of the Antarctic Ocean suggested that detrital food chains based on macroalgae are likely to play a significant role for the deep-sea benthos of high latitudes, given the extreme seasonality of phytoplankton sedimentation. With respect to the vegetation of perennial macroalgae (ZANEVELD, 1966a; FURMANCZYK and ZIELINSKI, 1982; DIECKMANN *et al.*, 1985) and the hydrographic conditions in the Bransfield Strait and northwest Weddell Sea (DEACON, 1979; WITTSTOCK and ZENK, 1983), this area appeared to be a favourable location to investigate the fate of macroalgal debris and its potential participation in deep-sea benthic food chains. The efficiency of detritus food chains based on red and brown macroalgae in the Antarctic marine environment has recently been confirmed (REICHARDT and DIECKMANN, 1985). Further observations made during a cruise of R.V. *Polarstern* (ANT II-3) indicate that benthic macroalgae are also available to deep-sea infauna.

MATERIALS AND METHODS

Box cores were taken from the Gibbs Island Basin in the northwest Weddell Sea (Sta. 233, 61°38.10′S, 54°46.82′W) at 2280 m water depth, and from the King George Basin in the Bransfield Strait (Sta. 274, 62°28.5′S, 57°46.82′W) at 1570 m water depth. The sediment column was entirely bioturbated down to a depth of 8–10 cm, and was devoid of any macroalgal fragments on the surface (Fig. 1).

The Reineck-type box core sampler had a maximal depth of penetration of 50 cm. Using metal boxes to punch out the sediment under a surface area of 116 cm^2, duplicate 30 cm long subcores (total volume: 3.48 dm^3) were obtained, sieved through a 1 mm sieve and preserved in 4% formaldehyde-seawater. Further subsamples were taken in plexiglass tubes (diameter: 6 cm), frozen and lyophilized for chemical analyses including carotenoid pigments. Algal fragments were collected and used for determination of ash-free dry weight, total organic carbon and nitrogen as well as for acridine orange epifluorescence microscopy (REICHARDT and DIECKMANN, 1985).

RESULTS AND DISCUSSION

The sieved residues contained fragments of brown and red algae (Fig. 2) amounting to 22.9 g m^{-2} (10.0 g m^{-2} ash-free dry wt) and 20.8 g m^{-2} (or 8.4 g m^{-2} ash-free dry wt) at Stas 233 and 274, respectively (Table 1). The fragments were tentatively classified according to microscopic details and HPLC-analysis of their carotenoid pigments (OLIE, unpublished data).

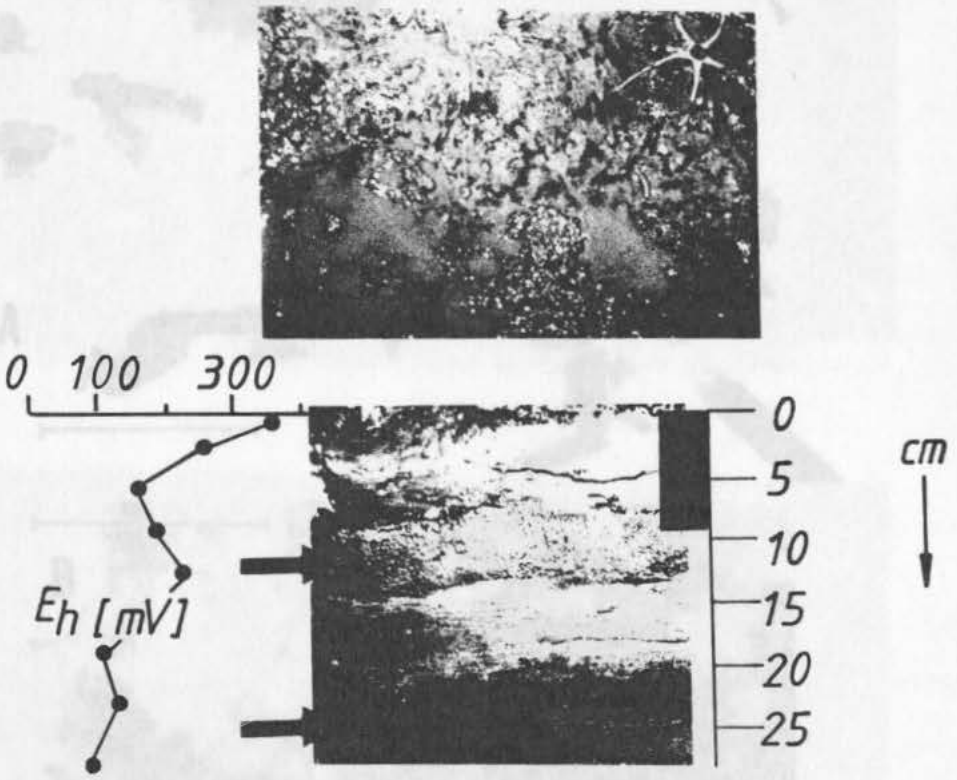

Fig. 1. Surface (top) and vertical profile (bottom) of sediment at 2280 m (Sta. 233). The black field marks depth of most intensive bioturbation; two turbidite layers are indicated by arrows.

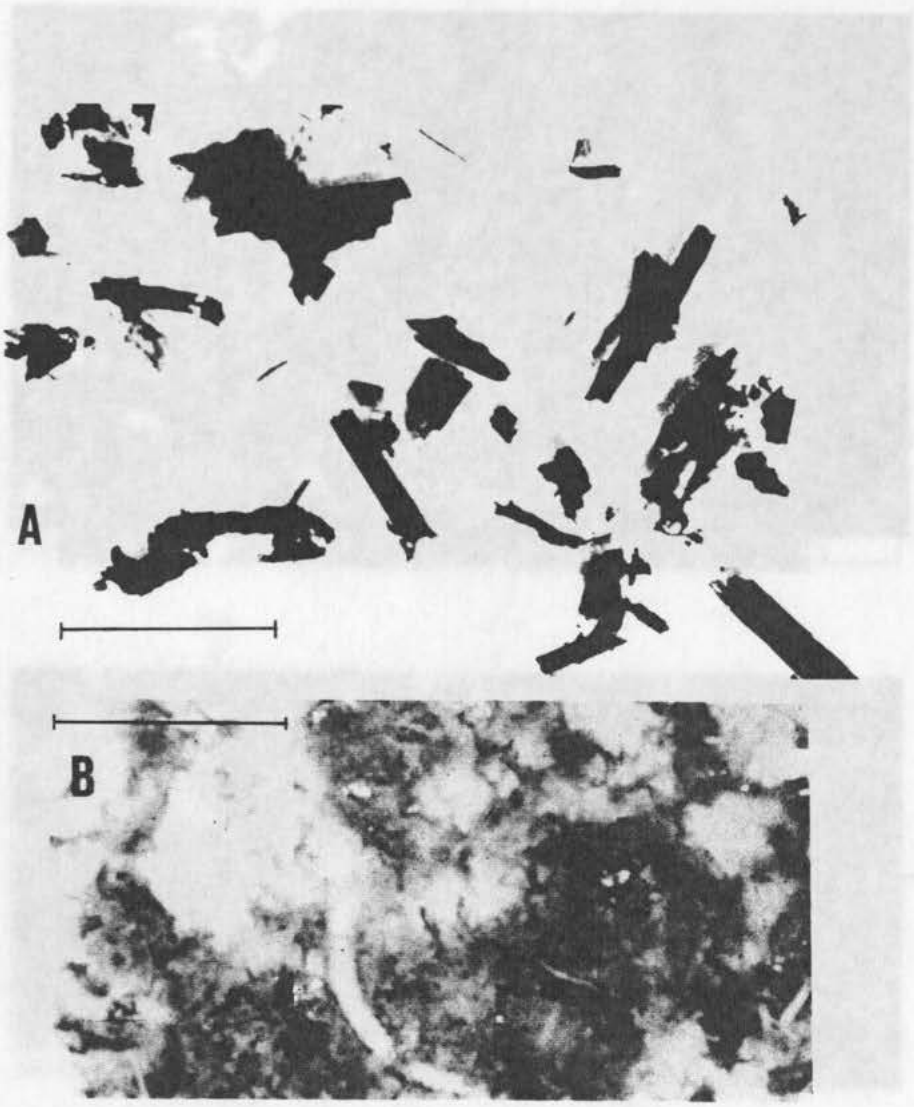

Fig. 2. Macroalgal fragments buried in sediment at (A) 2280 m (Sta. 233) and (B) 1570 m (Sta. 274). Bar = 1 cm.

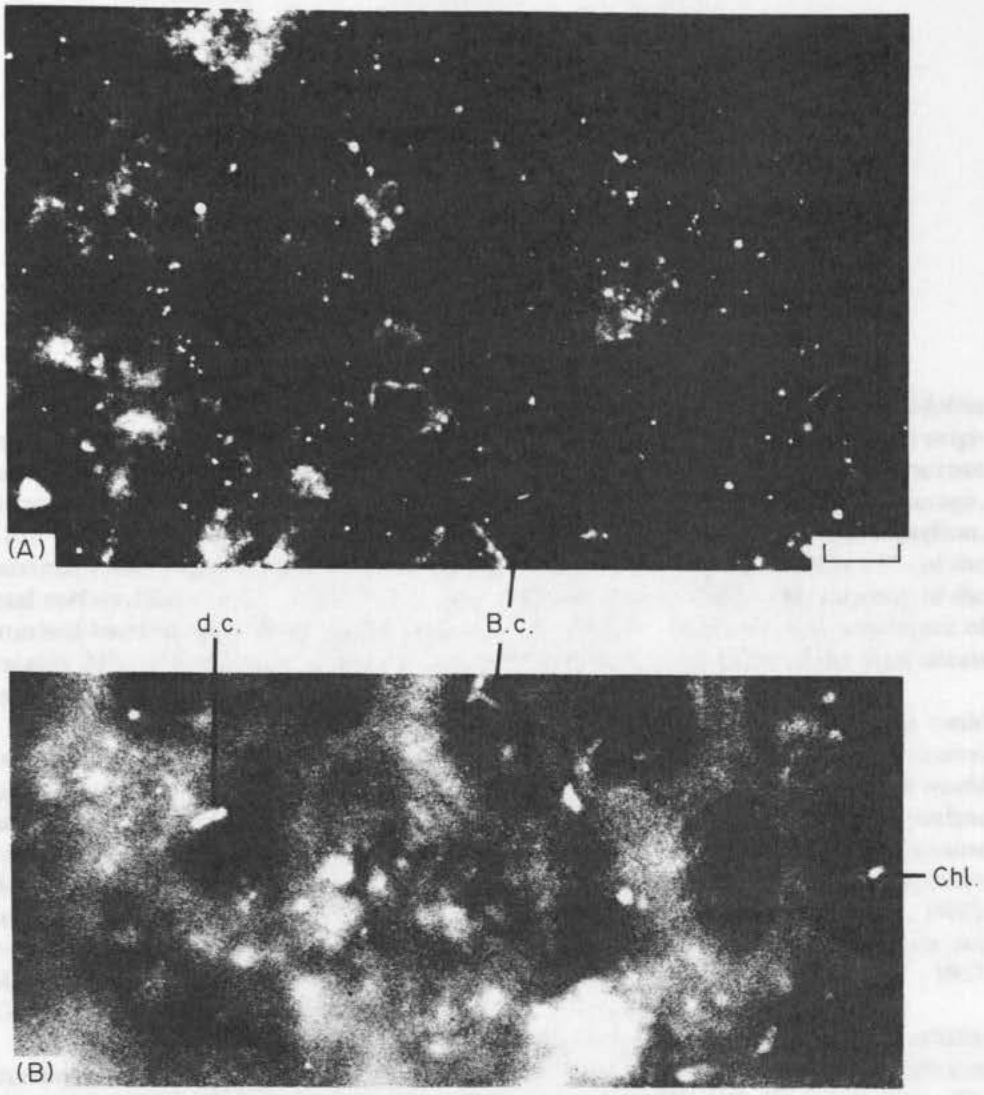

Fig. 3. Epifluorescence microphotographs of macroalgal fragments at 2280 m (Sta. 233) with autofluorescent chloroplasts (Chl) and acridine orange stained epiphytic bacteria (Bc). d.c., dividing cell. Bar = 10 μm.

Table 1. Description of sediment samples containing macroalgal fragments under their surface

	Station 233	Station 274
Location	61°38.1'S 54°46.8'W Gibbs Island Basin	62°28.5'S 57°37.3'W King George Basin
Water depth (m)	2280	1570
Predominant infauna (extrapol. number per m^2)	Polychaetes (173) (Ampharetidae, Capitellidae)	Polychaetes (260) (Maldanidae) Bivalves (78)
Macroalgal fragments >1 mm		
(a) g m^{-2} of ash-free dry weight	(a) 10.0	8.4
(b) C : N ratio	(b) 17.8	17.6

Thick, sturdy fragments from brown algae (most likely: *Himantothallus grandifolius* and *Desmarestia menziesii*) prevailed at Sta. 233, while membranaceous material originating mainly from red algae was predominant at Sta. 274 (Fig. 2). Epifluorescence microscopy revealed intact chloroplasts in both types of fragments. On the average, 0.8% of the total algal surface was covered by attached bacteria. For comparison, maximal values reported for bacterial colonization of macroalgae amount to 15% of the total surface (KOOP *et al.*, 1982; REICHARDT and DIECKMANN, 1985). The majority of the attached bacteria were short, partly coccoid, rods (Fig. 3). However, the occurrence of roughly 20% of dividing or elongated cells indicated that colonization of the algal debris by fast-growing bacteria had not ceased (Fig. 3).

The sediment at Sta. 233 was characterized by turbidite layers (Fig. 1). This could mean that the algal fragments had been carried by turbidity currents or submarine landslides (HEEZEN *et al.*, 1955; ANIKOUCHINE and LING, 1967). Such a mechanism would help to explain the burial of the plant material. Subsurface accumulation of macroalgae rarely has been reported or discussed before (HEEZEN *et al.*, 1955). This may be, because previous accounts of macrophytic material in the deep sea have been based on either sediment traps (WIEBE *et al.*, 1976; HONJO, 1978; HINGA *et al.*, 1979; HONJO *et al.*, 1982), bottom photography (MENZIES *et al.*, 1967; MENZIES and ROWE, 1969; SCHOENER and ROWE, 1979; ROWE and STARESINIC, 1979) or trawls and dredges (MENZIES *et al.*, 1967; GEORGE and HIGGINS, 1979).

Even if the macroalgal fragments had been carried and deposited with turbidity currents or slumping, most intensive bioturbation of a well-developed infauna indicated that biological transport mechanisms were also involved in the burial process. The infauna consisted predominantly of polychaetes (Ampharetidae and Capitellidae) at Sta. 233, and maldanide polychaetes (*Clymenella*) as well as different bivalves at Sta. 274 (Table 1). Burial and even "gardening" of macroalgae by polychaetes and bivalves are well documented for shallow coastal sediments (DALY, 1973; WOODIN, 1974, 1977; HYLLEBERG, 1975; HYLLEBERG and HENRIKSEN, 1980). In deep-sea environments, such feeding habits have not been reported, but they are particularly plausible at high latitudes in view of the highly seasonal energy supply via phytoplankton sedimentation. A specific form of "gardening" could have occurred by repeated ingestion and bacterial recoloniza-

tion of the algal fragments thus serving as a matrix for bacterial enrichment. According to C:N ratios (>17:1, Table 1) and bacterial colonization patterns (Fig. 3), previous utilization of the fragments as food seemed likely.

Initial stages of detritus from "Antarctic kelp" *Himantothallus grandifolius* were characterized by a low number of small, usually coccoid, epiphytic bacteria, whereas during advanced maturation, bacterial surface growth was stimulated and the frequencies of both dividing and larger bacterial cells increased (Reichardt and Dieckmann, 1985). These latter properties were also noted in the deep-sea samples; however, the overall cell densities were much lower. The deep-sea algal fragments had intermediate C : N ratios as compared with deep-sea trap data ranging from 8 to 33 (Hinga *et al.*, 1979; Rowe and Gardner, 1979; Honjo, 1980; Billett *et al.*, 1983). The value of 17.8 for brown algal fragments from Sta. 233, however, was high compared with those obtained with detritus derived from the same group of brown algae (Reichardt and Dieckmann, 1985). Both these results indicate that a considerable amount of the bacterial colonizers may have been stripped off from the deep-sea algal material, though the capacity to support the growth of fast-growing epiphytic bacteria was maintained.

Based on trap experiments, the relative contribution of large plant debris to the tidal POC flux usually has been considered as negligible (Honjo, 1978; Honjo *et al.*, 1982). In regions with extremely high macroalgal production, the maximum input of pelagic *Sargassum* into the deep sea amounts to 0.4 g C m^{-2} y^{-1}, or just 1/10 of the total POM caught in traps (Rowe and Staresinic, 1979). On the other hand, given the large POM flux at the Weddell Sea station 233 (2.2 g C m^{-2} y^{-1} according to sediment trap data of Von Bodungen *et al.*, 1986), accumulation of large algal debris (Table 1: 10 g of organic matter, or roughly 5.5 g of carbon) would have required considerably longer than $(5.5 : 2.2)^{\cdot} = 2.5$ years, which were the lower time limit for accumulation of POC, i.e. if this consisted 100% of macroalgal fragments. As this result appears most unlikely, it must be concluded that traps are inappropriate for recording the described transport of large fragments to the deep-sea benthic environment.

According to *in situ* measurements of oxygen consumption, total energy requirements of deep-sea benthos are estimated to be 40 mg C m^{-2} d^{-1} (Wiebe *et al.*, 1976). Under those conditions, the macroalgal fragments from Sta. 233 (10 g of organic matter) would be expected to provide the total benthic energy requirements for approximately 125 days. As yet, however, the energy requirements of the benthos at this Antarctic station are unknown. Therefore it remains unclear at which rate the macroalgal debris must be decomposed to keep pace with the heterotrophic energy consumption *in situ*.

Food chain relationships were further complicated by the occurrence of turbidites at Sta. 233. Hence, preservation of the debris in certain protected sediment layers should also be taken into account. Algal debris from Sta. 274 where no turbidites had been found, exhibited an advanced state of degradation (Fig. 2). As a result of decomposition processes that are characterized by a bacterial loop, macroalgal fragments are first leached (see Fig. 2) and then very slowly solubilized (Newell *et al.*, 1980; Koop *et al.*, 1982).

In conclusion, the input of macroalgal debris can constitute a substantial supply of energy to certain patches of the Antarctic sea floor. The availability of perennial macroalgae to food chains is much less dominated or limited by seasonal growth patterns as compared with the production of phytoplankton in the Antarctic Ocean (Zaneveld, 1966b). Therefore benthic detritus food chains based on macroalgae should reflect a less

seasonal periodicity than benthic responses to phytoplankton blooms (Deuser *et al.*, 1981; Billett *et al.*, 1983; Smith and Baldwin, 1984). Extended screening of Antarctic deep-sea sediments during all seasons for macroalgal fragments and their associated infauna is needed in order to draw further conclusions with respect to an overall budget.

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Differential temperature effects on the efficiency of carbon pathways in Antarctic marine benthos

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ABSTRACT: Enzymatic activities and metabolic rates, which play key roles in benthic carbon turnover, were characterized with respect to cold adaptation in aphotic Antarctic sediments. Temperature optima for CO_2 dark fixation and mineralization of glucose coincided largely with the maximal growth temperatures of obligate psychrophilic bacteria (at 20 °C and below). On the other hand, predominantly extracellular enzyme activities involved in the conversion of particulate organic matter (POM) into dissolved organic matter (DOM), such as scleroprotease and chitinase, had considerably higher temperature optima (40 to 55 °C). This lack of cold adaptation at the activity level was less strongly expressed in other hydrolases (sulfatase, alkaline phosphatase). Substrate affinities or activation energies were often inapplicable or insignificant as a potential measure of temperature adaptation. Alternative strategies of cold adaptation may be relevant at the level of synthesis of POM-solubilizing enzymes.

INTRODUCTION

As more than 90 % of the marine environment is permanently exposed to temperatures below 5 °C, the efficiency of biogeochemical cycles is affected by the extent of cold adaptation of their key processes. To understand the enzymatic control of carbon turnover at permanently low temperatures, it is important to know whether different biogeochemical pathways are dependent on different degrees of cold adaptation.

Numerous investigations have dealt with autecological aspects of cold adaptation (e.g. Arnaud 1977, George 1977, Baross & Morita 1978, Somero et al. 1983). However, little is known about the temperature characteristics of key biogeochemical processes in stable cold marine environments such as the Antarctic Ocean (Pomeroy et al. 1969, Holm-Hansen et al. 1977, Morita et al. 1977).

Utilization of dissolved organic matter (DOM) by Antarctic marine microheterotrophs exhibits a temperature dependence similar to growth rates of psychrophilic bacteria (Gillespie et al. 1976, Baross & Morita 1978, Hodson et al. 1981). An even more effective temperature acclimation seems to apply to photosynthetic processes (Neori & Holm-Hansen 1982, Li et al. 1984). Although the input of detrital particulate organic matter (POM) is extremely important for carbon budgets of aphotic sediments, information on temperature characteristics of the primary enzymatic processes involved in POM degradation is largely lacking. Investigations in freshwater environments seem to indicate that conversion of organic detritus particles to DOM is primarily a temperature-controlled process (Godshalk & Wetzel 1977).

It is conceivable that benthic carbon budgets are severely affected and controlled by different temperature adaptation on subsequent biogeochemical pathways. Lack of temperature compensation at the level of primary decomposition processes could turn marine sediments into sinks for POM. The permanently cold Antarctic benthic environment offered an ideal study site to determine the extent of physiologically extreme temperature adaptation for major pathways of the carbon cycle.

Parameters selected to characterize pertinent catabolic and anabolic processes of the sediment biota included POM-solubilizing and hydrolyzing enzyme activities in sediment extracts as well as *in vivo* rates of glucose mineralization and dark fixation of carbon dioxide. Temperature effects were studied with respect to both temperature optima and kinetic constants.

Poor cold adaptation was noted for the activities of hydrolytic enzymes related to key primary decomposition processes. Hence, decomposition of organic matter

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in the permanently cold benthic environments is likely to be restricted ('temperature-controlled') at its initial steps, unless the lack of enzymatic adaptation is compensated for by an increased production or accumulation of the pertinent enzymes.

MATERIAL AND METHODS

Sampling area. Sediment samples were obtained with a modified Reineck box core sampler on 2 cruises of RV *'Polarstern'* in the Bransfield Strait and NW Weddell Sea, Antarctica, from 28 Nov to 18 Dec 1983, and from 18 Nov to 4 Dec 1984 (Table 1).

Experiments. Subsamples were punched out with plexiglass tubes (6 cm inner diameter) for shipboard experiments in a refrigerated (0 °C) laboratory container. To obtain vertical activity profiles, sediment slices of 1 cm thickness were incubated at *in situ* temperatures (0 °). Material from the surface layers (0 to 2 cm) was used in temperature gradient experiments.

These were carried out in triplicate with duplicate blanks using a series of temperature-controlled water baths. These were run over both a narrow (−0 5 to 20 °C) and a wide (0 to 65 °C) temperature range at intervals of 3 to 4 C° and 5 to 10 C°, respectively. For comparison with environments that are only seasonally cold, late winter samples from Kiel Bay sediment (1 to 2 °C) were used in similar experiments.

Enzyme activities. Enzymatic activities were measured after cold extraction (30 min at 0 °C on a shaking machine) of 20 cm^3 of sediment with 80 ml of 2 % triton-X100 containing 2 g l^{-1} of polyvinylpyrrolidone in tris-HCl (10 mM, pH 7.5)-buffered artificial seawater. The following enzyme assays were selected because of the significance of these enzymes in the decomposition of organic detritus as well as for their feasibility under shipboard conditions.

Table 1. Survey of box core stations

Station number	Water depth (m)	Coordinates	
28 Nov–18 Dec, 1983			
270	113	62° 47 S	55° 24 W
249	244	60° 43 S	45° 48 W
264	468	61° 57 S	45° 60 W
254	4455	63° 57 S	44° 04 W
225	1948	62° 16 S	57° 38 W
233	2297	61° 38 S	54° 47 W
18 Nov–4 Dec, 1984			
A	1977	62° 20 S	57° 50 W
B	504	62° 14 S	58° 18 W
G	460	62° 09 S	58° 24 W
I	92	63° 12 S	58° 47 W
H	70	63° 15 S	56° 51 W

Activities of POM-solubilizing proteolytic, chitinolytic, and agarolytic enzymes were determined using dye release techniques described elsewhere (Rcichardt 1986). Following incubation for up to 20 h of 10 mg amounts of the solid substrates that were labelled with 'reactive' dyes, in 3 ml aliquots of the enzyme extract, the assay mixture was centrifuged (15 min at 6000 g) and the absorbance (660 nm) of the dissolved dye-labelled depolymerization products in the supernatant measured photometrically. Blanks were treated with 1 ml of 16 % formaldehyde.

Activities of alkaline phosphatase and sulfatase in the triton-X100 extracts were derived from the hydrolysis of 2 mM of *p*-nitrophenylphosphate and *p*-nitrophenylsulfate, respectively, (Morita & Howe 1957, Reichardt et al. 1967, King & Klug 1980) after incubation periods of 0.5 to 12 h. After the addition of 1 ml of 1 N NaOH the absorbance of *p*-nitrophenol was determined spectrophotometrically at 410 nm. Blanks contained 4 % formaldehyde.

***In vivo* rates.** Mineralization rates for ^{14}C (U)-glucose or equimolar amounts of glucose labelled in positions C-1 or C-3 in other experiments were determined in 50 ml serum flasks with 2 ml aliquots of sediment slurry, i.e. suspension of 20 cm^3 of sediment in 80 ml of 0.1 µm membrane filtered Antarctic seawater. The flasks were sealed with rubber serum bottle caps fitted with plastic rod and cup assemblies containing a fluted strip of Schleicher & Schüll chromatography paper (Hobbie & Crawford 1969, Morita et al. 1977). The slurries were incubated with 200 µl (9.3 kBq) of ^{14}C-(U)-glucose (New England Nuclear, 144 kBq µmole^{-1}) for 1 to 2 h (depending on the water depth). Samples treated with 4 % of formaldehyde served as blanks. The incubation was terminated by acidification, i.e. injection of 1 N H_2SO_4 to reach a final pH of 2.0, followed by injection onto the paper strips of 0.15 ml of phenethylamine as CO_2 absorbent. Flasks were put on a rotary shaker for 20 min and left for at least 6 h, before the paper strips were removed and placed in a toluene based scintillation fluid (Omnifluor, New England Nuclear). Radioactivity of the trapped $^{14}CO_2$ was measured in Beckman LS 100 and LS 1800 liquid scintillation counters. Quench corrections were based on external standards and channels' ratios.

Dark fixation rates of carbon dioxide were determined in 2 ml of sediment slurry, i.e. suspension of 20 cm^3 of sediment in 80 ml of filter-sterilized Antarctic seawater, using capped 10 ml polypropylene centrifuge tubes. From ampoules containing 370 kBq per ml of $NaH^{14}CO_3$ (specific activity = 2183 kBq µmole^{-1}) in 1.5 mM of non-radioactive $NaHCO_3$ adjusted to pH 10.2 with NaOH, 0.25 ml aliquots were mixed with

the slurry and incubated in complete darkness for 1 to 4 h, depending on the water depth. Blanks were poisoned with 20 mM iodoacetamide. Incubation was terminated by adding 0.1 ml of concentrated formaldehyde and 1 N H_2SO_4 sufficient to lower the pH to 2.0. After 2 rinses with ca 8 ml of sterilized seawater and subsequent centrifugation (15 min at 6000 g) the pellets were dried at 60 °C and stored in a freezer.

Weighed amounts were combusted using an automatic Packard ^{14}C-sample oxidizer for liquid scintillation counting. The radioactivity of the assimilated carbon was measured in a Beckman LS 100 liquid scintillation counter. Quench corrections were based on external standard and channels' ratios. Concentrations of total carbonate being available during the assay were derived from carbonate alkalinity according to Gargas (1980).

RESULTS

Vertical distribution patterns in the sediments indicated that most of the carbon turnover activities tested were limited to the bioturbated surface layers (1 to 10 cm, Fig. 1). In assays carried out at an approximate *in situ* temperature of 0 °C, activities of POM-solubilizing enzymes such as scleroprotease and chitinase were usually at or below their detection limits. Maximal levels of these enzymes as found at Stn 233 were correlated with high concentrations of organic matter (41 mg ash-free dry weight per g dry weight). A turbidite layer containing 20 mg of ash-free dry weight per g dry weight at 15 cm depth lacked any detectable activity. Obviously cell-free enzymes had not been spread beyond their production sites. Except for scleroprotease, largely parallel trends were noted for both catabolic and anabolic reactions. According to this distribution pattern which recurred basically at all sampling stations, the 0 to 2 cm surface layers were chosen to collect the presumably most active material for experiments in temperature gradients.

High thresholds of detection for POM-solubilizing enzymes at ambient temperatures were closely related to their temperature characteristics (Fig. 2 & 3). Scleroprotease showed the steepest increase before reaching its temperature optimum between 40 and 55 °C (Fig. 2). Similar temperature characteristics were noted for chitinase (Fig. 3) and agarase (not shown), although increments of activity at suboptimal temperatures were here less pronounced.

Cold adaptation of potentially extracellular hydrolases from Stn 264 was most pronounced for alkaline phosphatase with a temperature optimum around 30 °C (Fig. 4) compared with 45 °C for sulfatase (Fig. 5). Arrhenius constants for phosphatase and sulfatase amounted to 91.7 and 52.8 kJ $mole^{-1}$, respectively.

Carbon dioxide evolution, representing mineralization as the final process of organic matter decomposition, was characterized by temperature optima around 20 °C or lower (Fig. 6). This is far below the optimal temperature range of primary decomposition processes.

Even stronger cold adaptation with temperature optima around 10 °C was noted for dark fixation of CO_2. In contrast to mineralization, temperature characteristics of this anabolic process showed considerable variations between different stations (Fig. 7).

When incubated at 2 and 20 °C in a gradient of suboptimal substrate concentrations, none of the

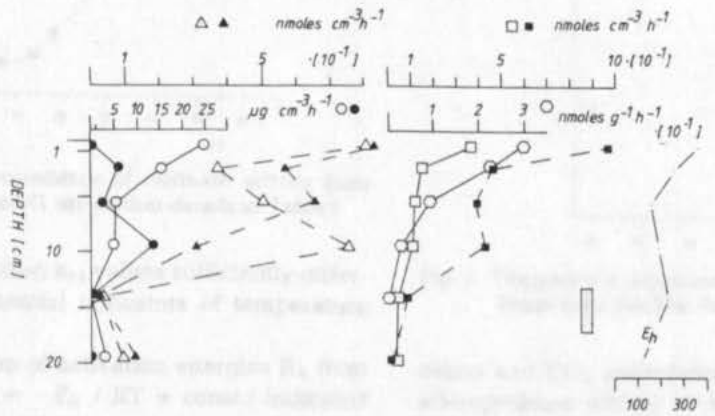

Fig. 1. Vertical profiles of different microbial activities (at 0 °C) contributing to carbon turnover in bioturbated sediment at 2297 m water depth (Stn 233). Chitinase (○), scleroprotease (●), alkaline phosphatase (△), sulfatase (▲), mineralization rates for glucose (□) and acetate (■), rates of CO_2 dark fixation (○). Position of turbidite indicated by small dotted column, redox potential (Eh)

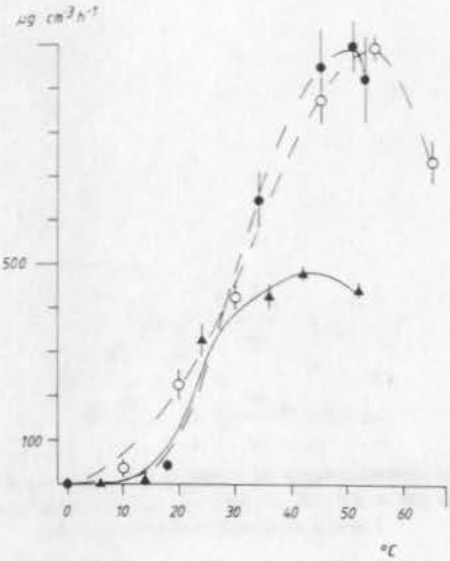

Fig. 2. Temperature dependence of scleroprotease activity from Stns 249 (○), H (●), and G (▲); further details in Table 1

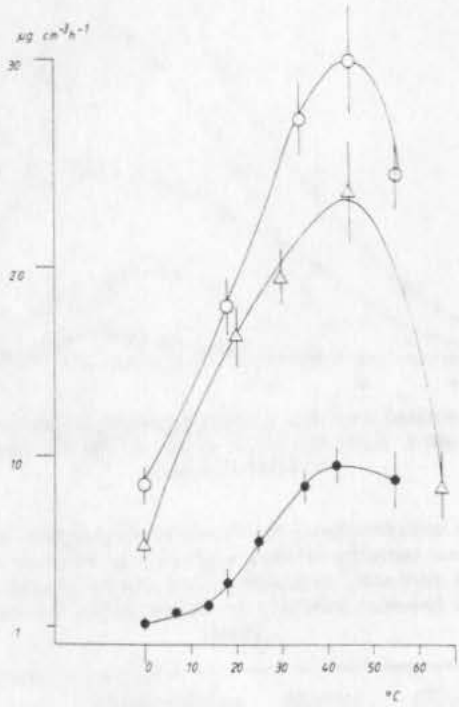

Fig. 3. Temperature dependence of chitinase activity from Stns 249 (△), H (○), and G (●); further details in Table 1

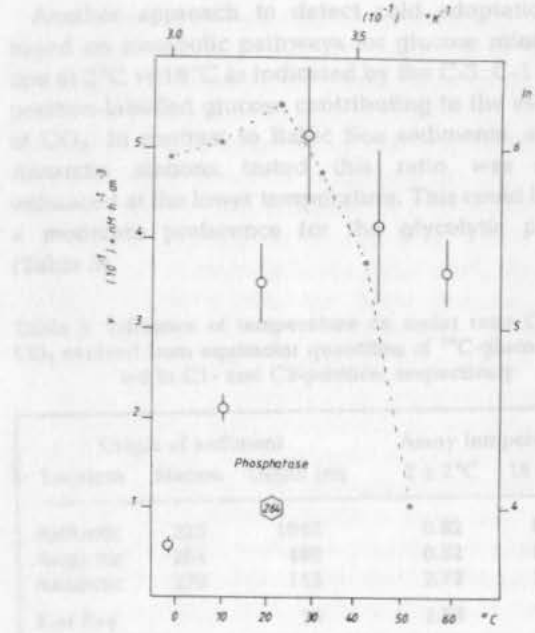

Fig. 4. Temperature dependence with Arrhenius plot for alkaline phosphatase from Stn 264; further details in Table 1

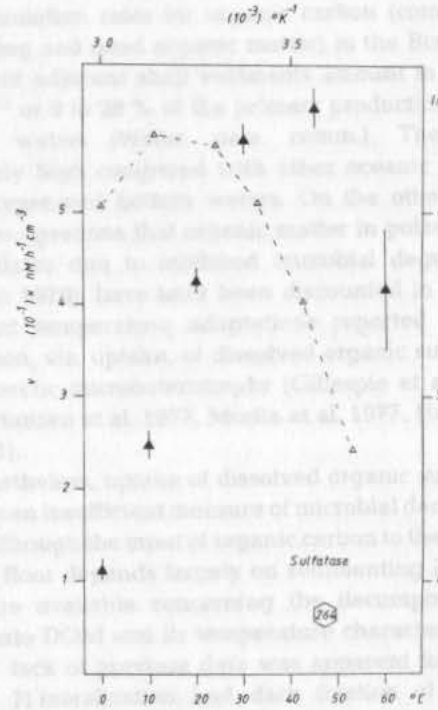

Fig. 5. Temperature dependence with Arrhenius plot for sulfatase from Stn 264; further details in Table 1

activities tested exhibited k_M values sufficiently different to qualify as potential indicators of temperature adaptation.

Likewise, calculation of activation energies E_A from Arrhenius plots ($\ln k = -E_A / RT + \text{const.}$) indicated insignificant differences between sediments from the Antarctic and the Baltic Sea, as far as glucose mineralization and CO_2 assimilation were concerned. Yet, for scleroprotease activity in Antarctic sediments, E_A-values were significantly higher, suggesting an unexpected 'negative' cold adaptation (Table 2).

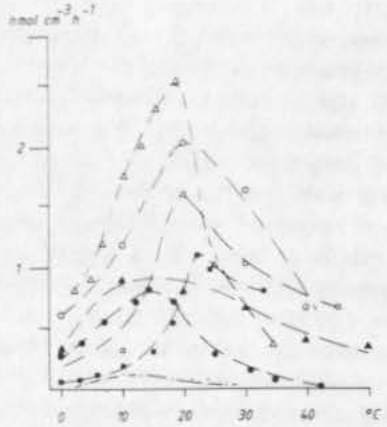

Fig. 6. Temperature dependence of mineralization rates for glucose at 7 stations: 225 (▲), 249 (○), 254 (□), A (■), G (△), H (●), I (•); further details in Table 1

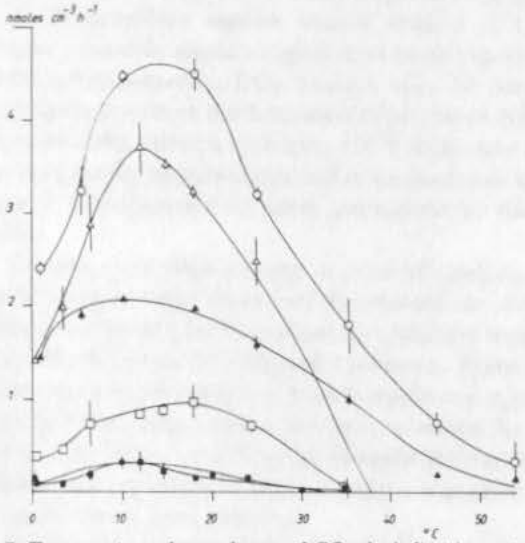

Fig. 7. Temperature dependence of CO_2 dark fixation rates at 6 stations: 254 (■), A (▲), B (□), G (△), H (□), J (●); further details in Table 1

Table 2. Temperature optima (T opt.) and activation energies (E_A) for selected catabolic and anabolic activities involved in carbon turnover in Antarctic sediments. Data from Kiel Bay sediment for end of winter (in brackets) included for comparison

	Scleroprotease activity	Glucose mineralization	CO_2-dark assimilation
Number of stations	10 (2)	9 (2)	8 (2)
T_{opt} (°C)			
Mean value	49 ± 7	17 ± 5	16 ± 7
SD	(50 ± 14)	(30 ± 3)	(20 ± 3)
E_A (kJ mole^{-1})			
Mean value	209 ± 157	44 ± 23	47 ± 10
SD	(37 ± 18)	(42 ± 27)	(50 ± 9)

Another approach to detect cold adaptation was based on metabolic pathways for glucose mineralization at 2 °C *vs* 18 °C as indicated by the C-3 : C-1 ratio of position-labelled glucose contributing to the evolution of CO_2. In contrast to Baltic Sea sediments, at the 3 Antarctic stations tested this ratio was slightly enhanced at the lower temperature. This could indicate a moderate preference for the glycolytic pathway (Table 3).

Table 3. Influence of temperature on molar ratio C3 : C1 of CO_2 evolved from equimolar quantities of ^{14}C-glucose labelled in C1- and C3-position, respectively

Origin of sediment			Assay temperature:	
Location	Station	Depth (m)	2 ± 2 °C	18 ± 2 °C
Antarctic	225	1948	0.82	0.75
Antarctic	264	468	0.52	0.40
Antarctic	270	113	2.72	2.33
Kiel Bay		18	1.03	1.35

DISCUSSION

Accumulation rates for organic carbon (comprising both living and dead organic matter) in the Bransfield Strait and adjacent shelf sediments amount to 17 g C m^{-2} yr^{-1} or 9 to 28 % of the primary production in the surface waters (Wefer, pers. comm.). These are extremely high compared with other oceanic regions with oxygenated bottom waters. On the other hand, earlier suggestions that organic matter in polar waters accumulates due to inhibited microbial degradation (Sorokin 1970), have later been discounted in view of excellent temperature adaptations reported for the utilization, viz. uptake, of dissolved organic substrates by Antarctic microheterotrophs (Gillespie et al. 1976, Holm-Hansen et al. 1977, Morita et al. 1977, Hodson et al. 1981).

Nevertheless, uptake of dissolved organic substrates alone is an insufficient measure of microbial decomposition. Although the input of organic carbon to the Antarctic sea floor depends largely on sedimenting POM, no data are available concerning the decomposition of POM into DOM and its temperature characteristics. A similar lack of previous data was apparent for carbon mineralization and dark fixation of CO_2 in aphotic Antarctic sediments.

Proof whether the carbon budget is balanced is difficult to obtain. This is also due to the spatial separation of primary production in the euphotic water column and remineralization often taking place at the sea bottom (Müller & Suess 1979). In principal, it is important whether equal efficiencies can be taken for

granted in both biosynthetic and catabolic biogeochemical pathways. To prove this, responses to temperature in particular qualify as an unambiguous indicator of biological activities (Radmer & Kok 1979).

Compared with temperature characteristics of individual enzymes or single organisms, little is known about cold adaptation at the holistic level concerning the major biogeochemical pathways in an ecosystem. Cold adaptation at this level is usually described by temperature optima and temperature-dependent increments of rates or enzyme activities which may be expressed as Q_{10} values or Arrhenius constants (e.g. Baross & Morita 1978, Fuhrman & Azam 1983).

As yet, temperature characteristics of anabolic pathways such as dark fixation of CO_2 have not been reported for Antarctic sediments. This mainly chemosynthetic pathway of primary biosynthesis revealed the highest degree of cold adaptation during the present investigation. Temperature optima usually around 10 °C and below were only slightly higher than those reported for chlorophyll *a*-specific CO_2 fixation rates of Antarctic phytoplankton from the Bransfield Strait (Neori & Holm-Hansen 1982, Tilzer & Dubinsky 1987). Still lower temperature optima (a plateau formed at temperatures higher than 0 °C) are known for Arctic phytoplankton (Li et al. 1984).

Despite their high degree of cold adaptation, CO_2 dark fixation rates were still suboptimal at ambient temperatures and far from showing a uniform temperature characteristic in different sediments. Particularly noteworthy is an extremely high temperature optimum (ca 55 °C) for dark fixation that was recorded for sediment from 1980 m depth in the Bransfield Strait where submarine volcanism is likely to play a role (Reichardt unpubl., Suess pers. comm.).

In Antarctic waters photosynthetic CO_2 fixation and microheterotrophic uptake of organic carbon share similar temperature characteristics (Gillespie et al. 1976, Holm-Hansen et al. 1977, Morita et al. 1977, Hodson et al. 1981). Corresponding data for carbon mineralization rates are lacking. With temperature optima around 20 °C, cold adaptation of glucose mineralization (Fig. 6) was only moderately expressed and less pronounced than previously reported for the uptake of organic carbon.

In psychrophilic bacterial isolates, key catalysts of mineralization such as NADH dehydrogenases possessed temperature optima between 20 and 25 °C (Takada et al. 1981). As psychrophilic bacteria may be considered as the chief microheterotrophs involved in organic matter mineralization in the investigated environment, it is noteworthy that their maximal growth temperatures (Morita 1975) coincide roughly with the temperature optima found for glucose mineralization rates.

Bacterial mineralization of POM requires conversio to dissolved compounds by extracellular solubilizin enzymes. Temperature optima between 45 and 55 °(for proteolytic and chitinolytic activities (Fig. 2 & 3 suggested a striking lack of cold adaptation. This cor curs with the poor cold adaptation of extracellular prc teases as well as chitinases in pure cultures of psy chrophilic bacteria (McDonald et al. 1963, Weimer & Morita 1974, Helmke & Weyland 1986). As compare with sediments from the Baltic Sea, no significar differences were noted with respect to temperatur optima. Increased activation energies for Antarcti scleroprotease indicated even a higher energy barrie for scleroprotein degradation in an environment beinç otherwise better adapted to cold temperature (Table 2). This would strongly support the idea tha conversion of POM to DOM is, more than other path ways of organic matter degradation, limited and con trolled by temperature (Godshalk & Wetzel 1977).

Side reactions such as desulfatation of organic com pounds (Weigl & Yaphe 1966, Fitzgerald 1976) wer(also affected by relatively high temperature optim (45 °C) of sulfatase in Antarctic sediments, althoug 60 °C temperature optima have been reported for sedi ments from lower latitudes (Oshrain & Wiebe 1979). considerably lower optimum (30 °C) characterize alkaline phosphatase from the same source (Fig. 4) Heat-sensitive alkaline phosphatases have also bee isolated from Antarctic marine bacteria (Koberi et a 1984). Consequently, there is evidence for mechanism of cold adaptation of certain hydrolyzing enzymes fro permanently cold environments that imply a down shift of their temperature optima.

Whereas temperature optima provide most essentia clues to thermal efficiencies of enzymatic reaction further aspects of temperature compensation must nc be neglected. Cold adaptation at the kinetic level ma not only affect Q_{10} values or activation energies, bu also substrate affinities for enzymes and their interac tions with allosteric effectors or inhibitors (Hochachk & Somero 1973). Attempts to compare Michaelis-Men ten constants (k_M) for alkaline phosphatase, sulfatas and scleroprotease in sediment extracts incubated at and 20 °C proved useless as long as the contribution t k_M of substrates already present in the extract remained unknown. In corresponding experiment with psychrophilic bacterial isolates, cold-induce increases of the substrate affinities ($1/k_M$) occurre occasionally, but increments were smaller than 1 orde of magnitude. Obviously, a more extended kineti approach is needed to evaluate the quantitative an qualitative changes affecting both enzymes and sub strates as a result of the *in situ* physico-chemical cond tions in sediments, where sorption plays a predominar role.

It has been postulated that temperature compensating changes affecting the enzyme activity are primarily associated with pathways of energy production (Hazel & Prosser 1974, Somero 1978). The differences noted for glucose mineralization in Antarctic and Baltic Sea sediments (Table 2) support this view.

For decomposition by extracellular enzymes, on the other hand, alternative pathways of temperature adaptation may be hypothesized. Instead of changing enzyme activities qualitatively, acclimation may be achieved by increasing the production of enzymes or iso-enzymes. Levels of scleroprotease, chitinase, alkaline phosphatase or sulfatase in sediments from the Antarctic and the Baltic, however, did not suggest that accumulation of enzymes was much stronger in the permanently cold environment (Reichardt 1986, unpubl.). Nevertheless, considering the inherent patchiness in these ecosystems, comparing activity levels in sediments appears to be an extremely insufficient approach to estimate enzyme production rates. On the other hand, there is experimental evidence for increased production of scleroprotease and, partly, chitinase at *in situ* temperatures by psychrophilic bacteria from investigated Antarctic sediments (Reichardt unpubl.). The existence of such mechanisms, which counteract a lack of temperature compensation at the activity level, may also be postulated for corresponding extracellular enzymes in the sediment.

Furthermore, acclimation to cold temperatures may be acquired by changing the pathways for key metabolic processes (Hochachka 1967). In particular, work on psychrophilic bacteria suggests a reduction of the electron transport system in favor of fermentative pathways (Rüger 1984). Limited proof of the significance of such mechanisms in permanently cold marine sediments can be gained from radiorespirometry using glucose labelled with ^{14}C in different positions (Katz & Wood 1960, Wang 1967). First results suggested in fact a slightly increased generation of CO_2 from the C-3 position at the lower temperature when the assays were carried out with Antarctic sediments (Table 3). Detailed enzymatic investigations are necessary to corroborate this indication of metabolic cold adaptation of fermentative pathways. As far as differences between glucose respiration in euhaline Antarctic sediments and estuarine Kiel Bay sediments are concerned, an increasing contribution of the Embden-Meyerhof pathway (C-3/C-1 > 1) may be expected at low salinities (Griffiths & Morita 1973).

Irrespective of thermal characteristics, there were fundamental differences between single depolymerizing enzyme activities involved in primary decomposition processes and the complex enzymatic machinery being responsible for CO_2 turnover. The largely membrane-bound functions of the latter required *in vivo* measurements, whereas extracellular enzymes had to be extracted from the sediment. Extracellular enzymes can be stabilized and survive in sediments over long time periods (Skujins & McLaren 1968, Nissenbaum & Serban 1987).

As discussed above, lacking cold adaptation at the activity level may be counterbalanced by cold-adapted enzyme synthesis. Due to various mechanisms of enzymatic regulation, interferences from variables other than temperature are likely to be expected in this case (Somero et al. 1983).

In conclusion, the consequences of the apparent lack of cold adaptation among certain extracellular POM-degrading enzyme activities become less severe, as the benthic carbon flow is controlled by temperature-independent mechanisms. This does not preclude, however, that, under certain circumstances, decomposition of detrital POM becomes a temperature-controlled process as stated by Godshalk & Wetzel (1977).

Four of the Antarctic stations sampled were at water depths below 1000 m where the impact of deep-sea hydrostatic pressure has to be taken into account. Under these conditions decomposition of POM is extremely slow, unless mediated by macrofaunal disruption and digestion (Jannasch et al. 1971, Sieburth & Dietz 1974, Hargrave 1976, Jannasch & Wirsen 1983). While biochemical adaptations to the hydrostatic pressure regime in the deep sea are well documented (Yayanos et al. 1979, Cahet & Sibuet 1986, Helmke & Weyland 1986), recalcitrance of sedimenting POM increases with residence time in the water column (Rice 1982) and with increasing water depth (Wakeham et al. 1984). In any case, solubilization rates for POM depend on the number of attachment sites for pertinent enzymes on the surface of their particulate substrates (Reese 1977). It is therefore conceivable that in the heavily bioturbated Antarctic seabottom, reworking of sediments and their POM load by burrowing infauna may play an even greater role for the efficiency of POM degradation than enzymatic cold adaptation at the activity level.

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Microbiological Aspects of Bioturbation

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Summary

Enhanced rates of microbial chemoautotrophic production in subsurface sediments are often linked to infauna burrows. Two independent methodological approaches revealed that bacterial populations that should be responsible for increased carbon dioxide dark fixation in burrow walls of the polychaete worm Nereis diversicolor, showed no enrichment effects. By using phospholipid fatty acid and hydroxy fatty acid biomarkers as biomass-specific signature compounds, it became clear that, in contrast to phototrophic eukaryotes (biomarker: ω20:5 3), bacteria including sulfate reducers (biomarker: w10 Me 16:0) as well as microfauna (biomarker: 20:5ω6) did not accumulate in the 1.5 mm thick superficially oxidized burrow walls. On the other hand, evidence for strong grazing was indicated by high levels of poly-ß-hydroxybutyrate (poly-ß-hydroxyalkanoates), a bacterial storage product, in the wall layer. This was in agreement with increased levels of partly extracellular enzyme activities such as scleroprotease and sulfatase.

Introduction

A variety of chemical changes in marine sediments as a result of macrofaunal activity are usually summarized as "bioturbation". Due to reworking of sediments by burrowing infauna, patterns of vertical stratification are destroyed. New structures such as burrows and tubes are formed. These function as barriers for the diffusion of dissolved nutrients and gases. The chemical environment is locally altered by the metabolic activities of sediment-inhabiting macro-invertebrates. By irrigation and aeration of subsurface sediment layers by particle transport as a feature of different feeding habits, by the excretion of nutrients and metabolites and by defecation, the burrowing macrofauna is able to form new potential niches for the enrichment of a variety of physiologically diverse microorganisms

(f.e. Yingst & Rhoads, 1980; Lee & Swartz, 1980).

Whereas bioturbate structures such as burrow walls and fecal casts have been recognized as potential enrichment sites for sediment bacteria (Driscoll, 1975; Yingst & Rhoads, 1980; Aller & Yingst, 1985; Kemp, 1987) quantitative evidence for this enrichment is scarce. This poor evidence may be attributed to strong grazing pressure that would keep bacterial populations at a relatively low density level. Yet, the number of pertinent studies is low, partly, because quantitative assessments of bacterial populations in sediments are hampered by methodological problems (White, 1985). Extreme aspects of the impact of marine sediment macrofauna on bacteria range from "gardening" (Hylleberg, 1975; Gerlach, 1978) to the excretion of antibiotic compounds (Ashworth & Cormier, 1967). If macrofaunal grazing controls bacterial densities in bioturbate structures, this may be largely food chain-mediated through meiofauna and microfauna; for certain investigations suggest to discount the idea of bacteria serving as the principal food source of macrofaunal detritovores in marine sediments (Pearson, 1982; Kemp, 1987).

The enrichment at polychaete burrows of meiobenthic bacteriovores is well documented (Reise, 1981; Scherer, 1985; Reise, 1987). Certain members of the benthic meiofauna such as Gnathostomulida are associated with sulfur bacteria in the redox potential discontinuity layer (RPD) of polychaete burrow walls (Reise, 1981). Sulfur-oxidizing, chemolithotrophic bacteria as their most probable source of food should find excellent conditions for growth, particularly at polychaete burrow walls in sulfide-rich sediments where reduced inorganic sulfur compounds ensure the fresh supply of electron donors for chemoautotrophs (Yingst & Rhoads, 1980, Reichardt, 1986a).

Since formation of oxidized internal surface and "horizontal" RPD-layers in reduced sediments is an important aspect of

macrofaunal burrowing, chemoautotrophic bacteria were considered as an appropriate "target" to evaluate the impact of bioturbation on the sediment microflora.

Current investigations of deep-ocean sediments have indicated an impact of bioturbation on carbon dioxide assimilation rates (Reichardt, in press). In addition to these shipboard studies, an easily accessible laggon sediment was chosen to follow small scale distribution patterns of microbial biomass and activities at and around bioturbate structures. This included a not yet conventional "fingerprint" approach to detect and quantify microbial biomass in marine sediments (White, 1985, Guckert et al., 1985).

Material and Methods

Sampling Sites

1) Box core samples from more than 30 cm sediment depth were obtained during a cruise of R/V "Poseidon" in the Vöring Plateau area of the Norvegian Sea at water depths of 653 m ($66^{o}54.11$ M, $8^{o}2.14$ E) and 1221 m ($67^{o}43.91$ N, $5^{o}54.99$ E).

2) A shallow lagoon at Stein (Kiel Fjord) was characterized by algal mats at its margins and dense populations of Nereis diversicolor and Corophium volutator inhabiting a sulfide-rich sandy sediment (51 % and 37 % coarse and medium sand, respectively) with an oxidized surface layer of only a few mm thickness. Burrows of Nereis diversicolor with a mean diameter of 35 mm represented 6 % of the total sediment volume from 0 to 10 cm depth. The burrow walls of 1.5 mm thickness were characterized by a light brown color and redox potentials ranging from +100 to 250 mV, whereas the ambient sulfide-rich sediment showed redox potentials ranging from -50 to +50 mV (Reichardt, 1986a).

Samples were dug out using metal boxes (7 x 16.5 x 30 cm). Immediately after sampling the box cores were broken open vertically, thereby halving annd exposing the burrows. Using concave metal spatulas, subsamples were scraped off from the burrow surface ("A") and occasionally subdivided into inner, medium and outer layers ("A1, A2, A3"), as well as from the ambient reduced sediment ("B") and the sediment surfaces "C"). Further samples were taken from sediment surfaces where visible enrichment ("lawns" or "mats") of Oscillatoriaceae, Beggiatoceae, or purple sulfur bacteria had developed ("Farbstreifensandwatt"). Together with an enrichment culture of thiosulfate-oxidizing bacteria ("S4") this material served as an aid for the tentative evaluation of phospholipid fatty acid biomarkers (see below).

Carbon dioxide dark fixation was determined in triplicate assays at in situ temperatures by incubating 0.5 cm^3 sediment sample for one hour with 0.1 ml of Na H $^{14}CO_3$, followed by acidification, combustion and absorption of the assimilated labelled carbon using a Packard Sample Oxidizer, and scintillation counting (Reichardt, in press).

Viable counts of thiosulfate oxidizing bacteria were determined using an MPN procedure in a liquid medium described by Tuttle & Jannasch (1972). Viable counts (colony forming units (CFU) of sulfate-reducing bacteria were determined on Postgate's (1979) medium "E" using an incubator with an argon atmosphere.

Enzymatic activities of scleroprotease and sulfate were measured in triplicate using extracts of 1 cm^3 sediment sample in 5 ml of ice-cold 0.01 molar tris-HCL buffer, pH 7.8, containing 12 $^{\circ}/_{\infty}$ of artificial seatwater, 2 % triton X-100 and 2 g/l Polyvinylpyrrolidone, with "hide powder azure" (Sigma; Reichardt, 1986b) and p-nitrophenylsulphate (King & Klug, 1980, respectively, serving as

substrates.

Subsamples taken for gaschromatographic analyses were frozen at - 30 °C and were subsequently lyophilized.

Lipid extraction

Lipid extraction for capillary gas chromatography followed the procedure described by Bobbie et al., 1980, White, 1985, and Guckert et al., 1985. 4-5 replicate subsamples of lyophilized sediment (1-10 g) or 20 g of freeze-dried bacterial cells were suspended in 50 mM phosphate buffer, pH 7.4, using 500 ml separation funnels where a two-phase lipid extraction with chloroform-methanol was carried out according to Bligh & Dyer (White et al., 1979). After drying the chloroform extracts (rotavap), the extracted lipids were redissolved in 0.1 ml of chloroform and applied on a silica gel column (unisil, 100-200 mesh). After elution with chloroform, aceton and methanol, the fractions of neutral lipids, glycolipids, and phospholipids were obtained. These were dried under nitrogen and stored at -20°C.

Analysis of phospholipids fatty acid methylesters

Methylation of the fatty acids of the phospholipid fatty acid fraction was performed by mild alkaline methanolysis (White et al., 1979). The phospholipids were dissolved and incubated in a mixture of 1 ml of methanol-toluene (1:1, v:v) and 1 ml 0.2 N KOH in methanol at 37°C for 15 min. After adjusting the pH to pH 6.0 with 1 M acetic acid followed by 3 times repeated shaking (5 min) with 2 ml of chloroform and 2 ml of aqua dest. and subsequent centrifugation, the phospholipid fatty acid methylesters (PFLAME's) were collected in chloroform and dried under nitrogen.

Thin layer chromatographic purification and separation of the

FAME's (RF = 0.3 - 0.36) and of the OH-FAME's (RF = 0.55 - 0.65) were performed on Whatman K6-250 µm silicagel plates with - hexane:diethylether. The two fractions were detected under uv light with 0.01 % Rhodamine B and quantitatively transferred into prepared pasteur pipettes in which FAME's were eluted with chloroform and OH-FAME's with chloroform-methanol (1:1). The eluates were dried under nitrogen and taken up in hexane for capillary gas chromatography.

Gaschromatographic quantification of the FAME's and their tentative identification were carried out using a Varian 3700 capillary gas chromatograph with FID detector by comparison with an internal standard (19:0) according to Bobbie & White, 1980). OH-FAME's were measured after derivatization with bis-(trimethylsilyl) fluoroacetamide. Final peak identification was performed on a Hewlett Packard 5996A gaschromatography-mass spectrometry (GC-MS) combination with a RTE-6 NM data system (Guckert et al., 1985, Nichols et al., 1986). Double bonds of monounsatured FAME's were confirmed after the formation of dimethylsulfide adducts (Nichols et al., 1986).

Poly-ß-hydroxybutyric acid (poly-ß-hydroxyalkanoates) were determined in the glycolipid fraction following the procedure of Findlay and White, 1983.

Results and Discussion

Vertical profiles of carbon dioxide dark fixation rates in Vöring Plateau sediments suggested a strong stimulation by macrofaunal burrowing (Fig. 1). A sampling station where burrowing Enteropneust and Echiuride worms were most abundant (at 1221 m water depth) showed the highest rates at greater sediment depths (9-15 cm) instead of near the sediment water interface, where microbial activities would usually be expected to peak (f.e. Rowe

& Deming, 1985). An increasing contribution of inorganic carbon to (chemoautotrophic) microbial production at macrofaunal burrow walls had already been found in coastal sediments (Reichardt, 1986 a). This increase could largely be attributed to a stimulation of thiosulfate-oxidizing chemoautotrophs in the burrow walls of Nereis diversicolor.

Enrichment of sulfur-oxidizing bacteria was difficult to assess, since cultivation techniques employed for so called viable counts of defined physiological groups of bacteria may not match the growth requirements of the indigeneous populations. Furthermore, colony forming units (CFU) or viable counts are at best an approximation to the "real world" of single cell densities. Therefore, lacking evidence for increased densities of sulfur-oxidizing (or other chemoautotrophic) bacteria inhabiting the burrow walls must be interpreted with caution (table 2).

In such a case, independent direct estimates based on biomass-specific signature compounds may be helpful (White, 1985; Guckert et al., 1985). Despite recent advances concerning biomarkers for sulfur-oxidizing bacteria (Kerger et al., 1986), our current knowledge is only based on cultures of Thiobacillus spp.. Hence, a thiosulfate-oxidizing bacterial enrichment culture from the study site ("S4") as well as natural microbial "lawns" from the "Farbstreifensandwatt" -sulfuretum of the lagoon were included in the search for known or potential phospholipid fatty acid fingerprints of sulfur-oxidizing microbial communities.

Of the most unique biomarkers for Thiobacillus spp. (Kerger et al., 1986) only cy 19:0 and OH-cy 19:0, besides traces of 10 Me 18:1ω6 (not shown in tabele 1), were detected in the lagoon sediment (table 1). The indigenous enrichment culture ("S4") was characterized by an extremely high concentration of 17:1ω8c. This fatty acid reached much lower levels in named cultures of thiobacilli (Kerger et al., 1986). As indicated by the natural enrichment samples of purple sulfur bacteria, the

biomarker hydroxy fatty acid OH-cy 19:0 may not be unique for chemoautotrophic sulfur-oxidizers. Table 1 shows finally that none of the known or potential indicators of sulfur-oxidizing chemoautotrophs was significantly enriched in the burrow walls, as compared with the ambient reduced sediment.

Hence, evidence for missing enrichment of sulfur-oxidizing bacteria in the burrow wall microhabitats could be provided by both viable counts and phospholipid fatty acid biomarkers (table 2). Rather unexpected was the peak of 10 Me 16:0, a biomarker for strictly anaerobic sulfate reducing bacteria, in the "oxidized" burrow walls (Fig. 2). This may indicate a close "consortia"-like association between sulfur-oxidizing and sulfate-reducing bacteria (Jörgensen, 1977). The existence of sulfate-reducing bacteria in the at least temporarily aerated burrow wall layers could further be used as an argument in favor of the existence of anaerobic "micropockets" in order to revive the "thiobios" discussion (Boaden, 1980).

There was no difference between the distribution patterns of the biomarkers fatty acid 10 Me 16:0 (Fig. 2); and more general bacterial fingerprints such as the ratio of i15:0 + a15:0/15:0 (Fig. 3). In contrast to this accumulation of bacterial biomarkers in subsurface layers, total phospholipid fatty acids were more concentrated at the euphotic sediment surface (Fig. 4). Phototrophic eukaryotes being detectable by their polyenoic biomarker fatty acid 20:5ω3 (White, 1985) had also penetrated into the burrows (Fig. 5).

The distribution pattern obtained for a microfaunal biomarker (20:4ω6) may indicate the absence of migration barriers despite the steep redox gradients (Fig. 6). However, it is more likely the result of (meiofaunal ?) grazing at the burrow walls. The same applies to the missing enrichment of bacterial biomass at this site (Fig. 3). There is some reason to explain this missing enrichment by grazing: Accumulation of poly-ß-hydroxybutyrate

which indicates unbalanced bacterial growth (Nickels et al., 1979) as well as grazing effects (Morrison & White, 1980) was peaking in samples from the burrow walls (Fig. 7).

Furthermore, it was noted that the inner surfaces of burrows from Nereis diversicolor were also the sites of enhanced extracellular enzymatic activities. This is illustrated for scleroprotease and and sulfatase (Fig. 8). Scleroprotease is needed to depolymerize and digest particulate proteins. Natural substrates for sulfatase are sulphomucopolysaccharides that are known as excretion products of polychaetes (Defretin, 1971).

In conclusion, certain macrofaunal burrows should be considered as both sites of increased (chemoautotrophic) bacterial production and, presumably, meiofaunal grazing, as well as sites of enhanced geobiochemical activity. Burrow walls may represent internal sediment surfaces of maximal biochemical activity. They help to explain "inverse" patterns of microbial activities in sediments that exhibit subsurface peaks.

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Figures Legend

Fig.1 Vertical patterns of CO_2 dark fixation rates at two stations of the Vöring Plateau. Intensive bioturbation by burrowing macrofauna at 1221 m

Fig.2 Distribution of biomarkers for sulfate-reducing bacteria in bioturbated lagoon sediment

Fig.3 Phospholipid fatty acid indicators of bacterial biomass in bioturbated lagoon sediment

Fig.4 Total phospholipid fatty acid content as indicator of total microbial (prokaryotic and eukaryotic) biomass in bioturbated lagoon sediment

Fig.5 Polyenoic fatty acid 20:5ω3 as indicator of phototrophic eukaryotes in bioturbated lagoon sediment

Fig.6 Polyenoic fatty acid 20:5ω6 as indicator of heterotrophic eukaryotes in bioturbated lagoon sediment

Fig.7 Molar ratio of the bacterial storage compound poly-ß-hydroxybutyrate (=poly-ß-hydroxyalkanoates) to total phospholipid fatty acids as indicator of unbalanced bacterial growth and grazing in bioturbated lagoon sediment

Fig.8 Enzymatic activities of scleroprotease (absorbance units/h cm^3) and sulfatase (nmoles/h cm^3) in bioturbated lagoon sediment (burrow walls, A1, A2; ambient reduced sediment,B; sediment surface, C).

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TABLE 1

Fatty Acid	Sources					
	S4, enrichment culture of $S_2O_3^{2-}$-oxidizing bacterium	Visible, in situ enrichment of: *Beggiatoa* spp.,	Purple S-Bacteria,	Oscillatoriacea	Burrow Walls (n = 4)	Ambient, reduced sediment
14:0	1.0	0.1	2.0	0.1	0.33 ± 0.20	0.71 ± 0.37
15:0	1.0	0.3	1.4	0.2	0.62 ± 0.04	1.02 ± 0.23
16:1ω7c	1.3	11.9	29.6	3.4	18.33 ± 0.48	22.31 ± 1.04
16.0	7.5	11.5	10.4	30.2	14.62 ± 3.27	18.12 ± 0.85
10 Me 16:0	1.2	0.2	-	-	0.63 ± 0.08	0.56 ± 0.08
17:1ω8c	13.1	0.6	0.7	0.4	1.36 ± 0.30	1.53 ± 0.21
17:1ω6c + cy 17:0	1.6	1.0	2.4	-	1.39 ± 0.22	1.38 ± 0.45
18:1ω7c	1.9	19.8	31.5	4.4	13.46 ± 1.47	12.77 ± 3.28
cy19:0	1.0	0.2	0.2	-	0.25	0.23
21:0	2.5	2.1	0.4	0.1	0.25 ± 0.11	0.42 ± 0.37
OH-cy 19:0	n.d.	0.62	6.64	-	0.34 ±	0.75

Phospholipid fatty acids and hydroxy fatty acid selected for their abundance (mole %) in microbial lawns of the "Farbstreifensandwatt" and their relationship to sulfur-oxidizing bacterial communities in the lagoon sediment. n.d.= not determined.

Fig. 1

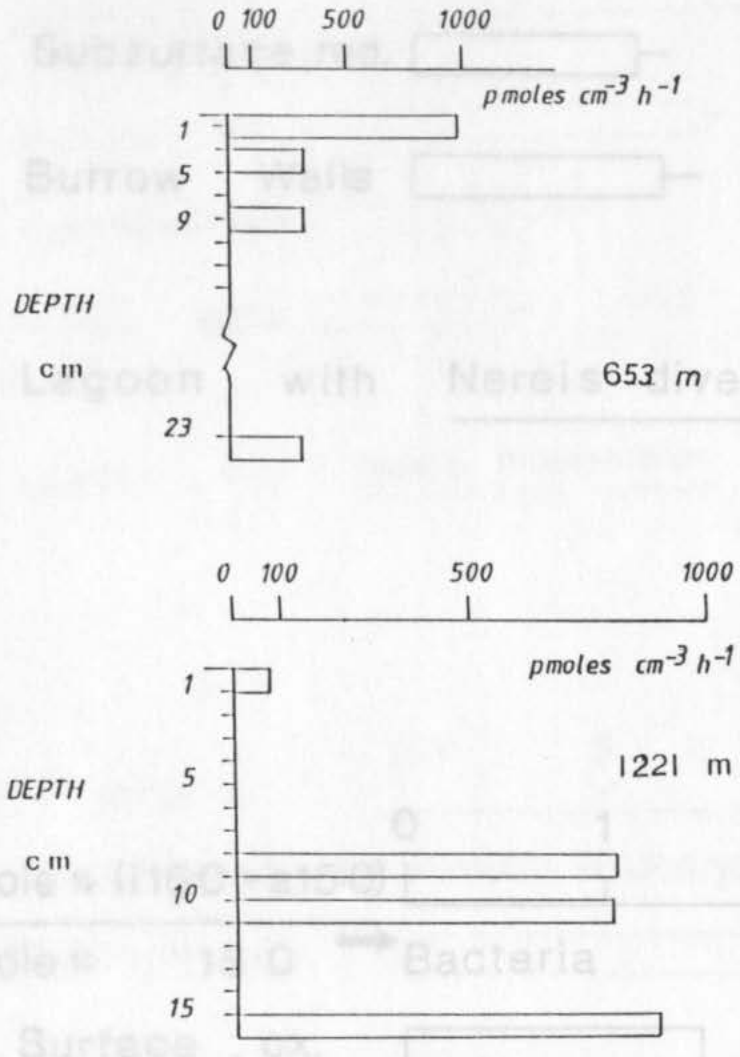

0 100 500 1000
pmoles cm-3 h-1
1
5
9
DEPTH
cm
653 m
23
0 100 500 1000
pmoles cm-3 h-1
1
5
1221 m
DEPTH
cm
10
15

Fig. 2

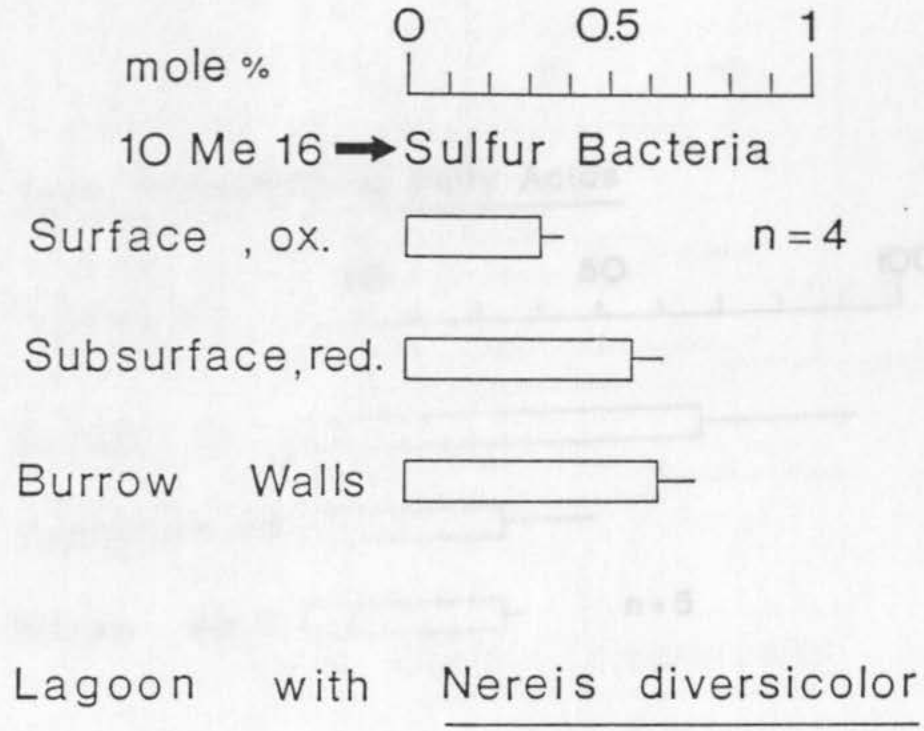

mole %
0
0.5
1
10 Me 16 → Sulfur Bacteria
Surface , ox.
n = 4
Subsurface, red.
Burrow Walls
Lagoon with Nereis diversicolor

Fig. 3

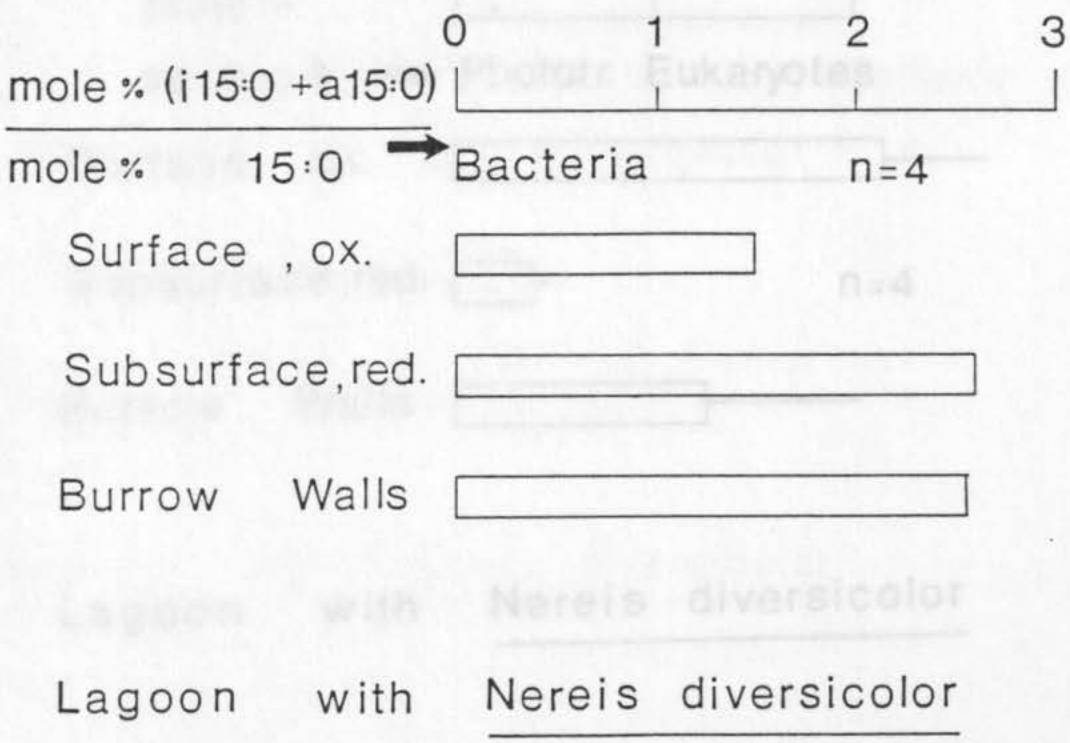

0
1
2
3
mole % (i15:0 + a15:0)
mole % 15:0
Bacteria
n = 4
Surface , ox.
Subsurface, red.
Burrow Walls
Lagoon with Nereis diversicolor

Fig. 4

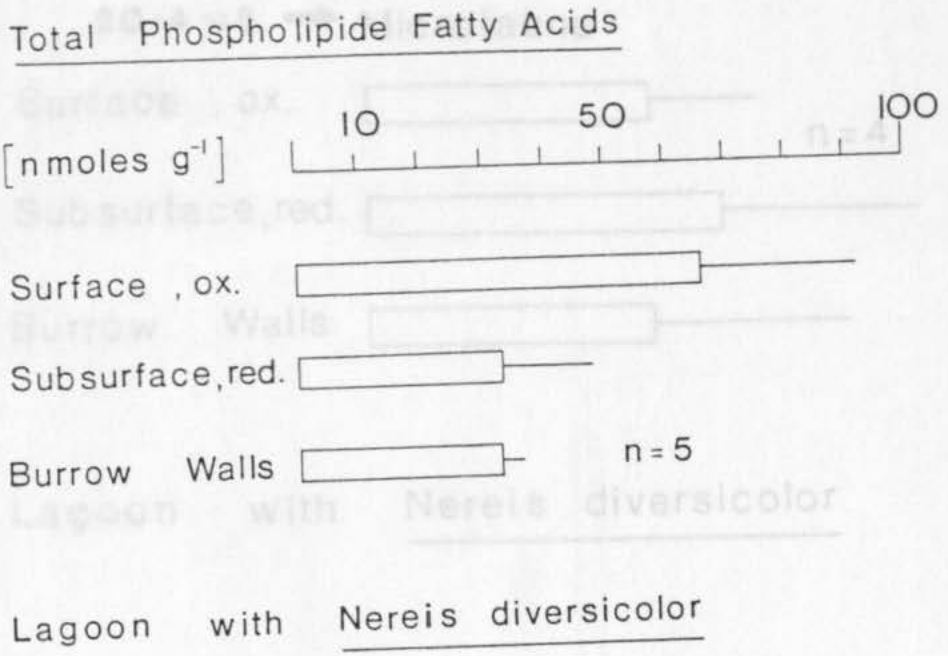

Total Phospholipide Fatty Acids
10
50
100
[nmoles g⁻¹]
Surface , ox.
Subsurface,red.
Burrow Walls
n = 5
Lagoon with Nereis diversicolor

Fig. 5

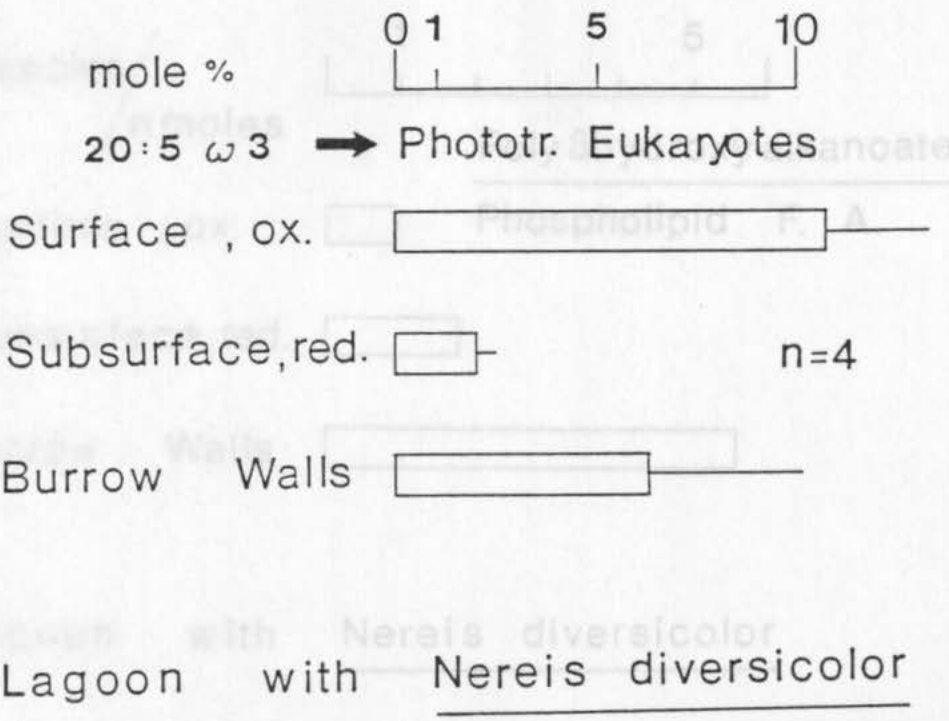

0 1
5
10
mole %
20:5 ω3 → Phototr. Eukaryotes
Surface , ox.
Subsurface,red.
n=4
Burrow Walls
Lagoon with Nereis diversicolor

Fig. 6

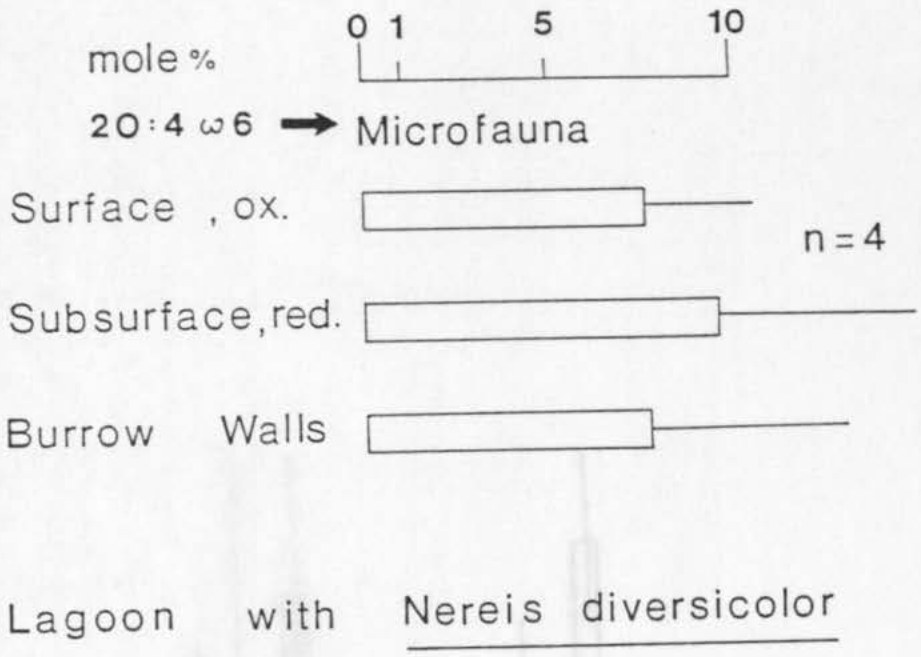

mole %
0 1 5 10
20:4 ω6 → Microfauna
Surface , ox.
n = 4
Subsurface, red.
Burrow Walls
Lagoon with Nereis diversicolor

Fig. 7

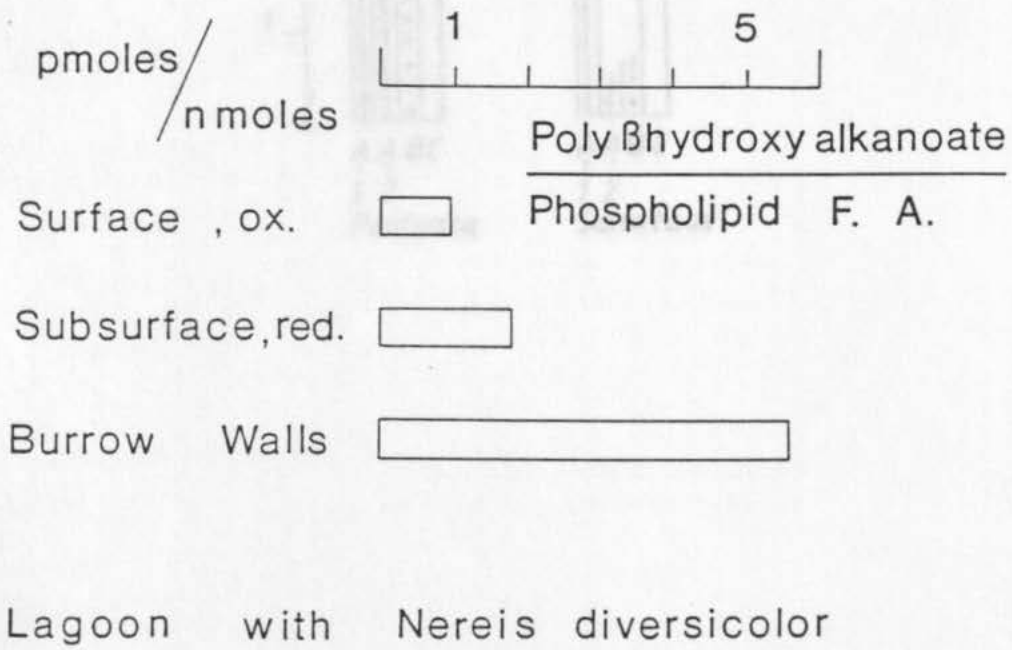

pmoles / n moles
1 5
Poly ßhydroxy alkanoate
Phospholipid F. A.
Surface , ox.
Subsurface, red.
Burrow Walls
Lagoon with Nereis diversicolor

Fig. 8

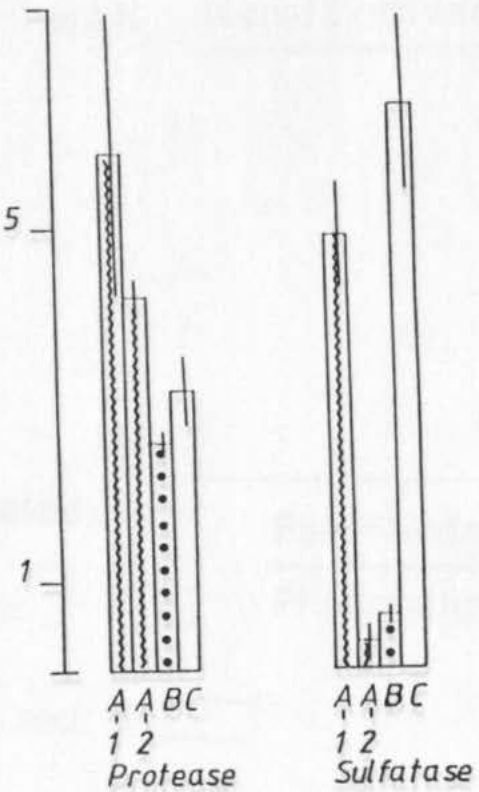
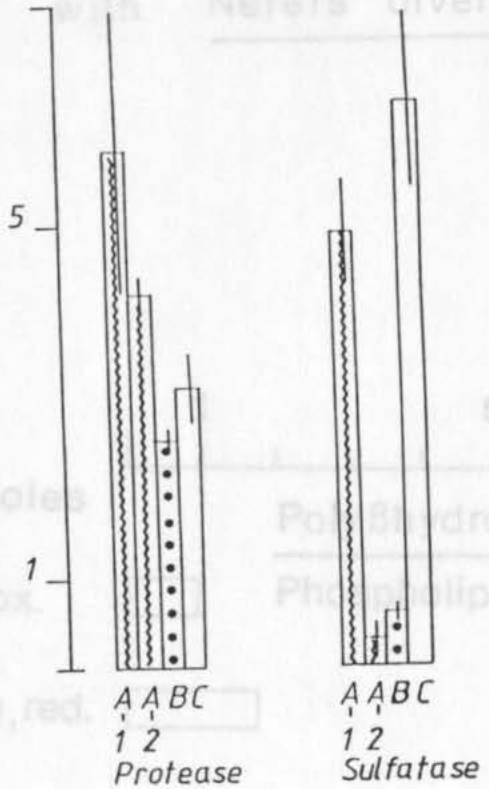
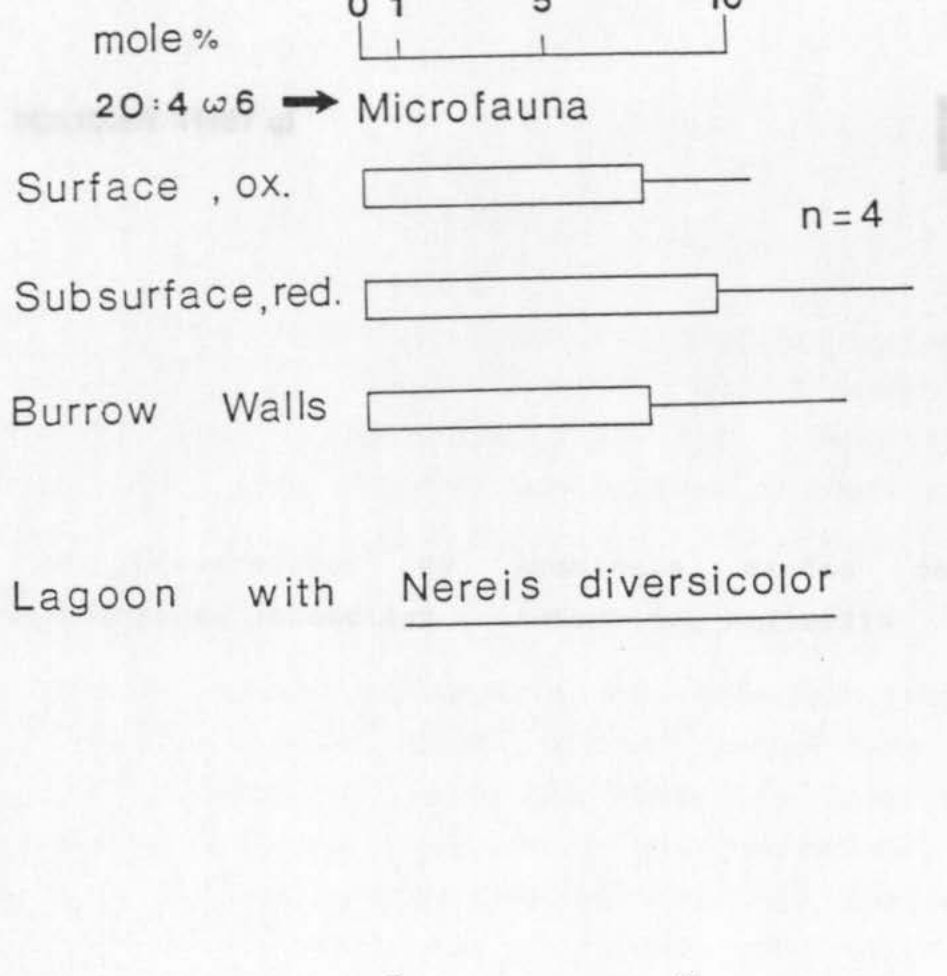


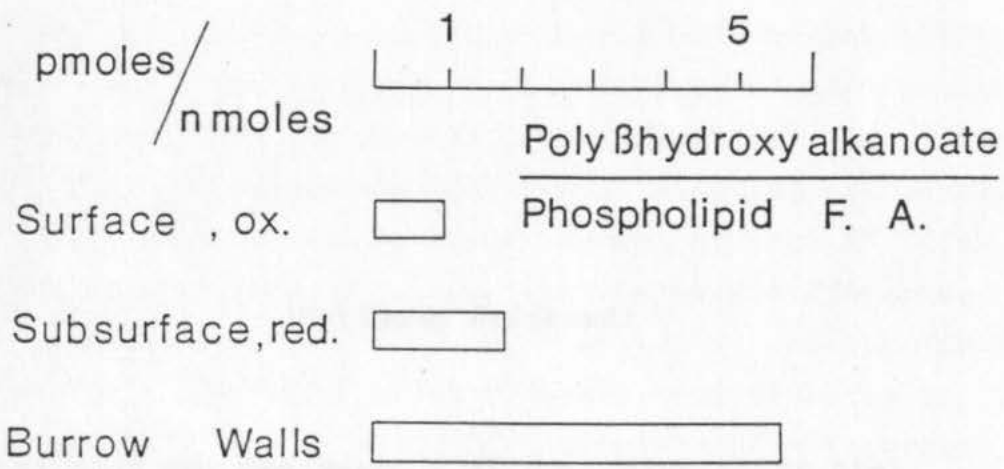
Fig. 8





Lagoon with Nereis diversicolor

ig. 7



Lagoon with Nereis diversicolor

Impact of bioturbation by Arenicola marina on microbiological parameters in intertidal sediments

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Abstract

Impacts of bioturbation by the lugworm Arenicola marina on sediment microflora and biogeochemical activities were investigated in intertidal sediments of the North Sea. Burrow walls and fecal casts were compared with unaffected surface and subsurface sediment surrounding the burrows. At the main study site burrow walls contained twice as much organic matter as the sediment surface. Viable counts of aerobic proteolytic and chitinolytic bacteria peaked at external and internal boundary layers. The latter (burrow walls) showed maximal bacterial production (incorporation of tritiated thymidine into DNA), maximal microheterotrophic activity (incorporation and mineralization of glucose and acetate) and highest levels of certain hydrolytic enzymes (alkaline phosphatase and sulfatase). Rates of chemoautotrophic CO_2 fixation in both burrow walls and surrounding subsurface sediment were 3-4 times higher than at the sediment surface, suggesting a short-circuited CO_2 turnover driven by an elevated catabolism. In a second, more polluted study area, burrow walls were again the main site of microheterotrophic activity, but only minor differences were noted at all for CO_2 fixation. Biogeochemical consequences of bioturbation by Arenicola could be understood essentially as a shifting of catabolic and anabolic microbial activity peaks from the top to subsurface layers where burrow walls showed the most conspicuous effects. Nevertheless, protease peaks in the fecal casts indicated also that this shifting pattern can be reversed for certain parameters.

Introduction

It is widely assumed that macrofaunal burrowing in marine sediments has a stimulating effect on microbial growth and metabolic activities (Hargrave,1970, Aller & Yingst,1978, Henriksen et al.,1980, Hylleberg & Henriksen,1980, Yingst & Rhoads,1980, Morrison & White,1980, Jones,1985, Reichardt,1986a, Kikuchi,1986), although exceptions from this rule are evident (Hanson & Tenore,1981, Bianchi & Levinton,1981, Pearson,1982, Alongi,1985a, Alongi & Hanson,1985).

In organically enriched environments,for example, macrofaunal control of the microbial production and activity may become negligible (Bianchi & Levinton,1981, Newell,1982,Alongi & Hanson,1985),indicating the loss of an important food chain linkage (Tenore & Hanson,1980, Newell,1982). This adverse influence of elevated nutrient levels was noted ,in particular, for pioneering surface deposit-feeders. In contrast to deep dwelling "equilibrium macrobenthos", the meiofauna and microflora associated with these early colonizers has also been shown to exhibit considerable resilience to physical disturbances (Christian et al.,1978, Alongi,1985a).

Arenicola marina, one of the most conspicuous macrofaunal inhabitants of the Wadden Sea sediment, is a funnel-type surface deposit-feeder (Krüger,1966) and occurs in pristine as well as in polluted intertidal sediments of the North Frisian Wadden Sea. Arenicola burrows are enrichment sites for unique meiofaunal assemblages (Reise,1981,1987, Scherer,1985). Though a direct impact of Arenicola on the microbial communities in Wadden Sea sediment can be postulated (Reise,1985), quantitative evidence for stimulating effects on microbial production and biogeochemical activities is lacking.

Most investigators of infauna - microflora relationships have focussed primarily on microbial production and related food chain

aspects (e.g. Hanson & Tenore,1981, Levinton & Bianchi,1981, Alongi,1985a,b, Alongi & Hanson,1985, Kemp,1987). Less attention has been paid to the biocatalytic potential of a macrofauna-controlled microflora (e.g. Hargrave,1970, Aller & Yingst,1978, Hylleberg & Henriksen,1980). Also with respect to the importance of ocean shelves and coastal sediments for microbial decomposition of pollutants, these biogeochemical aspects appear to deserve more attention (e.g. Lee & Swartz,1980).

Sediments with polychaete infauna have been noted for their increased production of CO_2 (Kikuchi,1986). It has also been claimed that significant losses of carbon from food webs were to occur in the absence of reversed processes such as CO_2-fixation by chemoautotrophic bacteria (Hanson & Tenore,1981). Therefore this investigation dealt with CO_2-production (mineralization) by microheterotrophs as well as CO_2-fixation by chemoautotrophic microbial communities. Instead of comparing large sediment areas with and without significant bioturbation (Hines & Jones,1985), a smaller scale approach was chosen to analyse exclusively microbial activities in sediment zones that were directly affected by the bioturbating animal (burrow walls, fecal casts) and in adjacent zones serving as controls. This included also CO_2 dark fixation as a measure of primary synthesis of organic matter by the sediment microbiota or, essentially, chemoautotrophic bacterial production. Finally, assays for extractable enzyme activities were designed to provide further biochemical clues concerning the degradation of organic matter. However, in this case differentiation between microbial and non-microbial sources was impossible.

Since the chosen study area (Westerhever Sand) appeared rather pristine, complementary investigations were carried out at a more polluted site (List) to study the impact of higher organic loads on microbial turnover of CO_2.

Materials and Methods

Sampling

Study sites were situated at Westerhever Sand (WHS), an intertidal sand flat off the Eiderstedt peninsula (54° 22.0`N, 8° 38.4`E) and at the northern tip of the island of Sylt (Königshafen-List, 54° 51.7`N, 8° 3.4`E) in the North Frisian Wadden Sea (North Sea). Samples from burrow sites of the lugworm Arenicola marina were collected within sampling areas of approximately 10000 m^2 during June and July, 1987, at water temperatures between 13° and 17° C.

During low tide, burrows with dark fecal casts were dug out to obtain sets of four different zones of sediment from each of n=6 (List) or n=10 (WHS) sampled burrow sites. The following four zones were considered for sampling: 1) fecal casts ; 2) an approximately 2 mm thick layer of the sediment next to them; 3) an approximately 2 mm thick brownish surface layer of the vertical tail shaft and beginning parts of the gallery (Reise,1981); and 4) the reduced (black or greyish) subsurface sediment surrounding the burrows at 1-5 cm depths.

For most analytical procedures samples were collected in sterile 1.0 cm^3 sawed-off syringes for distributing 0.1 cm^3 aliquots. Undisturbed subsamples with intact surfaces as used for CO_2 fixation measurements, were obtained as 1 cm^2 large discs punched out from 2-3 mm thick sediment layers by use of a cork borer and scalpellum, to be transferred into the fitting holes of tissue culture multiwell plates (Linbro).

Laboratory Analyses

Samples were processed within 30 min after sampling in laboratories located within a short walking distance from the sampling sites. Redox measurements in combined samples were done using an Ingold platinum electrode. Organic matter contents were determined as ash-free dry weights after combustion (24 hours at 500 °C) of dried (65°C) combined samples (5 cm^3). Combusted samples were further used for grain size fractionation according to the Wentworth scale. Protein concentrations were analysed in 0.1 cm^3 subsamples using a modification of Lowry`s method (Herbert et al.,1971) with bovine serum albumin as reference standard. Photometric absorbance at 500 nm was measured after centrifugation (5000 g, 15 min) of the reaction mixture .

Direct bacterial counts were based on a modification of the acridine orange epifluorescent technique by Rublee & Dornseif (1978), except that dispersion was accomplished by ultrasonication (3 x 5 sec at approximately 100 W).

Viable counts of proteolytic and chitinolytic bacteria were obtained according to Reichardt (1987b).

Incorporation of tritiated thymidine into bacterial DNA was determined by incubating 0.1 cm^3 of sediment with 20 µl (370 10^6 kBq) methyl-(^{3}H)-thymidine (140 10^6 Bq $\mu mole^{-1}$) for 30 min at in situ temperature, using samples containing 6 % formaldehyde as blanks. After two subsequent washes and centrifugation with 10 ml of 4 % formaldehyde, the concentration of thymidine incorporated into bacterial DNA was determined according to Findlay et al. (1984).

Rates of CO_2 dark fixation were measured in multi-well plates containing undisturbed surface samples of 2-3 mm thickness (see above). These were incubated for 30 min in the dark at in situ temperature with 200 µl (74 kBq) of ^{14}C-labeled sodium bicarbonate (specific activity: 2.18 kBq $mmole^{-1}$) in 1.5 mmolar

non-radioactive $NaHCO_3$, pH 10.2). Blanks were poisoned with 20 mM iodoacetamide, an inhibitor of ribulose-1.5-bisphosphate carboxylase (RubisCo) to exclude non-chemoautotrophic, RubisCo-independent CO_2 fixation as far as possible (Reichardt, unpublished). Incubation was terminated by adding 0.1 ml of concentrated formaldehyde and 1 N H_2SO_4 sufficient to lower the pH to 2.0. The sediment was taken up in 10 ml of filtered sea water, centrifuged (15 min, at 6000 g) and the pellets dried at 60°C. Weighed amounts were combusted using a Packard ^{14}C-sample oxidizer for liquid scintillation counting (Beckman LS 100). Quench corrections were based on external standard and channels` ratios. Total carbonate concentrations available in the assay were calculated from carbonate alkalinity of the pore water (Gargas, 1980).

Microheterotrophic activity was determined as simultaneous incorporation into macromolecular pools and mineralization to CO_2 of ^{14}C-labeled glucose or acetate. 0.1 cm^3 of sediment sample were incubated for 30 min at in situ temperature with 100 µl aliquots of ^{14}C(U)glucose (11.1 kBq; 163 kBq µmole^{-1}) and ^{14}C(U) acetate (11.1 kBq; 89 kBq µmole^{-1}) dissolved in sterile artificial sea water. Potential mineralization rates were measured according to Reichardt & Morita (1982); potential incorporation rates were obtained from the same assay after acidification, centrifugation, and combustion in a Packard ^{14}C-sample oxidizer (see: CO_2 fixation).

Enzyme assays were run with sediment extracts in ice-cold tris-HCl-buffered 2% triton-X-100 (Reichardt, 1986b). In assays for alkaline phosphatase and sulfatase (2-4 hours at 20 °C) the corresponding nitrophenylesters served as substrates (Degobbis et al., 1986; Oshrain & Wiebe, 1979). Scleroprotease activity was measured with the particulate "hide powder azure" (Sigma) substrate (Reichardt, 1986b).

Results

Physicochemical parameters and bacterial densities

The two study sites were characterized by different grain size distributions: fine sand prevailing at Westerhever Sand (WHS), and coarse to medium sand at List. In both areas, burrow linings contained a slightly higher portion of fine and medium sand than the surrounding subsurface zones (Table 1).

As indicated by ash-free dry weights, the coarser grained sediment at List was more reduced and contained higher amounts of organic matter accumulating below the surface at both sites, but to a greater extent at List (Table 2). Whereas the less polluted sediment (WHS) showed slightly higher organic matter content in the burrow walls than in the reduced surrounding zones, the opposite was found in the more polluted sediment (List).

At WHS, protein concentrations and ash-free dry weight showed similar patterns. Any correlation of these parameters with densities of (aerobic and facultatively anaerobic) proteolytic bacteria was missing (Table 3). Viable counts for protein- and chitin-degrading bacteria (Table 3; Fig.1) peaked at the top sediment layer ("S") as well as in the burrow walls("BW").

Viable counts and epifluorescent microscopic direct counts (AODC) were subject to great variations (Table 3). According to microcopical evidence, this was, at least partly, due to microscale heterogeneity of the distribution of bacterial cells. Especially in burrow wall samples, cells were often found in clumps adhering to pellicular substrata.

Anabolic bacterial activities

Rates of (iodoacetamide-inhibitable) CO_2 dark fixation peaked in

the subsurface layers of both study sites, where they showed a higher degree of variability than was encountered at the sediment surface (Fig.2). Differences between burrow walls and adjacent sediment were not regarded as significant. In the less polluted area (Fig.2 "B") fecal casts contributed to a local increase of CO_2 fixation on the sediment surface.

In contrast to CO_2 fixation as a measure of largely chemolithoautotrophic bacterial biosynthesis, incorporation of tritiated (methyl)-thymidine (tdr) into bacterial DNA peaked significantly in the burrow walls (Fig.3). This maximum of bacterial production exceeded rates in subsurface sediments surrounding the burrows by roughly one order of magnitude. Fecal casts revealed higher rates than subsurface sediment (where this material originated from); however, in contrast to CO_2 fixation (Fig.2:"B"), rates of tdr incorporation at the surface were higher than in fecal cast material.

Microheterotrophic utilization of glucose and acetate

Relative activities of incorporation and mineralization of glucose declined steeply from the surface toward subsurface layers at the less polluted site (Fig.4:"B"). This was much less pronounced at the more polluted site (Fig.4:"A"). In both cases, however, the burrow walls were most active in mineralizing glucose and incorporating it into microbial cells. A similar pattern of microheterotrophic activity was noted for acetate utilization at the less polluted study site ((Fig.5). The differences between surface sediment and fecal casts were rather insignificant for both substrates (Figs.4 & 5).

Enzyme activities

Activities of extractable alkaline phosphatase and sulfatase

followed essentially the same trend as microheterotrophic utilization of glucose and acetate. Fecal casts, however, turned out to be zones with the strongest enzymatic potential to degrade particulate (sclero-)proteins (Fig.6).

Discussion

Evidence for bacterial enrichment?

Disturbances are considered as a mechanism to keep parts of any ecosystem at a high state of productivity (Rhoads et al.,1978). Bioturbation of the sea floor by burrowing macroinvertebrates comprises physical as well as chemical changes in those zones of the sediment that are affected by metabolic activities of the infauna. Correspondingly, microbial enrichment processes in bioturbated marine sediments have been attributed to both physical disturbances (White et al.,1979, Findlay et al.,1985, Eckman,1985), and infaunal metabolism (Driscoll,1975, Lee & Swartz,1980, Yingst & Rhoads,1980, Alongi & Hanson,1985, Reichardt,1986a, 1987a). If bacterial enrichment is linked to certain macroinvertebrates, specific responses of these to physical disturbances have also to be considered (Woodin,1978, Thistle,1981).

Burrows of Arenicola marina are known as enrichment sites for meiobenthic bacteriovores (Reise,1981 & 1987, Scherer,1985). Therefore, food web relationships have to be considered in any assessment of bacterial enrichment related to these habitats.

Differences between bacterial cell densities obtained for sediment zones affected by the lugworm (burrow walls and fecal casts) and those serving as unaffected controls (sediment surface

and reduced bulk sediment) were rather weak (Table 3). On principle, this lack of strong apparent enrichment may be explained by a leveling influence of meiofaunal grazing pressure. However, especially in the burrow walls, bacterial counts were subject to high statistical errors caused by bacterial aggregates formed on flakes from the burrow lining material.

Total cell densities were in the range of several times 10^9 cm^{-3} which is common for intertidal sediments (Dale,1974, Rublee & Dornseif,1978, Cammen,1982, DeFlaun & Mayer,1983). As a static measure of an extremely heterogeneous group of organisms, total cell counts provide only little clues to the role of bacteria in food webs or nutrient cycling.

Sediment particles as potential carriers of attached bacteria showed slight selectivity for medium grain sizes; i.e., medium and fine sand accumulated in the burrow walls of both study areas (Table 1). Similar patterns have been reported for Nereis virens burrows (Kristensen et al.,1985). The opposite effect of more expanded grain size spectra was noted in fecal casts as compared with the adjacent sediment surface. Nevertheless, direct consequences of this redistribution of particles for bacterial densities seem unlikely. A correlation between grain sizes and total bacterial counts in intertidal sediments has only occassionally been observed (Dale,1974) and must not be generalized (Cammen,1982).

More likely, bacterial distribution patterns were directly linked to the metabolic activities of the polychaete. Thus, passage through the guts of sediment-feeding macrofauna is known as a selective force that reduces viable cell counts of certain bacteria (Wavre & Brinkhurst,1971). In the present study, viable counts for aerobic biopolymer-degrading bacteria in fecal casts were also reduced as compared to burrow walls and sediment surface, but not in relation to subsurface sediment surrounding the burrows (Table 2; Fig.1). Coincident peaks for viable counts

of proteolytic and chitinolytic bacteria at sediment surface and burrow walls suggested an intrusion from "external" onto "internal" surfaces. It seems unlikely that the moderate decrease of E_h (Table 2) accounted entirely for the drastic decline of aerobic viable counts (which include also facultative anaerobes) in the reduced zones.

Pool sizes of microbially available carbon in marine sediments are usually low (0.5 to 1 %) and may decrease rapidly with depth (Nedwell,1987). The coincidence of divergent organic matter (or protein) levels and similar viable counts in the top layer and the burrow walls could therefore indicate a minimum of available carbon below the surface. This would, however, only apply to the unlikely event that bacterial enrichment was not masked by micro- and meiofaunal grazing.

Thymidine incorporation

Production of bacterial biomass as measured by incorporation of tritiated (methyl)thymidine (tdr) into bacterial DNA peaked in the burrow walls. For comparison, sediment tubes of the capitellid polychaete Capitella capitata show also significantly higher rates of tdr-dependent bacterial production than adjacent parts of the sediment, although bacterial densities were not different (Alongi,1985b). In this case, protozoan rather than meiofaunal grazing has been suggested as predominant mechanism controlling bacterial densities.

Macrofaunal grazing of sediment bacteria is particularly known in connection with the feed back mechanism of "gardening" (Hylleberg,1975, Reichardt,1987b). Still, estimates of bacterial contribution to macrofaunal diet are rare. Bacterivorous holothurians may eat 20 to 50 mg of bacterial carbon per day, which is equivalent to 10 to 40 % of the total bacterial production in their habitat (Moriarty,1982, Moriarty et

al.,1985). Yet, such short-circuited bacteria-macrofauna food chains seem to play a minor role. Even in cases in which bacterial production rates in sediments exceed the carbon requirement of deposit-feeding polychaetes, low bacterial densities may limit the short-circuited utilization of bacterial carbon to less than 10 % (Kemp,1987). Hence, there is growing evidence against the notion of bacteria as a direct, principal food source of deposit-feeding marine infauna (Cammen,1980, Levinton & Bianchi,1981, Pamatmat,1983, Wetzel & Christian,1984, Alongi,1985, Kemp,1987). Considering the abundance of meiofauna in Arenicola burrows (Reise,1981, 1987), it seems most likely that also in this case macrofaunal grazing on bacteria could be ruled out as the primary mode of feeding.

In the poorly developed field of bacterial production measurements in marine sediments, short-term incorporation of tdr into DNA has almost become the method of choice (Moriarty & Pollard,1981, Moriarty,1982, Newell & Fallon,1982, Fallon et al.,1983, Pollard & Moriarty,1984, Moriarty et al.,1985, Alongi,1985b, Alongi et al.,1985, Kemp,1985). Nevertheless, various shortcomings inherent to this technique call for a cautious interpretation of the results obtained with it. The portion of actively metabolizing sediment bacteria being able to incorporate tdr is usually unknown and may be small (Douglas et al.,1987). Isotope dilution and recovery of DNA from different types of sediments mark further uncertainties (e.g., Moriarty,1984, Bell & Ahlgren,1987).

Based on a conversion factor of 1.1×10^{18} cells per mole of tdr incorporated into bacterial DNA (Riemann et al.,1987), the maximum of bacterial production in the burrow walls of Arenicola would account for 7.6×10^{7} cells $h^{-1} cm^{-3}$. Underestimates arising from greater densities of non-reactive anaerobes (Moriarty,1984) may be expected, in particular, for the more reduced zones. On the other hand, in a similar case of superficially oxidized burrow walls of another polychaete, the adjacent reduced sediment

showed no significant differences in terms of phospholipid fatty acid biomarkers for anaerobic and sulfate reducing bacteria (Reichardt, 1987c).

Carbon dioxide dark fixation

As sites of steep oxygen gradients and as a continuous source of potential electron donors, burrow walls may stimulate chemolithoautotrophic bacteria (Yingst & Rhoads,1980, Reichardt,1986a). Furthermore, measuring chemoautotrophic CO_2 fixation rates could also provide a production estimate of a selected group of bacteria.

At both study sites, burrow walls of Arenicola marina showed 1.6 to 3.7 times higher CO_2 fixation rates than the corresponding surface sediments (Fig.2). However, measurements in the burrow walls were subject to high variability, and the results obtained not significantly different from the surrounding sediment. This contrasted with earlier investigations in which burrow walls of Nereis diversicolor proved the most active sites of CO_2 dark fixation. This process was mainly driven by chemolithotrophic oxidation of reduced sulfur and followed a gradient even within the burrow wall layer of which the innermost, nearly anoxic layer showed maximal activity (Reichardt,1986a). Considerably thicker burrow wall samples were obtained in the case of Arenicola. Therefore existing activity gradients within the brown 2 to 4 mm thick walls (Reise,1981) might have been overlooked. E_h measurements (Table 2) proved rather inadequate to elucidate the nature of O_2 gradients.

Microelectrode measurements have shown that the diffusive oxygen flux from aerated water into sediments is limited by 0.2 to >1 mm thick diffusive boundary layers and shows a rapid decline within the topmost mm of the sediment surface (Jörgensen &

Revsbech,1985). With extremely high oxygen consumption rates to be antipated for major accumulation sites of degradable organic matter, even burrow wall surfaces may turn anoxic. Already Aller & Yingst (1978) noted a predominance of anaerobic activities in the outer burrow walls of a deposit-feeding polychaete.

In sediments with diurnal rhythms of hydrogen sulfide release (Revsbech et al.,1983), chemoautotrophic CO_2 fixation that is driven by the oxidation of inorganic sulfur compounds, would require bacteria which are still active under anaerobic or nearly anaerobic conditions. These requirements are fulfilled by obligate chemolithoautotrophs like Thiobacillus denitrificans (Taylor et al.,1971, Timmer ten Hoor,1975), but also by the more conspicuous filamentous and gliding marine members of the genus of Beggiatoa (Nelson et al.,1986). The latter show unique adaptations to the diurnal rhythms of H_2S evolution in coastal sediments (Jörgensen,1982). Since CO_2 dark fixation rates peaked in the reduced and (despite enhanced E_h) presumably largely anoxic zones (Fig.2), predominance of sulfur-oxidizing chemoautotrophs seemed very likely, yet has still to be proven.

On the other hand, most of the scarce evidence being available for enhanced chemoautotrophic activities in polychaete burrows is related to nitrification (Henriksen et al.,1980, Sayama & Kurihara,1983, Kristensen et al.,1985). In these investigations potential turnover rates of the electron donors were measured, but related data on CO_2 assimilation do not exist. For an attempt to quantify the contribution of chemoautotrophs to carbon fluxes in benthic marine food webs, however, such information would be indispensable.

Microheterotrophic activities

Close-circuited bacteria-based food chains would require CO_2 consuming chemoautotrophs to maintain their carbon flow (Hanson &

Tenore,1981). Microheterotrophic mineralization of DOM constitutes a major source of CO_2. Potential CO_2 production from glucose or acetate in the burrow walls proceeded at 3 to 8 times higher levels than in the ambient sediment (Figs.4 & 5). In addition to these microheterotrophic activities, CO_2 output from the polychaete itself must be taken into account. E.g., the polychaete Neanthes japonica contributed 36 % to the total flux of CO_2 (Kikuchi,1986). Finally, anaerobic pathways of detritus mineralization seem to produce particularly high amounts of CO_2 (Hansen & Tenore,1981).

The present investigation deals with potential rates of CO_2 evolution from glucose and acetate as model substrates for dissolved organic matter (DOM). Thus, only a relative measure was obtained instead of real in situ mineralization rates. But these would have required a better knowledge of the availability and pool size (isotopic dilution) of the substrates used in the assay (Gocke,1981, King & Berman,1984, Nedwell,1987). Measured rates of CO_2 production from the two substrates employed should be referred to as "apparent" mineralization to avoid misleading conclusions about the carbon flow in situ (King & Berman (1984). The percentage of CO_2 of the total amount of glucose incorporated ranged from 26 to 39 %. This corresponded with similar measurements in coastal marine sediments (Novitsky & Kepkay,1981, Novitsky,1983). Yet, estimates based on mass balances may be twice as high (King & Berman (1984). Further potential underestimates of real CO_2 production in situ resulted from measuring only net rates.

Heterotrophic utilization of glucose and acetate revealed quite similar distribution patterns (Fig.4 & 5). They coincided also with bacterial production estimates based on incorporation of tdr into DNA (Fig.3). Great differences of microheterotrophic activities occurring between burrow walls and surrounding sediment (Figs.4 & 5) did not correspond with total organic matter or protein contents (Tables 2 & 3). This may indicate that

the heterotrophic microflora in the burrow walls was particularly adapted to large fractions of easily available DOM.

Activities of hydrolyzing enzymes

Part of the microbial substrates accumulating in burrow linings are excretion products of the burrow inhabitants (Johannes,1964, Zola,1967, Defretin,1971, Daly,1973). Enzymes which can hydrolyze such compounds, are not confined to microbial sources (Zottoli & Carriker,1974, Reichardt,1986b). Excretion by Arenicola marina of the three enzymes investigated in the present study, is thinkable (Johannes,1964, Oshrain & Wiebe,1979), and for proteases most likely (Zottoli & Carriker,1974).

Contrasting activity patterns obtained for alkaline phosphatase and sulfatase on one side, and scleroprotease on the other, were most striking (Fig.6). Proteases are hypothesized to play a role in purging inner polychaete tube surfaces (Zottoli & Carriker,1974). In the present study, distribution patterns for scleroprotease activities pointed to the gut of the polychaete as main enzyme source. Activities in the fecal casts reached five- to threefold higher levels than in burrow walls and sediment surface, respectively. This may indicate the existence of a potent output mechanism for an essential biocatalyst which is based on bioturbation-driven transport from subsurface zones to the primary accumulation site for sedimenting particles.

Alkaline phosphatase and sulfatase, on the other hand, peaked in the burrow walls. Notwithstanding the general tendency of the biodegradative potential being concentrated in the burrow walls, the likely excretion of pertinent enzyme substrates by polychaetes should also be considered as factors controlling the levels of these particular enzymes. For some polychaetes

excretion of sulphomucopolysaccharides and phosphate-rich polysaccharides is documented (Defretin,1971; Zola,1967).

General aspects

Bell jar experiments near one of the study sites (List) show a significant influence of Arenicola marina on the remobilization of nutrients (Asmus,1986). According to field studies of other shallow water sediments, bioturbation by polychaetes and bivalves may account for three- to fivefold increases in nutrient turnover that is mediated by bacteria (Hines & Jones,1985). The present study aimed at the impact of one predominant bioturbator on microbial and biogeochemical activities. This implied also a smaller scale approach that had already proven useful in tracking meiobenthic populations (Reise,1987).

As to be expected (Aller & Yingst,1978, Yingst & Rhoads,1980, Reichardt,1986a), most of the biogeochemical parameters showed peaks in the burrow walls. Steep E_h gradients at these "microsites" have been cited as main determinants of bacterial, and particularly, chemoautotrophic growth (Yingst & Rhoads,1980, Reichardt,1986a). Nevertheless, the influence of other environmental factors should not be underestimated, as for example diffusive permeabilities and molecular sieve effects of mucoid burrow linings (Aller,1983), and the accumulation of organic matter at the burrows ((Tables 2 & 3).

Stimulation of anabolic processes in the burrow walls was mainly documented by elevated rates of tdr incorporation into bacterial DNA. Further considerations of the role of bacterial production in burrow wall- linked food webs, however, were beyond the scope of this study which focussed on microbial biogeochemical activities. Nevertheless, abundance of bacterivorous meiofauna in Arenicola burrows (Reise,1981, 1987) indicates the existence of an efficient "small food web" (Kuipers et al.,1981).

In a previous investigation (Reichardt, 1987c), intensive grazing on bacteria in burrow walls of Nereis diversicolor was indicated by elevated levels of poly-ß-hydroxybutyrate. At the same time, phospholipid fatty acid biomarkers for bacteria and microfauna (protozoa) reflected no increments of the standing crops. This may suggest that meiofaunal grazing played an important role in those benthic environments. Yet, considering the conflicting views of the role of benthic meiofauna in bioturbated sediment structures (Alongi,1985b), general conclusions can not be drawn.

Whereas a positive feed back mechanism between deposit-feeding burrowing macrofauna and bacterial decomposers (Yingst & Rhoads,1980) seems to function only when the detritus supply is low (Hanson & Tenore,1981), higher detritus availability may cause an uncoupling (Bianchi & Levinton,1981, Alongi & Hanson,1985). A comparison of the two study areas supports this view at least with respect to anabolic activities represented by CO_2 dark fixation . On the other hand, heterotrophic activities in the sewage affected study area indicated still some macrofaunal control. At both places, subsurface peaks of major anabolic and catabolic processes could be explained by bioturbation-driven downward shifting of microbial and enzymatic activities. Elevated scleroprotease levels in the fecal casts indicated further that, for at least certain processes, the direction was reversed.

The relative significance of physical perturbation and nutrient availability in bioturbated sediments for microbial communities are still discussed (Aller & Yingst,1978, Christian et al,1978, Howarth & Hobbie,1982, White,1983, Alongi,1985a). The present study emphasizes the importance of lugworm burrows as transport and accumulation sites for organic matter, thus favoring a short-circuited CO_2 turnover between heterotrophic and chemoautotrophic microbial communities.

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Figure legends

Fig.1: Viable counts (aerobic colony forming units ,CFU) of chitin-degrading bacteria in: fecal casts ("FC"), surface layer ("S"), reduced subsurface layer ("RS"), and burrow walls ("BW") of Arenicola marina study sites at List (A) and Westerhever Sand (B). Bars mark standard deviations of a given number (n) of individual burrow sites sampled.

Fig.2: Rates of (iodo-acetamide-inhibitable) CO_2 dark fixation in four selected sampling zones at burrow sites of Arenicola marina. Further details in Fig.1.

Fig.3: Rates of incorporation of tritiated (methyl)thymidine into bacterial DNA in four selected sampling zones at burrow sites of Arenicola marina (Westerhever Sand). Further details in Fig.1.

Fig.4: Potential rates of utilization of ^{14}C-(U)glucose in four selected sampling zones at burrow sites of Arenicola marina. Further details in Fig.1.

Fig.5: Potential rates of utilization of ^{14}C -acetate in four selected sampling zones at burrow sites of Arenicola marina (Westerhever Sand). Further details in Fig.1.

Fig.6: Activities of hydrolytic enzymes after extraction with triton-X-100 from four selected sampling zones at burrow sites of Arenicola marina (Westerhever Sand). Further details in Fig.1.

Table 1. Grain size distribution in combusted sediments from 4 sampling zones at burrow sites of Arenicola marina (Westerhever Sand = WHS, and List). Percent dry weight in combined subsamples.

Grade limits	W H S				List			
(µm)	FC	S	RS	BW	FC	S	RS	BW
>1000	-	-	-	-	6.7	3.8	1.5	1.4
500 -1000	5.8	0.9	3.5	1.0	55.7	62.6	47.9	41.3
250 - 500	21.6	22.9	14.5	16.2	31.5	31.6	43.1	49.5
125 - 250	65.1	70.8	63.1	68.8	4.1	1.6	4.5	6.3
63 - 125	7.5	5.4	17.0	12.5	1.4	0.3	2.2	1.4
<63	-	-	1.9	1.5	0.6	0.1	0.8	0.1

FC= fecal casts; S= sediment surface; RS= reduced subsurface sediment; BW= burrow walls

Table 2. Redox potential (E_h, mV) and total organic matter (O.M. as mg cm^{-3} of ash-free dry weight) in 4 sampling zones at Arenicola marina burrow sites at Westerhever Sand (WHS) and List, (mean values of n= 3 pooled samples).

Sampling zone	WHS	List	WHS	List
	(E_h)		(O.M.)	
Fecal casts	365	295	11.0	14.9
Sediment surface	425	290	8.9	12.1
Reduced subsurface sed.	150	130	13.2	23.5
Burrow walls	370	260	16.8	19.7

Table 3. Protein content (mg cm^{-3}), proteolytic viable counts (CFU), and acridine orange epifluorescent direct counts of bacterial cells (AODC) in 4 sampling zones at Arenicola marina burrow sites in intertidal flats at Westerhever Sand. S.D.= standard deviation for given number (n) of samples.

Sampling zone	Protein (n=4)		CFU x10^6 cm^{-3} (n=5)		AODC x10^9 cm^{-3} (n=5)	
		(S.D.)		(S.D.)		(S.D.)
Fecal casts	1.53	(0.38)	1.12	(0.81)	4.9	(0.7)
Sediment surface	1.06	(0.22)	7.04	(3.94)	2.5	(0.4)
Red.subsurface sed.	1.90	(1.29)	0.40	(0.61)	3.5	(1.7)
Burrow walls	2.27	(1.30)	4.89	(3.30)	4.6	(6.7)

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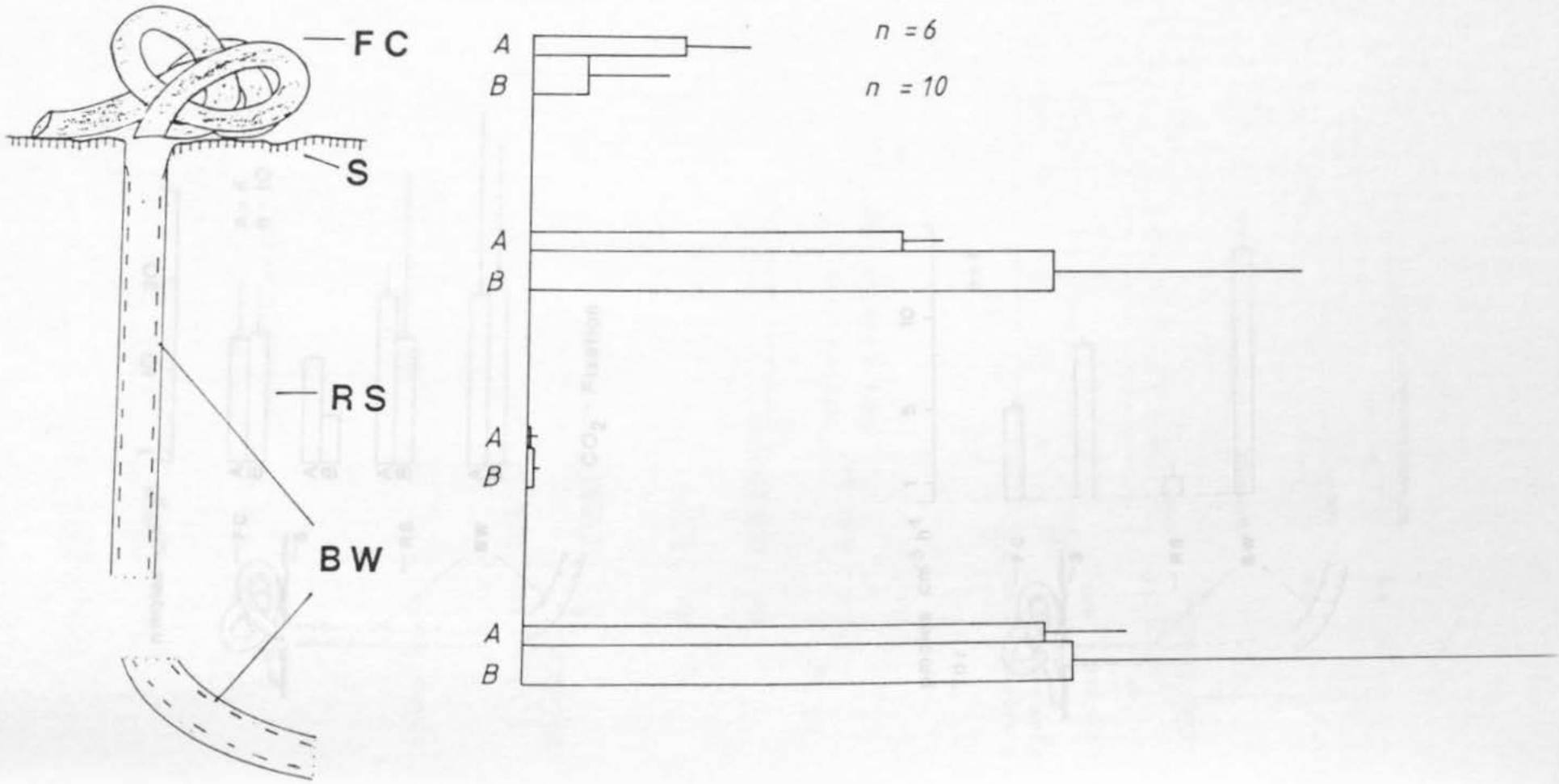

1 2 3 4 5 6 7 8 9 10
x 10^4
CHITINOLYTIC C F U cm^-3
FC
S
RS
BW
A
B
n = 6
n = 10

Fig. 2

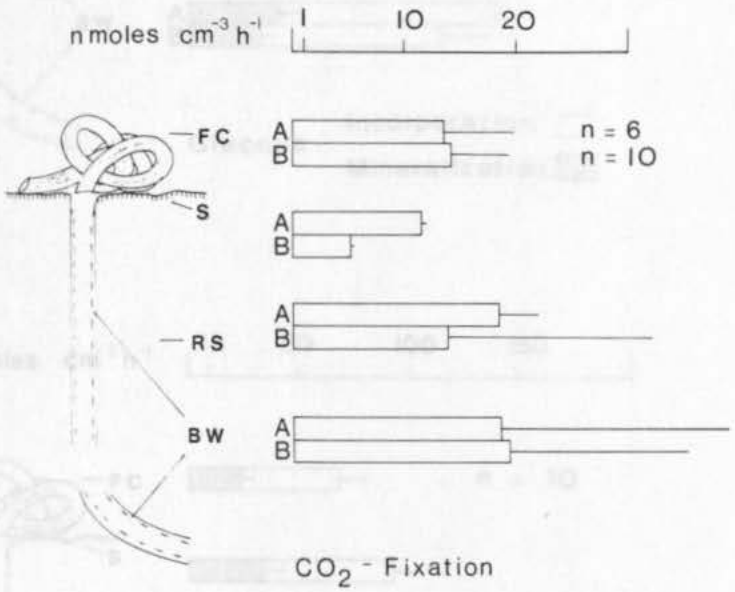
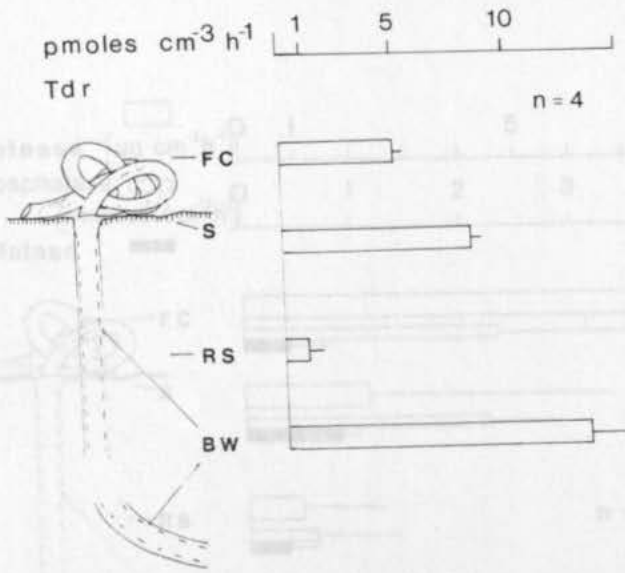

nmoles cm⁻³ h⁻¹
1
10
20
FC
S
RS
BW
A
B
n = 6
n = 10
CO2 - Fixation

Fig. 3


pmoles cm⁻³ h⁻¹
1
5
10
Tdr
n = 4
FC
S
RS
BW

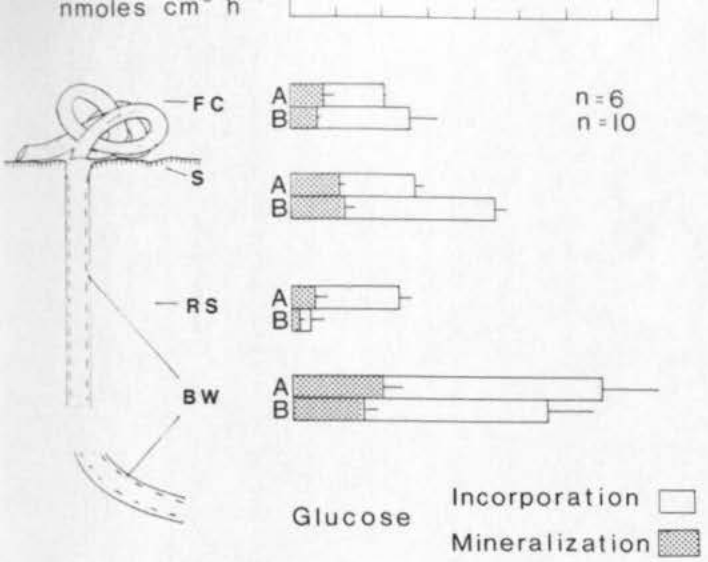

nmoles cm⁻³ h⁻¹
FC
S
RS
BW
A
B
n=6
n=10
Glucose
Incorporation
Mineralization

Fig. 5

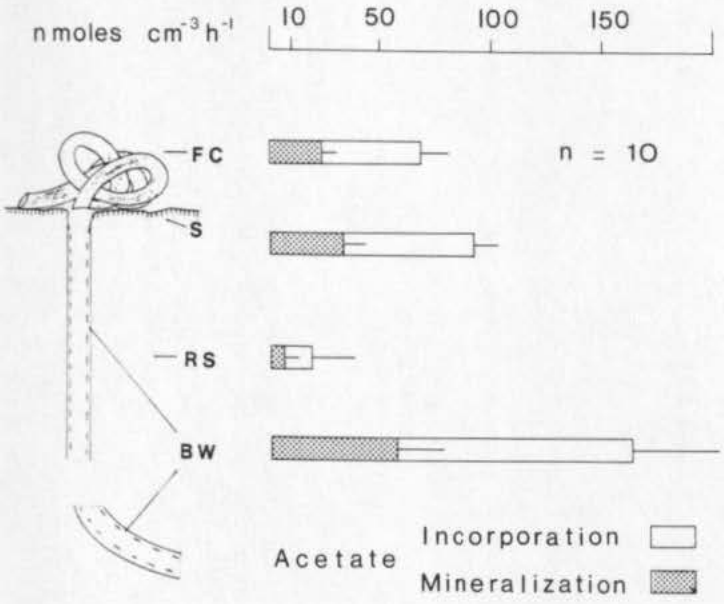
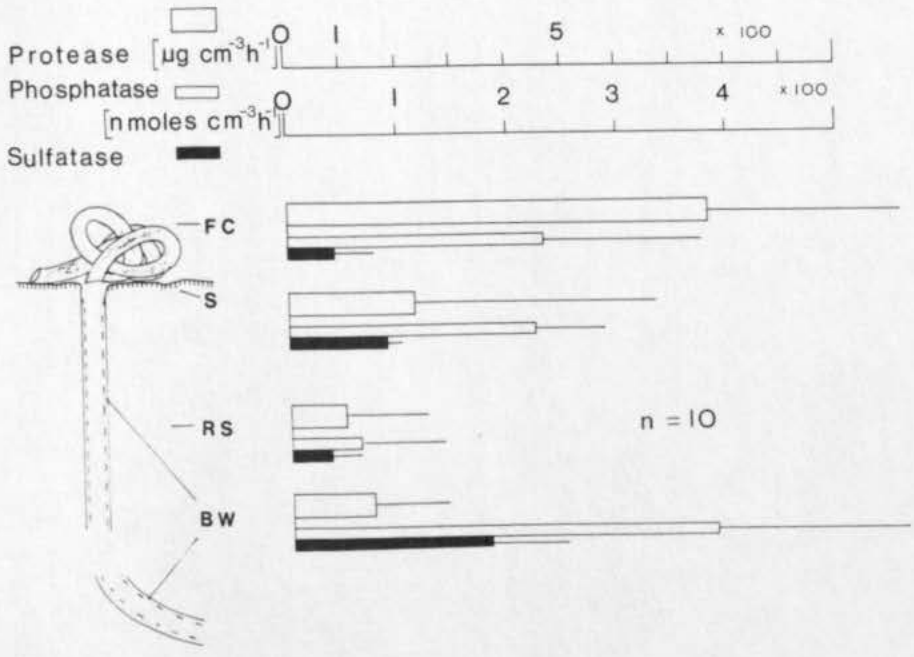

n moles cm⁻³ h⁻¹
10
50
100
150
FC
S
RS
BW
n = 10
Acetate
Incorporation
Mineralization

Fig. 6


Protease [µg cm⁻³h⁻¹]
Phosphatase
[n moles cm⁻³h⁻¹]
Sulfatase
0
1
5
x 100
0
1
2
3
4
x100
FC
S
RS
BW
n = 10