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Mucous aggregates under natural and laboratory conditions: a review

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Abstract

During the years 1991 and 1992 several experiments were carried out in order to study the phenomenon of mucous aggregates in the Northern Adriatic Sea. Samples of surface and bottom sea water were treated in different ways, according to the various experiments, and then placed in 10 or 20-l tanks. The tanks were stored in thermostatic cells at a temperature of 20°C and a 16:8 h light:darkness period. After 8–10 days the formation of mucous filaments occurred. The genus *Nitzschia* was essential for the formation of mucous filaments, under the conditions of light and temperature applied during the experiments. Appropriate concentrations of phytoplankton and nutrients seemed to be very important also. It was possible to observe a sequence of species in the formation of mucilaginous filaments. There was a transition from species belonging to centric diatoms toward pennate diatoms. The centric diatoms (genus *Chaetoceros)* formed the first small aggregates which later, with the colonisation of the genus *Nitzschia*, developed into real filaments.

Keywords: Mucous aggregates; Microcosms

1. Introduction

In early summer 1991 large amounts of mucous aggregates reappeared in the Northern Adriatic Sea. The phenomenon seems to be of a similar nature to those recorded in the same area during the last century (Fonda Umani et al., 1989).

Mucous aggregates are of complex compositions and may have different origins (Alldredge and Silver, 1988; Alldredge and Gotschalk, 1989; Beers et al., 1986; Lochte, 1991; Prezelin and Alldredge, 1983; Turley, in press).

Although several recent studies (Andreoli et al., 1992; De Angelis et al., 1992; Fanuko et al., 1989; Guerrera et al., 1992; Marchetti, 1990; Marchetti et al., 1989; Monti et al., 1992b; Pettine et al., 1992; Revelante and Gilmartin, 1990; Stachowitsch et al., 1990) have examined the composition of the mucous aggregates which appeared in the Northern Adriatic, there are still many doubts concerning their origin and the causes of their formation (Herndl and Peduzzi, 1988; Herndl et al., 1992).

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It is, however, difficult to establish the original composition of aggregates because many factors could influence and modify their original nature, e.g. the zone where they are formed, how and where samples were collected, how long they have been in the sea, weather, sea conditions, etc.

In order to improve our understanding of the phenomenon, several laboratory experiments were carried out between 1991 and 1992. The aim of the work was to reproduce mucous filaments under controlled conditions (Casaretto et al., 1992; Monti et al., 1992a; Monti et al., 1994) and, as far as possible, to limit secondary causes which influence and modify mucous aggregates in the sea.

2. Materials and methods

During the period of research, samples of water, sediment and mucus were collected at a station (AA3) located ~12 Km offshore from the eastern coast, between Punta Salvore and Punta Tagliamento. In May 1992, samples were also collected at a station (M1) situated ~1.5 miles offshore from the Marine Biology Laboratory of Trieste (Fig. 1).

Water samples were collected using tanks for surface water and a 5-l Niskin bottle for intermediate and bottom depths. Chemical and physical parameters (temperature, salinity, pH, dissolved oxygen) were also recorded by means of an Idronaut Ocean Seven mod. 401 multiparameter probe.

The water samples, having been treated in different ways according to the experiment concerned, were placed in 10 or 20-l tanks and stored in thermostatic cells at a temperature of 20°C and a 16:8 h light:darkness period. Both the temperature and the light:darkness cycle reflected conditions present in nature when the samples were first collected (July 1991), during an abnormal production of mucus. In successive experiments the same conditions were maintained in order to obtain comparable data.

Temperature, salinity, dissolved oxygen and pH values were recorded daily by means of laboratory probes (WTW mod. OXI 196, WTW mod. LF 196, ORION mod. SA 250 equipped with a RHOSS electrode).

Chlorophyll *a* concentration was determined spectrophotometrically following concentration on cellulose acetate filters and extraction in a solu-

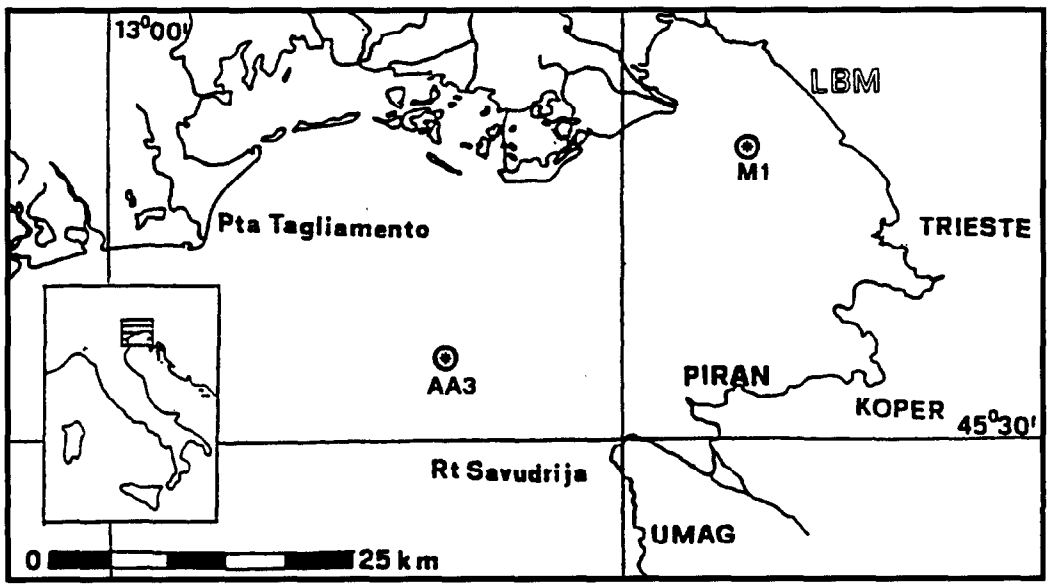

Fig. 1. Map of the sampling area.

tion of 90% acetone (Panella and Magazzù, 1978).

Nutrient concentration ($P\text{-}PO_4$, $N\text{-}NO_2$, $N\text{-}NO_3$ and $N\text{-}NH_3$ and $Si\text{-}SiO_2$) were measured by means of an Alliance mod. Integral., continuous flow analyser according to the analytical procedures of Strickland and Parson (1972).

Water subsamples were taken during every experiment in order to analyse phytoplankton. The samples were fixed with 4% formaldehyde buffered with sodium tetraborate at a slightly alkaline pH and observed under an inverted Zeiss microscope according to the Utermöhl method (1958).

The sediment samples, for analysis of the microphytobenthos, were collected by means of a core with a diameter of 5 cm. One sample of 5 cc taken from the surface layer (1 cm), was fixed with 25 cc of 4% formaldehyde buffered with hexamethylenetetramine at a slightly acid pH. Subsamples of 20 μl were observed under an inverted Zeiss microscope and all algal cells were counted.

The natural mucus was collected by SCUBA divers using a 500-ml plastic syringe.

Qualitative analyses were carried out on mucus formed in the tanks and collected at sea.

Part of the material was stained using Ruthenium Red (Blanquet, 1976) and Alcian Blue 8GX (Barka and Anderson, 1963) in order to obtain a histochemical characterization of the polysaccharide components.

Samples were examined using the following microscopic techniques: light microscope, inverted light microscope, epifluorescence microscope, scanning electron microscope (SEM) and transmission electron microscope (TEM).

Samples for examination under light and epifluorescence microscopes were not fixed and were placed on specimen slides.

Material for examination under SEM was filtered on 0.8 μm Nuclepore filters, gradually dehydrated with ethanol and examined using a Philips 201 electron microscope following metallization with gold/palladium. Material for examination under TEM was cleaned with sodium dodecyl sulphate (Dr. A.M. Schmid, pers. commun.), placed on copper grids, 300 mesh, coated with formvar film and observed under a Philips 201 electron microscope.

The most characteristic species were measured and photographed using a Wild Photoautomat camera.

2.1. Year 1991

July. Surface sea water was collected, placed in 10-l tanks and stored in a thermostatic cell at 20°C and at a 16:8 h light:darkness period (Fig. 2). Further samples of surface water were filtered through meshes of different diameters (150 μm, 20 μm, 10 μm, 1.2 μm) and then treated as above.

At the same time as the water samples were taken, divers collected samples of mucus at three different depths (0, 10 and 20 m), and surface sediment for analysis of the microphytobenthos.

August. Surface sea water and sediment samples were collected. On this occasion, samples of unfiltered water and of water filtered through meshes of 100 μm and 10 μm were used in the experiments. Replicates were treated with a mixture of antibiotics (streptomycin, cloramphenicol and penicillin at a ratio of 5:2:5).

The surface sediment, 2 cm thick, was placed on the bottom of 10-l tanks which were then filled, in one case with surface water filtered through a mesh of 10 μm, and in the other with

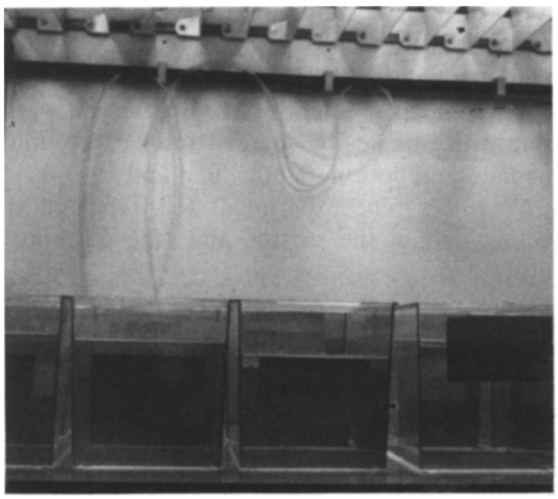

Fig. 2. Sea water samples under controlled conditions.

synthetic seawater filtered at 0.8 μm. The tanks were then stored in thermostatic cells under the same conditions of light and temperature as the previous experiments.

A further experiment was carried out using synthetic water. This was placed in 10-l tanks and inoculated with *Nitzschia closterium* (> 400 000 cell/dm^3) which had been isolated from the filaments formed during the August experiment. The cells were isolated using micropipettes and kept in f/2 culture (Guillard, 1975). The tanks containing the inoculated water were stored under the same conditions of light and temperature as the previous experiments.

October. The experiment of August was repeated and the sea water collected was filtered at only 10 μm. Streptomycin and cloramphenicol were added to unfiltered and filtered water at a ratio of 5:1. A replicate tank containing 10 μm filtered sea water was inoculated with *N. closterium* as described above.

2.2. Year 1992

April. The surface sea water samples were placed in three plastic 10 l tanks and stored this time at temperatures of 7°C, 15°C and 20°C and at a 16:8 h light:darkness period.

May. Water samples were collected both from the surface and from the sea bottom (16 m). Samples of marine snow, measuring only a few centimeters, were also collected at the same time.

Every 3 days subsamples of the water were taken from the tanks in order to analyse phytoplankton, chlorophyll *a* and nutrients.

June. The experiment of May was repeated, this time with samples of water from surface, intermediate and bottom depths (0, 8 and 19 m).

November. The experiment was repeated collecting only at the surface and the sea bottom layer.

3. Results and discussion

3.1. Year 1991

July. In all the experiments concerning unfiltered water and water filtered at > 150 μm, mucous filaments appeared after ~ 1 week. These filaments looked like a whitish cobweb and

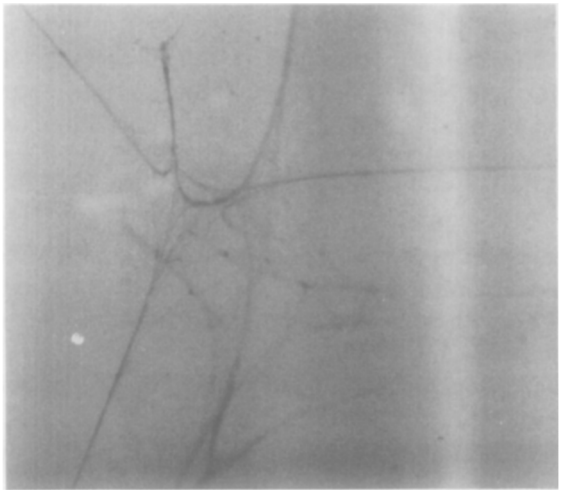

Fig. 3. Mucuous filaments developing in the tanks (— = 1 cm).

were distributed from the surface to the bottom of the tank, with some adhering to the tank walls. The diameter of the filaments ranged from 0.2 to 0.5 mm and their maximum length was the same as the height of the water in the tank (~ 30 cm) (Fig. 3).

No mucous filaments appeared in the water samples filtered through meshes < 150 μm. Qualitative analysis of the newly formed mucus, under light microscope and SEM, showed that the genus *Nitzschia* sp. (Figs. 4 and 5) and both coccoid and filamentous bacteria (Monti et al., 1992) were dominant. TEM was used for a precise taxonomic identification: it was thus possible to classify the pennate diatom *N. closterium* (Ehr.) W. Smith. This method of observation highlighted the raphe ridge, the puncta or fibulae and the irregular undulatae striae (Casaretto et al., 1992) (Fig. 6) of the species.

Further analyses were carried out to verify if there was any correspondence between the species present in the newly formed filaments, those present in the phytoplankton and microphytobenthos populations and those included in the natural mucous aggregates. Analysis of the phytoplankton populations indicated a good correspondence between the species included in the mucous filaments and those present in the water

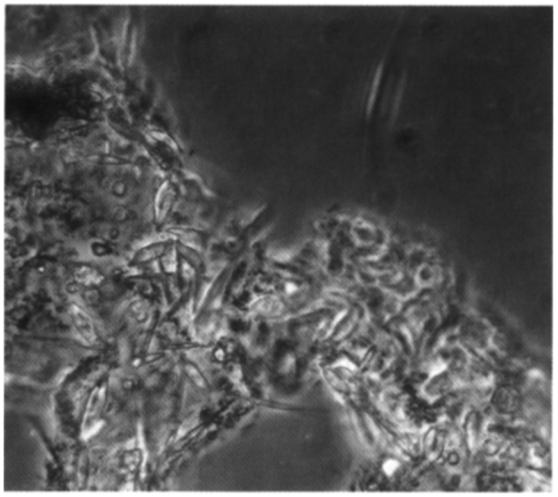

Fig. 4. *Nitzschia closterium* (Ehr.) W.Sm. embedded in mucous filament (×500).

samples. Analysis of the phytoplankton populations present in the sea enabled us to identify pennate diatoms belonging to the genus *Nitzschia* (570 000 cell/dm^3), to the genus *Navicula* (100 000 cell/dm^3) and microflagellates (850 000 cell/dm^3).

Analysis of the microphytobenthos population indicated that the genus *Nitzschia* with *N. closterium* was dominant.

Examination of the mucus collected at three depths under light microscope revealed the presence of pennate diatoms (*N. closterium*, *Synedra* sp., *Navicula* sp.), centric diatoms (*Cyclotella* sp.), dinoflagellates (*Prorocentrum* sp., *Dinophysis* sp. and *Gymnodinium* sp.), coccolithophorids (*Rhabdosphaera* sp.), coccoid cyanophyceae, filamentous bacteria and exuviae of Copepods. Examination by SEM provided further information for the classification of the various organisms. The results showed that the three depths examined were substantially homogeneous whereas the composition of the phytoplankton populations was seen to be significantly different along the water column. Only the genera *Navicula* and *Nitzschia* were found in the bottom water, in the sediment and in the mucus (Monti et al., 1992b).

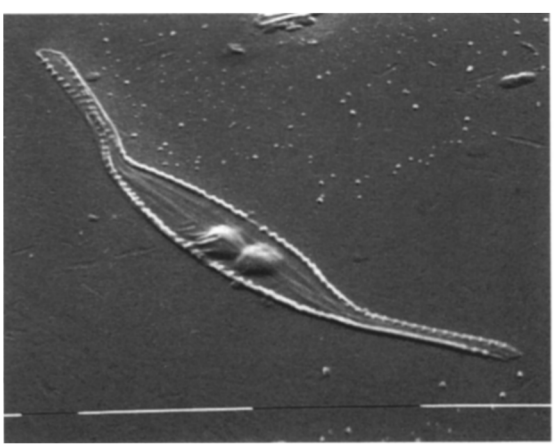

Fig. 5. *Nitzschia closterium* (Ehr.) W.Sm. SEM micrograph (×3000).

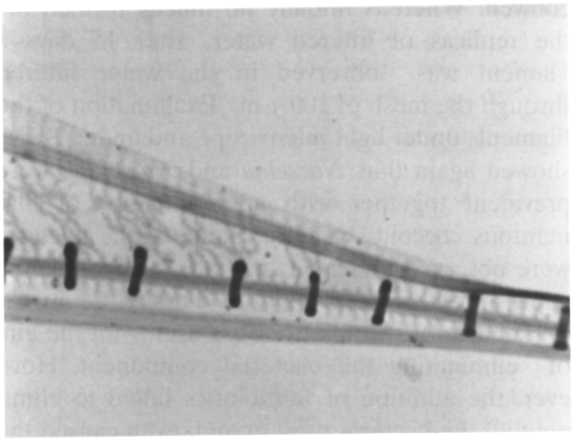

Fig. 6. *Nitzschia closterium* (Ehr.) W.Sm. TEM micrograph of the frustule (×20 000).

The size of *N. closterium*, present in the water, mucus (both natural and formed under controlled conditions) and sediment, were, on average, larger in the water and in the sediment (80–100 μm long) than in the mucus (50–60 μm long).

August. Analysis of the phytoplankton populations present in the water revealed a drop in the genus *Nitzschia* (180 000 cell/dm^3) in comparison with the previous month, whereas the amount of microflagellates remained constant (930 000 cell/dm^3).

Although few diatoms were present, after 1 week a mucous filament formed in the unfiltered water and developed into a fairly thick mucous

cobweb. Whereas initially no mucus formed in the replicas of filtered water, after 15 days a filament was observed in the water filtered through the mesh of 100 μm. Examination of the filaments under light microscope and under SEM, showed again that *Nitzschia* and *Navicula* were prevalent together with an abundance of filamentous coccoid bacteria. Some of the bacteria were observed under an epifluorescence microscope and it was possible to classify them as cyanobacteria. Antibiotics were used with the aim of eliminating the bacterial component. However, the addition of antibiotics failed to eliminate all the bacteria present and even caused the growth of two resistent forms.

After 1 week a filament was observed in the tank with antibiotic treated unfiltered sea water.

An experiment was conducted using water and sediment in order to understand whether *N. closterium*, which was also present in the sediment, but where the genera *Navicula*, *Pleurosigma*, *Gyrosigma* and *Cymbella* were more abundant, was able to migrate and colonise the layers of water lying above. Undisturbed sediment was placed at the bottom of the tanks and synthetic seawater and seawater filtered through a mesh of 10 μm was added. The only colonisation observed was that by *N. closterium* in the overlying water with initial values of 15 400 cell/dm^3 and final values of 260 000 cell/dm^3 in the synthetic water and maximum values of 143 000 cell/dm^3 in the filtered seawater, after 12 days. These samples of water and sediment were then left undisturbed for ~1 month. The water (but not the sediment) was then transferred into new tanks and stored at the same conditions of light and temperature. After ~20 days, a mucous filament formed in both tanks and later developed into a mucous cobweb. One filament was removed and examined. The presence of *N. closterium*, cocci and rod bacteria was noted.

In a further attempt to obtain an environment as free from bacteria as possible, an experiment was performed using synthetic water. This water, placed in 10-l tanks under the same conditions of light and temperature as in the previous experiments, was inoculated with a concentration of ~400 000 cell/dm^3 of *N. closterium*. After 1 week a fine mucous filament appeared.

October. A further experiment was performed using unfiltered water, unfiltered water + antibiotic and water filtered through a mesh of 10 μm to which was added an inoculum of 400 000 cell/dm^3 of *N. closterium*. After 1 week a fine filament was observed in the tank containing the filtered water with the inoculum of *Nitzschia*. A qualitative analysis of seawater revealed an abundance of the genus *Chaetoceros* and of microflagellates whereas the genus *Nitzschia* was almost completely absent. In the filament there was almost exclusively *N. closterium*.

3.2. Year 1992

April. Samples of surface sea water were examined at different temperatures. The difference in temperature (7, 15 and 19°C) did not, however, lead to any difference in results and, after 1 week, small mucous lumps appeared in all the tanks. These lumps then collapsed after a further week: they did not give rise to any mucous filaments. The aggregates presented pennate diatoms and embedded microflagellates.

Phytoplankton analysis revealed an increase in all the components up to the formation of the mucous lumps (average data showed an increase of diatoms from 14 295 cells/dm^3 to 558 354 cells/dm^3, microflagellates from 923 145 cells/dm^3 to 2 819 689 cells/dm^3 and dinoflagellates from 31 266 cells/dm^3 to 151 084 cells/dm^3).

Initially nutrient concentrations were very low ($P\text{-}PO_4$ = 0.08 μmol/dm^3, $N\text{-}NO_2$ = 0.14 μmol/dm^3), only $N\text{-}NO_3$ reached a value of 3.70 μmol/dm^3. $Si\text{-}SiO_2$ concentration was 0.84 μmol/dm^3. After 8 days all values slightly increased.

May. To assess the influence of light and temperature on water from different depths, seawater samples from surface and bottom (16 m) were collected.

After 1 week, small lumps of mucus appeared in the tank containing water from the sea bottom. Analysis of the mucus under light microscope and SEM revealed that the genus *Chaetoceros* (Fig. 7) was prevalent and that the

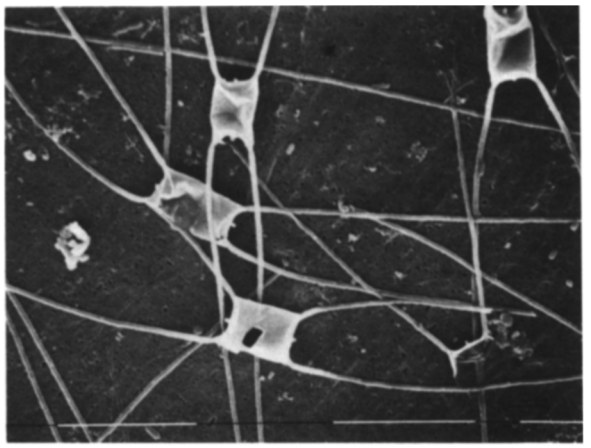

Fig. 7. *Chaetoceros* sp. Ehr. SEM micrograph (×750).

genus *Cyclotella* was sporadic. After another 2 days a mucous filament developed in the tank. This aggregate contained centric diatoms as well as pennate diatoms belonging to the genera *Nitzschia* and *Thalassionema*. The filaments were periodically removed and examined. The phytoplankton populations found in the filaments revealed a gradual transition from centric diatoms, mainly represented by the genus *Chaetoceros*, to pennate diatoms of the genera *Nitzschia*, *Navicula* and *Amphiprora*.

Specific stains were used to reveal the histochemical nature of the molecules in the mucous aggregates. Anionic polysaccharides were present in all the samples thus confirming that the histochemical nature of the aggregates collected under natural conditions was substantially uniform (Welker, 1994).

Analysis of the small, whitish (Seguy scale n° 235) flakes of marine snow (5–7 cm long and 0.3–0.5 cm in diameter) revealed the presence of *Chaetoceros*, *Leptocylindrus* and in particular the species *N. closterium*. Moreover dinoflagellates, coccolitophorids and cyanobacteria were always present.

Analysis of nutrients in the tanks highlighted that $P\text{-}PO_4$ tended to fluctuate. These fluctuations corresponded with those of $N\text{-}NO_2$, $N\text{-}NO_3$ and $N\text{-}NH_3$ in surface water, whereas in bottom sea water they were out of phase only with $N\text{-}NO_3$. The formation of lumps and filaments coincided with a drop to ~ 0 in $P\text{-}PO_4$ values which then rose again 3 days after the formation of filament. The ratio N/P, obtained by summing nitrate azote + nitrous azote + ammoniac azote/orthophosphate ($N\text{-}NO_2$ + $N\text{-}NO_3$ + $N\text{-}NH_4/P\text{-}PO_4$) in the water from the sea bottom varied greatly over the course of the experiments.

$Si\text{-}SiO_2$ followed a similar trend in all the different tanks until filaments were formed. However, the values in water from the sea bottom were lower and reached their minimum level after the formation of filaments, and then increased slightly during the following 3 days.

Chlorophyll *a* was measured the day the samples were collected and on the last day of the experiments. Concentrations in surface water and in water from the sea bottom were similar and in both cases there was a relevant increase. Values increased from 0.34 mg/m^3 to 15.5 mg/m^3 in surface water and from 13 mg/m^3 to 15.6 mg/m^3 in water from the sea bottom.

Phytoplankton analysis led us to the identification of centric diatoms of the genus *Cyclotella* and of pennate diatoms of the genera *Nitzschia*, *Navicula*, *Cylindroteca*, *Thalassiotrix* and *Thalassionema*. Dinoflagellates were always present of the genus *Gymnodinium* and the species *Prorocentrum micans*. No great difference was observed between species present in surface and bottom water. Quantitative analysis showed no values $> 300\,000$ cell/dm^3. On average, surface water values were higher than those in the bottom water, with a prevalence of dinoflagellates compared with diatoms. Only 1 week after the beginning of the experiment an increse in diatoms in the bottom sea water was observed (249 000 cell/dm^3). During the days when the filament formed phytoplankton decreased to 3000 cell/dm^3.

June. The experiment performed in May was repeated, and again only the bottom sea water gave rise first to small lumps of mucus and then to a proper filament. Qualitative examination of aggregated material under light microscope revealed the presence of two distinct species of *Chaetoceros*, *N. closterium*, *N. longissima*, *P.*

minimum and coccolithophorids. We again observed a gradual transition from centric diatoms present in the lumps, to pennate diatoms embedded in the mucous filaments.

Phytoplankton analysis showed low values of diatoms in all samples. *N. closterium* was present only in the bottom sea water sample (4060 cells/dm^3). In the same sample dinoflagellates decreased from 58 881 cells/dm^3 to 4460 cells/dm^3 after 14 days, when mucous filaments occurred.

During the experiment all nutrient concentration values were very low (P-PO_4 = 0.01 μmol/dm^3, N-NO_3 = 0.04–1.28 μmol/dm^3, N-NO_2 = 0.04–0.38 μmol/dm^3, Si-SiO_2 = 0.90–5.07 μmol/dm^3).

November. The previous experiment was repeated and after 1 week small lumps (0.5–1 cm) occurred in the bottom sea water. One week later these lumps had collapsed and no more mucous filaments were formed. The lumps which had collapsed aged and turned a yellowish colour (Seguy scale, n° 262). These lumps contained *Chaetoceros*, *Amphiprora* sp., *Fragilaria* sp., *N. closterium*, *N. seriata*, *T. nizschioide*, *Rhizosolenia* sp. and *Cyclotella* sp.

Phytoplankton analysis showed a decrease in all the components. Microflagellates decreased from 1 002 556 cells/dm^3 to 310 648 cells/dm^3, diatoms from 38 577 cells/dm^3 to to 16 243 cell/dm^3 and dinoflagellates from 4210 cells/dm^3 to 4060 cells/dm^3.

N-NO_3 concentration ranged from 0.62 to 2.88 μmol/dm^3 while the other nutrient concentration values were constantly low. Si-SiO_2 was more abundant only in the bottom sea water sample (max. value, 4.29 μmol/dm^3).

4. Conclusions

Our experiments led to the formation of mucous filaments under controlled conditions of light and temperature (Table 1).

Filtering water through different sized meshes enabled us to identify the size class of phytoplankton involved in the formation of the filaments. Filaments were formed only in tanks containing either unfiltered seawater or water filtered through meshes of 150 μm and 100 μm. It may therefore be deduced that the formation of mucous filaments is closely related to the

Table 1
Filament formation under controlled conditions

			Synthetic water	Unfiltered water	< 150 μm	< 100 μm	< 20 μm	< 10 μm	< 1.2 μm
July 1991	0 m			+	+		–	–	–
August 1991	0 m	Without antibiotic		+		+		–	
	0 m	Antibiotic treated		+		–		–	
	0 m	With sediment	+						
	0 m	With inoculum[b]	+						
October 1991	0 m	Without antibiotic		–					
	0 m	Antibiotic treated[a]		–					
	0 m	With inoculum[b]						+	
April 1992	0 m	19°C		–					
	0 m	15°C		–					
	0 m	7°C		–					
May 1992	0 m								
	16 m			+					
June 1992	0 m			–					
	8 m			–					
	19 m			+					
November 1992	0 m			–					
	19 m			+					

[a]Axenic conditions were not obtained.
[b]Inoculum with *N. closterium* Ehr. (W. Smith).

passage of organisms with a size > 100 μm through the meshes used for filtering.

It seemed that the genus *Nitzschia* was essential for the formation of mucous filaments under the conditions of light and temperature applied in our experiments. This genus was able to reproduce inside a mucilaginous matrix and lead to the formation of filaments. This hypothesis was further supported by the experiments performed with synthetic water. In fact, formation of a filament was observed in the synthetic water inoculated by *N. closterium* or colonized by *N. closterium* ascending from the sediment. In the first case a minimal concentration was necessary for inducing mucus formation. We can also suppose that in the natural environment a minimum concentration of this species (or alternatively others) limited to a few specific months, would be necessary to start the hyperproduction of mucus.

For the same reason, the different concentration of phytoplankton in surface sea water in comparison with that present in bottom sea water, together with the concentration of nutrients, may have limited the formation of filaments.

Development of appropriate concentrations of phytoplankton depends on nutrient availability. In bottom sea water a sharp increase of N/P ratio was observed in the days coinciding with the formation of filaments. There was a change from a N-limited situation to P-limitation. The same phenomenon was observed in surface water, only 3 days later; in this case the fact that mucous filaments failed to form seems to be due to the scarce presence of *N. closterium*.

It was also possible to observe a sequence in the dominant species during the formation of mucilaginous filaments. There was a transition from centric diatom species, towards pennate diatoms. The centric diatoms (genus *Chateoceros*) formed the first small aggregates which later developed into real filaments due to the colonisation of the genus *Nitzschia*. In particular, *N. closterium* incorporated in the filaments was of a smaller size than in the water and the sediment.

Our experiments should be regarded as a first step towards specific research with special regard for possible genetic and physiological modifications in some species, such as *N. closterium*, which are particularly significant in the phenomenon of hyperproduction of mucous material.

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