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Beggiatoa leptomitiformis – a Filamentous Sulfur-oxidizing Bacterium Colonizing Laboratory-made Aggregates*

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With 3 Figures and 3 Tables

Key words: Aggregates, estuary, *Beggiatoa leptomitiformis*, sulfate-reducing bacteria

Abstract

The colonization of laboratory-made aggregates by the filamentous sulfur-oxidizing bacterium, *Beggiatoa leptomitiformis*, was investigated over a period of 14 days during winter and spring. The aggregates were generated from natural water from the freshwater zone of the Elbe Estuary using a tilting tube roller. Experiments were carried out under natural environmental conditions in a climate chamber. Within one hour after the beginning of the experiments, macroaggregates had already started to form. They reached a maximum size of 1 mm during the experiments. Both in winter and in spring, the water in which aggregates developed remained well saturated with oxygen. From the first day of the experiments, the sulfur-oxidizing bacterium, *Beggiatoa leptomitiformis*, was detected living in the aggregates and in the surrounding water. In the aggregates, the number of these bacteria was in the order of 10^6 ml^{-1}, whereas in the surrounding water, they were three orders of magnitude less abundant. In addition to the sulfur-oxidizing *Beggiatoa*, sulfate-reducing bacteria were detected within the aggregates.

Introduction

Aggregates are ubiquitous in aquatic environments, including the ocean (SUZUKI & KATO 1957), lakes (GROSSART & SIMON 1993), rivers (BERGER et al. 1996) and estuaries (EISMA 1986). They are formed from suspended organic and inorganic matter in the water column (ALLDREDGE & SILVER 1988) and produced by biologically-enhanced physical processes (MCCAVE 1984; JACKSON 1990). Because they originate to a large extent from zooplankton and phytoplankton, their composition varies with the seasons. Aggregate sizes range from a few microns to many centimeters (ALLDREDGE & SILVER 1988; GROSSART 1995; BERGER et al. 1996; ZIMMERMANN & KAUSCH 1996), depending on the environment in which they occur, their composition and the shear forces which determine aggregate formation. Aggregates are rich in nutrients, a fact that enhances their colonization by communities of bacteria, phytoplankton, protozoans and sometimes metazoans. Since aggregates contain such an enriched microbial community, they are important sites in the water column on which processes of photosynthesis, decomposition and nutrient regeneration occur at highly elevated levels (ALLDREDGE & SILVER 1988).

Aggregates from the limnetic zone of the Elbe Estuary, Germany, contain a rich microbial community of bacteria, protozoans and even metazoans (ZIMMERMANN & KAUSCH 1996; ZIMMERMANN 1997; ZIMMERMANN-TIMM et al. 1998). Filamentous sulfur-oxidizing bacteria of the genus *Beggiatoa* were also frequently found as colonizers (WÖRNER 1997). Members of this genus form colourless, gliding filaments. The filaments are composed of a linear series of individual cells and are usually filled with sulfur granules. *Beggiatoa* can be observed in a variety of environments, all of which are characterized by the presence of a detectable level of hydrogen sulfide. *Beggiatoa* is common in freshwater habitats as well as in brackish and marine environments. It is found mainly on the surfaces of sediments or on decomposing plant material. For example, a variety of eutrophic lakes and ponds contain benthic mats rich in *Beggiatoa* spp. (WINOGRADSKY 1887; KEIL 1912). In marine environments, *Beggiatoa* is enormously enriched due to the great abundance of sulfate. The surface sediments in shallow seas, brackish fjords, salt marshes and intertidal sand flats may be covered by mats rich in *Beggiatoa* spp. (ANKAR & JANSSON 1973; JØRGENSEN 1977b; LACKEY et al. 1965). Hydrothermal vents in the deep sea and in shallow waters are also habitats for this genus (POWELL et al. 1983; KARL 1987).

* This paper is dedicated to Prof. Dr. HARTMUT KAUSCH on the occasion of his 60th birthday.

Since *Beggiatoa* oxidizes hydrogen sulfide, members of this genus grow naturally where both hydrogen sulfide and oxygen are present. Hydrogen sulfide is not stable in oxic waters due to auto-catalytic oxidation, so the habitat of *Beggiatoa* is restricted to the transition zone between the oxic and anoxic environment (JØRGENSEN 1977b). Originally, members of the genus *Beggiatoa* were considered to be true chemoautotrophs, deriving energy from the oxidation of hydrogen sulfide to sulfuric acid and obtaining organic carbon from the fixation of carbon dioxide (WINOGRADSKY 1888). Other investigations, however, showed that pure cultures grow best chemoorganotrophically on compounds such as acetate and succinate, though the oxidation of hydrogen sulfide seems to stimulate growth (PRINGSHEIM 1964).

In order to conduct a detailed study of the microbial community on aggregates in the Elbe Estuary, we produced aggregates in the laboratory using natural water within a period of 14 days. Two experiments were carried out, one in winter and one in spring, in order to study seasonal differences in aggregate formation, the composition of the aggregates and their colonization by *Beggiatoa leptomitiformis*. Aggregates were generated using a tilting tube roller, a system on which glass bottles filled with water were both rolled and tilted. In preliminary experiments, this system was found to be most suitable for generating aggregates similar to those in the natural environment. The associated protist fauna and their succession on the aggregates have been described (WÖRNER 1997; WÖRNER et al. submitted). Because of the occurrence of *Beggiatoa* on the aggregates, we suspected that hydrogen sulfide was present within them. For this reason, we tested aggregates with sulfate-reducing bacteria twice during the experiments, at the beginning and toward the end.

Materials and Methods

General design and field collection

Aggregates were produced in the laboratory from natural water of the Elbe Estuary during two different times of year. Each experiment was carried out over a period of 14 days, once during February and again during May and June. The environmental temperature and illumination in each season were maintained in a climate room (Table 1).

Water for the laboratory experiments was obtained from the shallow, slow flowing Hahnöfer Nebenelbe, which is situated near Hamburg, Germany, in the freshwater zone of the Elbe Estuary. It was collected at the surface during high tide using a horizontal tube (Hydro Bios, Kiel, Germany). Temperature, oxygen concentration and conductivity were determined simultaneously using portable WTW OXI-96 and WTW LF-96 devices. The chlorophyll *a* concentration was determined according to the method of NUSCH (1980). Within an hour after collection, the water was delivered to the laboratory and placed in sterile 1 l screw-cap glass bottles. Two replicates were made (Bottle 1 and Bottle 2). Aggregates were generated using a tilting tube roller (Stuart Scientific Co LTD). In this system, glass bottles filled with water were simultaneously rolled at 11.5 r.p.m. and tilted at 35 cycles per minute on parallel rubber bars. One tilting cycle entails one movement forward and one backward.

Table 1. Conditions in the climate room during the experiments.

	Winter (Feb. 4–Feb. 20)	Spring (May 22–June 7)
Temperature [°C]	3.0	14.0
Illumination [Quanta sec^{-1} cm^{-2}]	0.2×10^{16}	0.12×10^{17}

Daily sampling

Samples were taken at 24 hour intervals over a 14 day period in order to determine aggregate abundance and volume as well as the abundance of *Beggiatoa,* both within the aggregates and in the surrounding water. Abundance and volume of the aggregates were determined in a 5 ml subsample under a dissecting microscope at 25×. These subsamples were removed carefully using an Eppendorf pipette with an extra wide tip in order to avoid breakage of the aggregates. For determining the volume, the sizes of at least ten aggregates were measured using an ocular micrometer. The volume was calculated by measuring three dimensions of each aggregate and multiplying the values. In winter, abundance and volume of the aggregates in Bottle 2 were determined for a period of only eight days. In determining the abundance and volume, only aggregates at least 0.2 mm in one dimension were considered. In order to determine the abundance of *Beggiatoa* within the aggregates, subsamples of three to five aggregates were collected using a very narrow Pasteur pipette. The volume of each aggregate was determined. The aggregates were then fixed in formalin at an 8% final concentration. The bacteria were dislodged from these preserved aggregates by ultrasonic vibration for 5 minutes using a Sonorex TK 52 from Bandelin Electronic. Then the sample was whirled for 10 seconds. The abundance of *Beggiatoa* was determined by counting the organisms after staining with DAPI (PORTER & FEIG 1980) under a Leitz Diaplan epifluorescence microscope at a magnification of 400×. In order to be sure of the identification, only filaments of *Beggiatoa* at least 35 µm in length were counted. The abundance was determined per unit volume. Subsamples were also taken for determining the abundance of *Beggiatoa* in the surrounding water. These water samples were passed through a 30 µm sieve and fixed in formalin at a 4% final concentration. Then they were stained with DAPI, and the filaments of *Beggiatoa* were counted under the epifluorescence microscope as described above.

Aggregates were also examined alive under a phase contrast microscope at a magnification of 200× to 1000× in order to determine their composition and to observe filaments of *Beggiatoa* associated with them. The oxygen concentration in the surrounding water was determined three times during the experiments using an oxygen electrode (WTW OXI-96).

Determination of sulfate-reducing bacteria

The number of sulfate-reducing bacteria in the aggregates was determined using the M.P.N. technique (JACOBS & GERSTEIN 1960). By this method, the most probable number (M.P.N.) of living cells is determined.

For this method, samples were taken from Bottle 1 at the beginning on the first day and toward the end on the tenth day of the experiment. For the experiment in winter, 15 ml water samples were removed from the glass bottles using a sterile pipette and placed in a sterile test tube. Within two hours, the aggregates settled to the bottom. The overlying water was discarded, and 3 ml of the concentrated aggregates were whirled under anaerobic conditions for one minute. An inoculum of 0.5 ml was then placed in each of three test tubes containing 4.5 ml of nutrient medium E for sulfate-reducing bacteria (POSTGATE 1984). From this suspension, dilutions of 1 to 10, 1 to 100, and 1 to 1000 in 0.9% sodium chloride were prepared. The test tubes were incubated in an anaerobic work station (MK 3, dw Scientific) for four weeks at 24 °C. A positive reaction was indicated by a black coloration of the test tube, which is caused by FeS-precepitation. The number of sulfate-reducing bacteria was calculated using the M.P.N. table of JACOBS & GERSTEIN (1960), which is based on the number of positive test tubes at each of the different dilutions.

Since we found very few sulfate-reducing bacteria in winter, perhaps due to an incomplete disintegration of the aggregates, we modified the method in spring, when 15 ml water samples were taken and centrifuged for 20 minutes at 8000 r.p.m. in a Heraeus-Christ-Hämofuge. All but 1 ml of the supernatant water was discarded. Then, 9 ml of a buffer (CAMPER et al. 1985) used to disintegrate biofilms was added. The sample was then spun 3 times for 30 seconds in an Ultra-Turrax, Type T25, from Janke & Kunkel at 10000 r.p.m. From the resulting suspension, an inoculum of 0.5 ml was placed in each of three test tubes. The subsequent treatment was the same as that used in winter.

Results

Table 2 shows the abiotic parameters and the chlorophyll *a* concentration in the Hahnöfer Nebenelbe on the two dates of sampling in winter and in spring. Water temperature was about 11 °C higher in spring. The chlorophyll *a* content increased from 3.33 µg l^{-1} in winter to 15.65 µg l^{-1} in spring.

Table 2. Biotic and abiotic variables in the Hahnöfer Nebenelbe at sampling time.

	Winter (Feb. 4)	Spring (May 22)
Water temperature [°C]	3.0	14.0
Illumination at a water depth of 10 cm [Quanta sec^{-1} cm^{-2}]	0.36×10^{16}	3.6×10^{16}
Oxygen concentration		
[mg l^{-1}]	14.2	11.7
[% saturation]	104	129
Conductivity [µS cm^{-1}]	930	791
Chlorophyll *a* concentration [µg l^{-1}]	3.33	15.65

Aggregate-formation and oxygen concentration

Visible aggregates were formed within one hour after the water was placed in the tilting tube roller. Figure 1 shows the abundance and volume of the aggregates during the experiments. In winter, the number of aggregates > 200 µm varied between 26 and 93 ml^{-1} in Bottle 1 and between 5 and 30 ml^{-1} in Bottle 2. However, in Bottle 2 only days one to eight were considered. Maximum abundance of aggregates was recorded on the 12th day in Bottle 1. In spring, the aggregates were considerably less abundant than in winter and ranged from 9 to 25 ml^{-1} in Bottle 1 and from 5 to 22 ml^{-1} in Bottle 2. Samples taken during this season showed the greatest abundance on the 11th and 8th days, respectively.

The mean volume of the aggregates > 200 µm produced in Bottle 1 reached a maximum of 34.93×10^6 μm^3, while these in Bottle 2 remained smaller with a maximum mean volume of 12.22×10^6 μm^3. However, as mentioned above, only days one to eight were considered. In spring, the mean volumes of the aggregates were in the same range as in winter. In Bottle 1, a maximum value of 34.4×10^6 μm^3 was

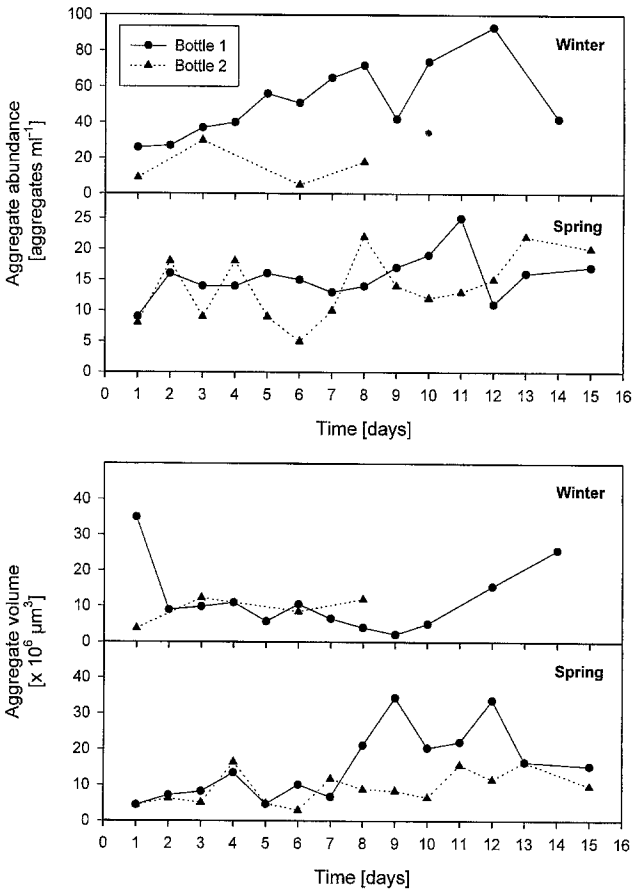

Fig. 1. Aggregate abundance [ml^{-1}] and volume [$\times10^6$ μm^3] during the experiments.

Table 3. Oxygen concentration in the surrounding water during the experiments in mg l^{-1} and in percent of saturation.

	Day of experiment					
	1		10		14	
	mg l^{-1}	% Satur.	mg l^{-1}	% Satur.	mg l^{-1}	% Satur.
Winter						
Bottle 1	13.7	128	11.7	113	13.0	121
Bottle 2	12.0	110	11.5	108	12.1	110
Spring						
Bottle 1	13.4	166	13.6	167	13.5	167
Bottle 2	–*	–*	12.3	142	12.2	144

* Not determined

reached, wheras in Bottle 2, a lower average of 16.36×10^6 μm^3 was calculated. During both winter and spring, about 90% of the aggregates were less than 1000 µm in diameter. Aggregate shape varied from elongate to nearly spherical, and some had comet-like tails.

The oxygen concentration during the experiments is shown in Table 3. In both seasons, the oxygen concentration remained lower in Bottle 2. In winter, a maximum value of 13.7 mg l^{-1} was reached in Bottle 1, wheras in Bottle 2 the highest concentration was 12.1 mg l^{-1}. In spring, a maximum concentration of 13.6 mg l^{-1} was reached in Bottle 1, while in Bottle 2, a maximum of 12.3 mg l^{-1} was determined. In all cases, both in winter and in spring, the oxygen saturation of the water did not drop below 100%.

The composition of the aggregates depended on the season. In winter, the main components were detritus, mineral particles and a few centric diatoms. The percentage of organic matter was about 80%. In spring, the aggregates contained many centric diatoms, green algae, fecal pellets of copepods, loricae of tintinnids and molts of cladocerans and rotifers. In this season, the aggregates contained about 95% organic matter.

Abundance of *Beggiatoa leptomitiformis*

Observations of unfixed aggregates under a phase contrast microscope revealed that in addition to a rich fauna of protozoans, about 60% of the aggregates were colonized by filamentous bacteria of the genus *Beggiatoa* (Fig. 2). The filaments all had a uniform width of about 1.5 µm. On the basis of this characteristic, they were identified as *Beggiatoa leptomitiformis* (MENEGHINI) TREVISAN. The filaments reached a maximum length of about 150 µm and contained sulfur droplets. The individual filaments were motile and glided constantly through the aggregates.

The abundance of filaments of *Beggiatoa leptomitiformis* both in the aggregates and in the surrounding water is shown

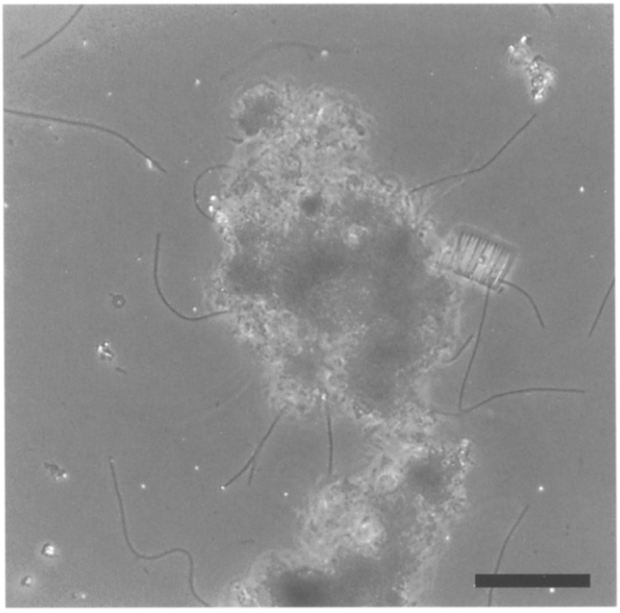

Fig. 2. *Beggiatoa leptomitiformis* colonizing an aggregate. Scale bar = 50 µm.

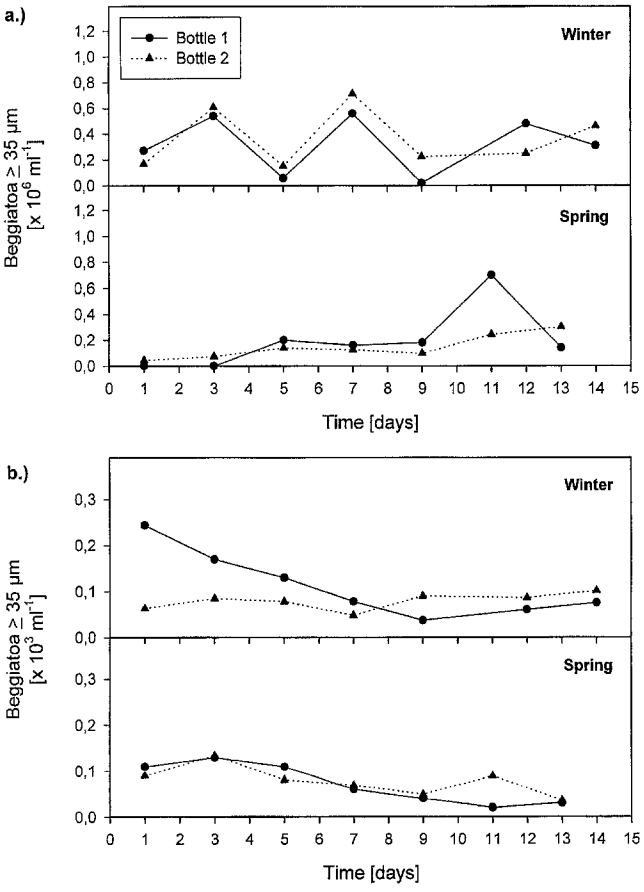

Fig. 3. Density of *Beggiatoa leptomitiformis* within the aggregates (**a**) and in the surrounding water (**b**).

in Fig. 3. Within the aggregates (Fig. 3a), the number of filaments >35 µm reached in winter was 0.56×10^6 ml^{-1} in Bottle 1 and 0.71×10^6 ml^{-1} in Bottle 2. Two peak values above 0.4×10^6 ml^{-1} were reached on the 3rd and 7th days in both replicates. Bottle 1 also reached a peak on the 13th day. In spring, the number of filaments of *Beggiatoa leptomitiformis* within the aggregates reached a maximum of 0.7×10^6 ml^{-1} in Bottle 1 on the 11th day. In Bottle 2, a lower maximum of 0.3×10^6 ml^{-1} was reached on the 13th day.

In the surrounding water (Fig. 3b), the number of filaments was approximately three orders of magnitude less than within the aggregates. In winter, a maximum number of 0.24×10^3 ml^{-1} was reached in Bottle 1 whereas in Bottle 2, a lower maximum of 0.1×10^3 ml^{-1} was determined. In Bottle 1, the maximum was reached on the first day, whereas in Bottle 2, it was determined at the end of the experiment on the 14th day. In spring, the filaments developed similarly in both bottles, reaching densities between 0.02×10^3 and 0.14×10^3 ml^{-1}. In each case, the highest values were detected on the third day.

Determination of sulfate-reducing bacteria

During both winter and in spring, we were able to detect sulfate-reducing bacteria in the aggregates.

In winter, 4 cells ml^{-1} were detected on the first day of the experiment. On the tenth day, toward the end of the experiments, 1 cell ml^{-1} was recorded. In spring, on the first day, only 1 cell ml^{-1} could be detected. Toward the end, on the tenth day, no living sulfate-reducing bacteria were found.

Discussion

In most cases, the abundance of aggregates was low at the beginning of the experiment, and it was highest toward the end. In winter, Bottle 2 contained fewer aggregates than Bottle 1. In spring, the abundance of the aggregates remained in the same range in the two replicates during the course of the experiment. Nevertheless, the values differed greatly from time to time, indicating that aggregate formation is very dynamic, and comparisons of the different bottles is not easy. In spring, aggregates were considerably less abundant than in winter, although more organic material for aggregate formation was available during spring. It is likely that rotifers and other metazooplankters of the spring community decimated the aggregates in our microcosms. Aggregates in all systems tended to have greater volumes toward the end of the experiment (Wörner 1997; Wörner et al. submitted). As in the case of aggregate abundance, the volume also differed widely at times in the two replicates.

The composition of the aggregates depended on the season and reflected the composition of the planktonic community, as described in previous publications (Grossart & Simon 1993; Zimmermann-Timm et al. 1998).

Among the organisms larger than 20 µm that are associated with the aggregates or suspended in the water, not only the protists are remarkable (Wörner et al. submitted). Filaments of bacteria in the genus *Beggiatoa* were observed in the aggregates and the surrounding water as early as the first days of the experiments (Fig. 2). According to conventional nomenclature, these were identified as *Beggiatoa leptomitiformis*. The species of this genus are identified almost exclusively by the width of their filaments. Although this is an insufficient basis for distinguishing species because strains of equal diameter need not be identical (Pringsheim 1964), other characteristics have not yet been recognized. Further investigations should focus on genetic methods, such as *in situ* hybridization.

As filaments of *Beggiatoa* were so enormously enriched within the aggregates, we assume that the aggregates produced in the laboratory provide a good habitat for them. Another explanation is that the filaments were resuspended from the sediment during high tide, when water was collected for the experiments, and then concentrated in the aggregates due to shear in the rolling tanks. However, our microscopic observations on living *Beggiatoa* revealed, that they actively penetrate the aggregates and seldom stay in the water surrounding them.

In winter, the number of *Beggiatoa* within the aggregates fluctuated considerably in both replicates during the course of the experiment. The abundance fluctuated between highs and lows, wheras in the surrounding water, such fluctuations could not be detected. The conditions within the aggregates did not seem very stable, perhaps due to disintegration of the aggregates or enhanced protozoan grazing.

The proliferation of *Beggiatoa* in the surrounding water during winter and spring seems to be similar. In most cases there was a tendency for these bacteria to become less abundant toward the end of the experiment, beginning on the 9th day.

The oxygen concentration in the water containing the aggregates never dropped below 11 mg l^{-1}. In all cases, the oxygen concentration in Bottle 1 remained higher than that in Bottle 2. We suppose that Bottle 2 was shaded by Bottle 1 and was therefore less illuminated. Thus, less oxygen was produced by photosynthesis. Due to the greater photosynthetic activity, the oxygen saturation of the water in nearly all cases was greater in spring.

Since *Beggiatoa* has been described as a microaerophilic organism (Jørgensen & Revsbech 1983) and hydrogen sulfide occurs only under anoxic conditions due to its rapid autocatalytic oxidation in oxygenated water, microniches within the aggregates in which strong reducing conditions prevail are likely. Møller et al. (1985) demonstrated a negative chemotactic response by *Beggiatoa* to oxygen, a behavior that is of ecological importance because it helps the filaments to locate the hydrogen sulfide-oxygen interface.

Shanks & Reeder (1993) frequently found reducing microzones within diatom frustules. They were also able to de-

tect sulfide in marine snow. Similar microenvironments within the aggregates and planktonic colonies of microalgae have also been studied by the use of micro- and minielectrodes and by the addition of tetrazolium salts (PEARL & BEBOUT 1988; RICHARDSON et al. 1988; PEARL et al. 1989; LUBBERS et al. 1990; GROSSART 1995). Recent investigations, however, revealed that the descriptions of the microzones are doubtful (PLOUG & JØRGENSEN 1999) because they were not investigated under natural conditions. JØRGENSEN (1977a) found anoxic microzones in oxidized sediment and identified them as fully or partially disintegrated fecal pellets in which hydrogen sulfide was produced and from which it diffused into the surrounding pore water. Furthermore, the large organic content of the aggregates supported a high microbial metabolism and a depletion of oxygen. JØRGENSEN (1977a) suggested that the shell of a dead foraminiferan or a cell in a plant fragment might also contain reduced microniches since diffusion from them is limited. Frustules of diatoms and molts of cladocerans and rotifers were components of our artificial aggregates, especially in spring. However, the existence of microniches with strong reducing conditions within the aggregates remains unconfirmed. Further investigations with microelectrodes are needed.

In the natural estuarine environment, the situation is different. There are always variations in the processes of sedimentation and resuspension of particles. Changes in the locations of the particles cause changes in diffusion and thereby change the concentrations of the chemical components. Perhaps anoxic microzones exist in resuspended sediment material.

Not only the concentrations of oxygen and hydrogen sulfide but also grazing by phagotrophs may influence the abundance of *Beggiatoa*. We observed an amoeba consuming part of a filament. In the marine environment, some ciliates as well as a nematode graze on filamentous sulfur bacteria (BERNARD & FENCHEL 1995).

In anoxic marine sediments, sulfide is produced mainly by bacterial reduction of sulfate (JØRGENSEN 1977b). Another source is the hydrolytic decomposition of proteins in detritus. We detected sulfate-reducing bacteria within the aggregates, during both winter and spring. Since sulfate-reducing bacteria are reportedly strict anaerobes (POSTGATE 1984), we assume that they must have occupied anoxic microniches within the suspended particles.

On the other hand, the oxygen tolerance of sulfate reducing bacteria has been under discussion continuously since they were reported to be obligate anaerobic organisms. Two strains of sulfate-reducing bacteria grew in an aerated chemostat where the oxygen concentration was low (GOTTSCHAL & SZEWZYK 1985). In the oxic layers of a marine sediment, many sulfate-reducers were found, and sulfate-reduction was detected (JØRGENSEN & BAK 1991). It is therefore possible that anoxic conditions are not as necessary as previously assumed. Furthermore, we detected sulfate-reducing bacteria during both winter and spring, but the quantitative data recorded remain doubtful. According to the M.P.N. table of JACOBS & GERSTEIN (1960), the highest density we determined was 4 cells ml^{-1} of water in which the aggregates were present. Because the number of sulfate-reducing bacteria was low, it does not seem that the aggregates made in the laboratory were a very good habitat for them. On the other hand, it should be remembered that the nutrient medium we used is not suitable for all sulfate-reducing bacteria, and it supported the growth of species mainly in the genus *Desulfovibrio*. Furthermore, the M.P.N. method is not very precise and often results in an underestimation of abundance (CARON et al. 1989; MASSANA & GÜDE 1991). Another explanation for the low abundance of sulfate-reducing bacteria is that reducing microzones were few and small. The anoxic zone in an aggregate suspended in oxygenated water only occupies about 8% of its total volume (PLOUG et al. 1997). It was remarkable that during both winter and spring, the number of sulfate-reducing bacteria decreased toward the end of the experiments. In spring, we did not observe any positive test tube at this phase of the experiment. Perhaps the sulfate content had decreased within the aggregates. Another factor controlling the rate of sulfate-reduction is the availability of organic substrates (HADAS & PINKAS 1992). Protozoan grazing can also cause structural changes in an assemblage of bacteria (JÜRGENS & GÜDE 1994). According to the results of our experiments we assume that sulfate-reduction by sulfate-reducing bacteria and oxidation of hydrogen sulfide by species of the genus *Beggiatoa*, processes which have been observed mainly in sediments, may also occur ephemerally in the pelagic environment. The existence of a sulfur cycle within aggregates must be demonstrated by further investigations.

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