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Chitin Biodegradation in Marine Environments: An Experimental Approach

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Key Word Index—Chitin; biodegradation; sediments; microborers; biogeochemical cycles.

Abstract—Chitin biomasses and production in marine environments are quite high. Planktonic biocenoses are the main producers and one should expect that sediments, mainly organoclastic ones, will constitute some kind of reserve compartment for the biogeochemical cycle of this polymer. In fact, this is not the case. The low chitin biomass in most marine sediments can only be explained if chitin is weathered at the same rate as it is produced. In order to test this hypothesis, we developed an experimental approach to chitin biodegradation in marine environments. In open water conditions, zooplanktonic remains are first degraded by autolytic processes making most organic compounds readily susceptible for further hydrolysis by extrinsic decomposers. Different populations (with high densities and various hydrolytic potentials) follow each other. The sequence of hydrolytic activities optimizes the recycling of most detritic compounds including nearly 90% of the chitin produced. At sediment–water interface, the remaining material appears to be pulverized and incorporated into the aerobic sedimentary layers while the decomposer community changes once again. Sediment chitinoclasts are opportunistic and densities react quickly to chitin input. In sediments, oxic and anoxic, chitin appears essentially present in the form of chitinoproteic matrices inside mineralized skeletons. A rich population of microborers develops on these matrices by secreting extracellular hydrolases. Densities of microborers of 250–450×10^3 cm^{-2} are currently encountered. Anaerobic decomposers are more adapted to refractory compounds than aerobic ones. This leads to a nearly complete mineralization of the chitinoproteic matrices embedded in the biotic sedimentary layers (more than 90% of the chitin weathered within less than two years).

Introduction

The biomasses and production of chitin in marine environments have been thoroughly studied and the results published in several recent well-documented papers [1–3]. The main producers are to be found among zooplanktonic organisms, mainly euphausids in higher latitudes and copepods in temperate and tropical conditions. Benthic biocenoses on hard substrates, even quite rich in chitin, appear to contribute to a lesser extent due to the wide surfaces occupied by less productive sediment areas.

Chitin produced in marine environments should thus accumulate in sediments as these are the "reserve compartment" of most biogeochemical cycles. This should be true especially in organoclastic ones as most animal skeletons are built on a chitinoproteic matrix more or less calcified [4, 5]. This actually appears not to be true. In preceding papers [5–7], we have reported screening of approximately 100 samples of sediments covering a wide diversity of climatic areas, ecological settings and sedimentological conditions. Most sediments have low or very low chitin content (67% of samples are under 100 μg chitin per gram decalcified sediment). This observation can only be explained by very active degradation processes of chitinous compounds so that chitin disappears at the same rate as it is produced. As chitin is considered as one of the most important biogenic polymers produced at the earth's surface, this reveals the utmost importance of biodegradation processes in the oceans affecting the planet.

In order to understand chitin biodegradation processes in general biogeochemical cycles of carbon and nitrogen, we broached the problem under the formalism of a box-model. The model discussed will take four boxes into account (Fig. 1), namely an open water compartment, an interface compartment, an oxic sedimentary layers

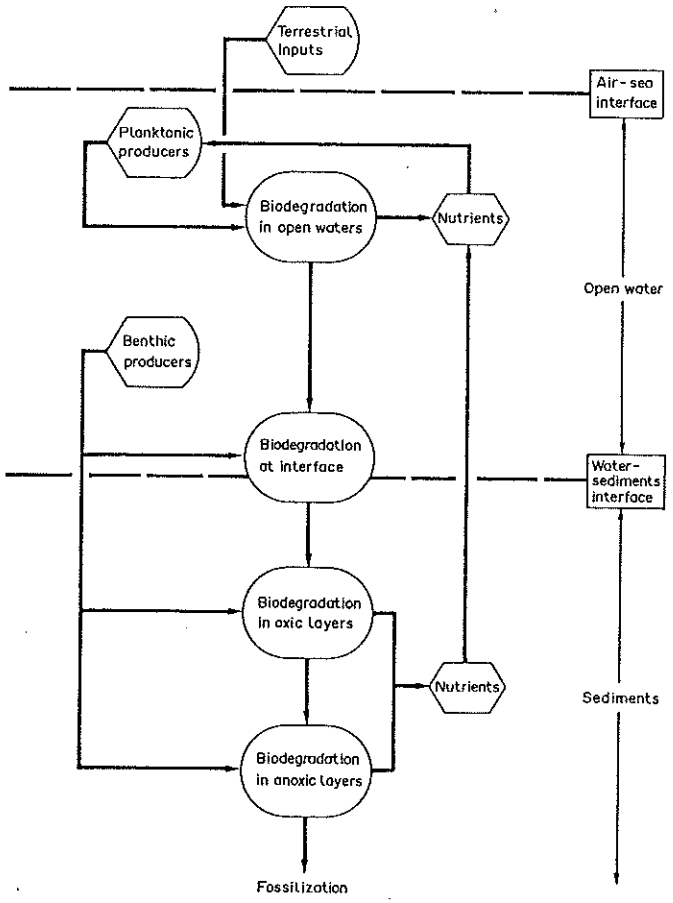

FIG. 1. GENERALIZED BOX-MODEL OF MARINE CHITIN BIOGEOCHEMICAL CYCLE (SEE TEXT FOR FURTHER EXPLANATIONS).

compartment, and an anoxic sedimentary layers compartment. Any of these boxes is fed by several chitin inputs. In any of these boxes, chitin undergoes biodegradation processes or is transferred to another compartment. Actually, all these boxes were considered separately for our *in situ* experimental approach and will be considered separately in the following account.

Materials and Methods

This study is primarily based on experimental studies of the early biodegradation of chitinous material.

The fresh, carefully cleaned material (wrapped in 180-µm nylon mesh bags) was laid down at and below sediment–water interface in different settings and recovered periodically by diving. Anaerobic samples were obtained by confinement of samples in closed jars filled with natural sediment and incubated *in situ*. Open water biodegradation processes affecting zooplanktonic remains were studied the same way: a container closed by 180-µm mesh net filled with freshly killed planktonic specimens suspended well above sediment was used. For further details concerning experimental procedures see Refs 5 and 8–14. The experiments were performed in the Mediterranean sea (Calvi, Corsica), off the coast of Brittany (Roscoff, France) and in the Virgin Islands (Ste Croix). Moreover, samples were collected from a wide variety of sites in order to clarify the

weathering patterns of organoclastic remains in different environmental conditions in comparison with experimental material [10].

The experimental material from open water consisted of freshly collected Mediterranean zooplankton samples from the centre of the Calvi bay (Corsica), composed approximately of 95 to 98% copepods [15] and gently killed by slow refrigeration. Macrozooplanktonic organisms were removed before the beginning of the experiment. In sedimentary layers, the experimental material was made up of several kinds of carefully cleaned animal skeletal structures (crustacean cuticles, mollusc shells, echinoid plates, etc.).

From a morphological point of view, the recovered samples were examined with SEM and TEM. For SEM, fixation was performed with 4% glutaraldehyde in freshly 0.22-µm filtered seawater (16 h at 4°C and pH 8.2) then 1% osmium tetroxide in distilled water for 2–4 h. After several washings, the material was freeze-dried at −55°C under 15 mT absolute pressure. Alternatively, the material was dehydrated through a graded ethanol series and embedded in a fluorocarbon PELDRI II resin (Ted Pella Inc.) at room temperature (24°C) then polymerized at 15°C. After sublimation of the resin at 15°C in vacuum, the material to be examined was glued onto aluminium stubs with TEMPFIX (Neubauer Chemikal), gold–palladium sputtered (10–15 nm layer with a cool-diode BALZERS SCD20 device) and examined with a scanning electron microscope operated at 20 kV. For TEM, the material was simultaneously fixed and decalcified in a 0.28 M EDTA-Na salt in 4% glutaraldehyde in 0.2 M isotonic cacodylate buffer, pH 7.8 (24–96 h at 4°C). After postfixation in osmium tetroxide (1% in distilled water, 1 h at 4°C), the material was dehydrated through a graded ethanol series, embedded in either Spurr or Epon 812 resin and cut with a diamond knife on an ultramicrotome Porter Blum MT2B. The material was observed with a transmission electron microscope operated at 80 kV.

From a biochemical point of view, enzymatic hydrolytic activities were estimated with APIZYM tests (Biomerieux) allowing 19 determinations on very small samples. Chitin was estimated with the enzymatic specific method of Jeuniaux [4, 7, 16] using 1 mg ml^{-1} solutions of SIGMA 6137 chitinase in Na_2HPO_4 1.2 M–citric acid 0.6 M buffer at pH 5.2 to which was added a chitobiase solution (lobster serum diluted 10 times).

From a microbiological point of view, the decomposers were counted after dilution in sterile sea-water and inoculation on sheet media (PETRIFILM 3M, "Total count" and "Yeasts and molds" films were used) incubated at 37°C both in aerobic and anaerobic conditions (anaerobic jars or Generbag BIOMERIEUX). Strains were characterized microscopically and metabolically (API 20A strips BIOMERIEUX).

Results and Discussion

Chitin biodegradation processes in open waters

Planktonic communities are especially rich chitin producers due to the abundance of holo- and meroplanktonic crustaceans [3, 15, 17–19]. Through mechanical and biodegradation processes, this chitinous material is partly incorporated in the particulate matter in suspension in the oceans [8, 9].

Zooplankton biodegradation in open water is a very effective and well structured process. From a morphological point of view, the organisms are first degraded from inside by autolytic processes [9]. These processes induce the lysis of most muscles and organs during about 50 h *post mortem*. This autolysis results from the activity of the hydrolytic enzymes of the digestive tract and other organs and of the symbiotic microorganisms in the intestine. This stage leads also to the opening of the cuticle, generally at the level of an articular membrane between two sclerites (where the cuticle is less hardened) [8]. This happens 6–20 h *post mortem*. Through this gap, heterotrophic microorganisms can invade the corpse and further degrade it. At 50–100 h *post mortem*, the chitinoproteic layers of the cuticle are deeply altered so that pieces of appendages (legs, antenna, etc.) are shed away. These pieces can form macroscopic aggregates often known as "marine snow", falling relatively fast through the water column. This material involves a rich heterotrophic microorganism community, carrying on the biodegradation processes during sedimentation.

From a biochemical point of view, a wide variety of hydrolases have been detected during biodegradation processes. The importance of each enzymatic activity varies with time.

Lipolytic activities are first to appear, culminating after 12–40 h [9]. This activity is mainly autolytic. Proteolytic activities and glycolytic activities develop next, and culminate 60–120 h *post mortem* [9]. These activities contribute in preparing the cuticles for hydrolysis by chitinolytic activities (maximum between 100 and 150 h *post mortem*). All these activities are mainly due to the development, on the experimental

material, of several different populations of heterotrophic microorganisms [20]. Autolytic processes appear negligible after 50 h of the experiment [9].

The number of active bacterial strains on the experimental material increases according to a logarithmic curve during the first 100–150 h *post mortem* (Fig. 2) then decreases slowly, according to the progressive hydrolysis of metabolizable substrates [20].

The hydrolytic activities described above lead to a quick decrease of the organic content of the animal remains (Fig. 3). The decrease of organic content happens as a negative exponential kinetic curve. As far as chitin is concerned, the process appears essentially similar. After a short delay during which proteins and lipids gradually disappear, the chitin content of the material reduces quickly (maximum variation between 50 and 120 h *post mortem*). After about 150 h, approximately 90% of the chitin initially present has disappeared [9].

Chitin biodegradation processes at water–sediment interface

The material of planktonic origin settling onto the sediments has thus probably already undergone an extensive biodegradation. As most decomposer microorganisms of open water remain attached to the settling material, the matter deposited at the sediment surface carries on the same biodegradation processes as in open water [20]. The apparent slowing down observed in the phenomena is mainly due to the prior loss of most labile compounds. The material is now essentially constituted of more or less refractory molecules, little chitin remains at this stage. In shallow water, mechanical disruption and abrasion occur, due to hydrodynamic forces (waves, swell, internal waves, currents, tide effects, etc.) resuspending the material in

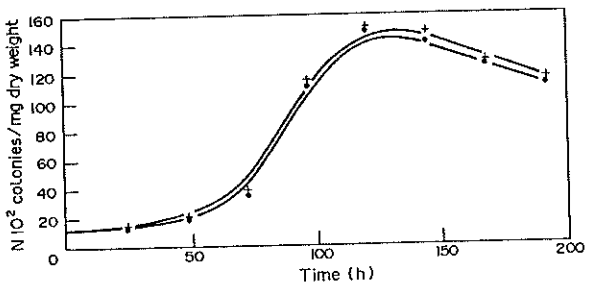

FIG. 2. BACTERIA COUNTS ON PLANKTON (*IN SITU* AEROBIC EXPERIMENT). —•— 24 h incubation; — + — 48 h incubation. Mean of six triplicate counts.

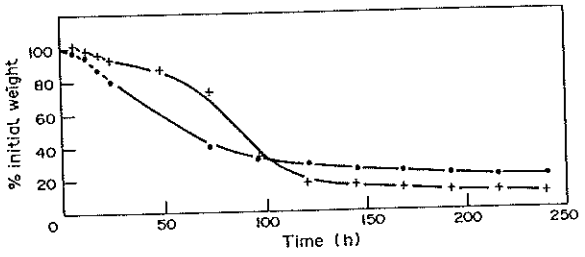

FIG. 3. ZOOPLANKTON BIODEGRADATION (*IN SITU* AEROBIC EXPERIMENT). —•— Total weight; — + — chitin. Mean of six experiments (Calvi, Mediterranean sea) summers 1986–1990.

close contact with sediment particles [20]. The weakening of remaining cuticle leads to a complete disruption and pulverization of the planktonic material (Fig. 4).

Such mechanical disruption of the material does not occur deeper in the oceans and chitin incorporation into the oxic sedimentary layers should be possible. These depths are not readily accessible by diving and, thus, direct experimental evidence is difficult to obtain. Further work should elucidate this problem.

Whatever the results, it has been shown [7] that the number of chitinoclastic bacterial strains is a logarithmic function of the chitin content of the sediment at the interface (Fig. 5).

This means that a slight increase of the chitin content readily available should induce an immediate growth of chitinoclastic bacterial populations and thus a quick degradation of this input. In such conditions, the bacteria behave as typically opportunistic organisms.

As particulate chitin accumulation appears unlikely, chitin content of sediments is typically related to their composition: organoclastic sediments typically harbour much more chitin than terrigenous, volcanic or authigenic ones [7]. Mollusc shells and bryozoa are the main contributors to sedimentary chitin.

Chitin biodegradation processes in oxic marine sediments

In the oxic layers of marine sediments, biodegradation of the chitinoproteic matrices of animal skeletons occurs mainly through the activity of microborers. A well-defined sequence of microorganisms settle onto the detritic skeletal pieces and some of them are able to bore holes of 1–150 μm in diameter [5, 21]. Blue-green algae are abundant,

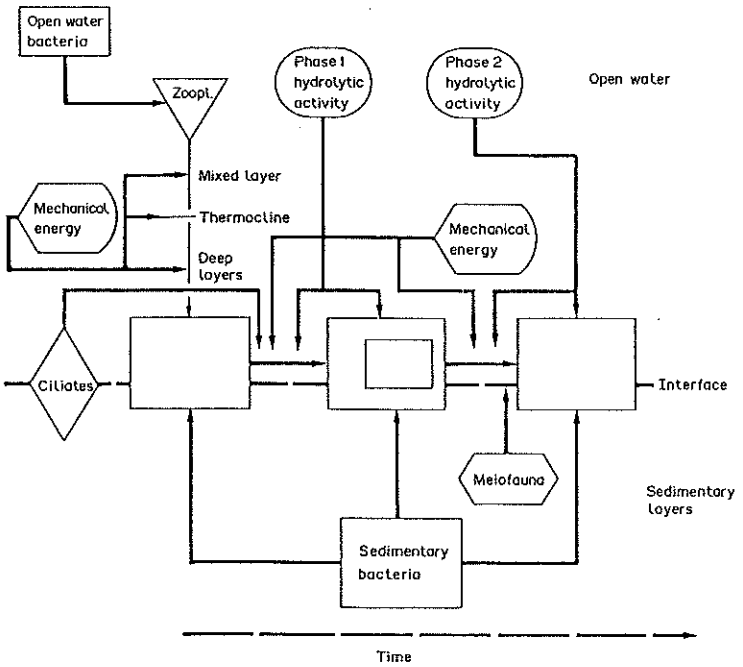

FIG. 4. GENERALIZED BOX MODEL OF FACTORS AFFECTING DETRITIC MATERIAL AT SEDIMENT–WATER INTERFACE.

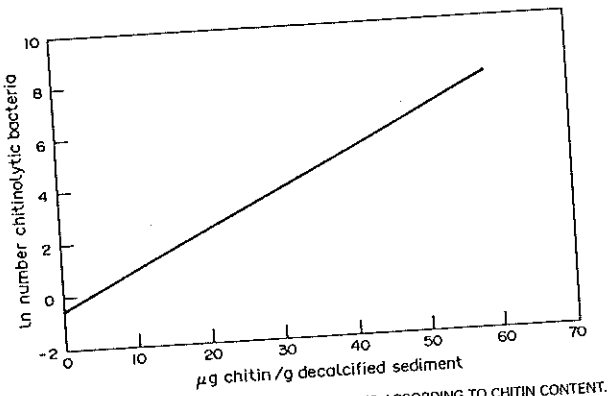

FIG. 5. NUMBER OF BACTERIA STRAINS IN SEDIMENTS ACCORDING TO CHITIN CONTENT.

but bacteria, distributed within the organic matrices [22], and fungi are the main contributors to biodegradation activities.

After one to four years of experiment, typical densities of boreholes have been found to be of the order of 150,000 to 400,000 per square centimeter (Fig. 6) [10]. There is no significant difference between experimental settings, namely Mediterranean sea, the English Channel or the Caribbean Virgin Islands. As most boreholes are empty (only 10–15% are effectively occupied by the microorganism that realized it (Fig. 6) the empty borings are used by bacteria as a means of reaching the organic veils inside the skeletons [10, 22].

With the remarkable exception of blue-green algae, most microorganisms isolated from weathered skeletons are able to secrete extracellular hydrolases and proteolytic and chitinolytic enzymes (Table 1) when cultivated *in vitro* [11]. These hydrolytic activities were also directly detected inside detritic skeletons. Enzymatic activity estimations inside weathering skeletons show a great diversity of hydrolases (Table 2). The hydrolytic activity levels are quite high [11]. As far as chitin is concerned, mollusc shells and crab cuticles), the chitinolytic activity is important [10–12, 21] and can explain a quick decrease of the chitin content of the experimental samples (Fig.

In the three experimental settings, chitin disappears following the same kinds kinetics: after a first step during which the microborers settle onto the experimental material and invade the cortical layers (low hydrolytic activity), we observe a fast decrease of the chitin content, synchronous with the exponential growth of endolithic populations (Fig. 6). The kinetics are of negative exponential type, just as are most

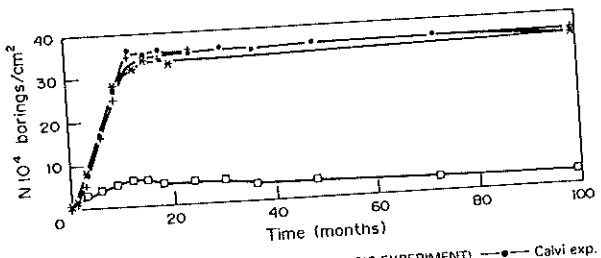

FIG. 6. MICROBORERS COUNTS IN MOLLUSC SHELLS (*IN SITU* AEROBIC EXPERIMENT). —•— Calvi exp. setting; — Britain exp. setting; — * — Virgin Isl., — □ — Live organisms. Means of six triplicate counts in three experimental water (8–37m) settings.

TABLE 1. HISTOENZYMOLOGICAL DEMONSTRATION OF PROTEOLYTIC AND CHITINOLYTIC ACTIVITIES OF MICROORGANISMS ISOLATED FROM EXPERIMENTALLY WEATHERED SHELLS (MOTHER-OF-PEARL, CALVI EXPERIMENTAL SITE, 18 MONTHS IMMERSION)

Organism	Chitinase	Protease
Cyanobacteria		
Hyella sp.	O	+
Mastigocoleus testarum	O	+
Fungi		
Oomycete Strain 1	++	++
Oomycete Strain 2	+++	++
Ascomycete unidentified	+	++
Bacteria		
Bacilli Strain 1	+	++
Bacilli Strain 2	+	++
Bacilli Strain 3	+	++
Cocci Strain 4	+++	+
Cocci Strain 5	++	+++
Chlorophyta		
Oestrobium sp. (*quecketti?*)	O	O?

O = no detectable activity; +, ++, +++ = increasing hydrolytic activitites.

TABLE 2. HYDROLYTIC ACTIVITIES IN EXPERIMENTAL SKELETAL MATERIAL WEATHERED UNDER AEROBIC AND ANAEROBIC CONDITIONS (CALVI EXPERIMENTAL SITE, −37 M)

Material	Aerobic conditions ($n = 6$)	Anaerobic conditions ($n = 6$)
Mother-of-pearl		
Phosphatases	1311–2380	1867–3551
Lipases	1166–2088	1120–2663
Proteases	777–2720	467–3373
Glycosidases	1020–3934	653–6215
Sum of activities	3982–10393	4108–15801
Echinoid test		
Phosphatases	340–777	360–365
Lipases	291–1238	360–469
Proteases	341–631	102–130
Glycosidases	147–1020	0–104
Sum of activities	1117–2428	822–1200
Crab cuticle		
Phosphatases	17–579	29–242
Lipases	0–331	0–97
Proteases	0–620	0
Glycosidases	0–909	0
Sum of activities	17–2438	29–338
Fish vertebrae		
Phosphatases	800–2538	130–854
Lipases	160–1177	194–854
Proteases	360–1904	0–1067
Glycosidases	360–3808	0
Sum of activities	1680–9429	324–2774

All activities are calculated after a three-month summer immersion as nM hydrolysed substrate per hour and gram material (wet weight basis).

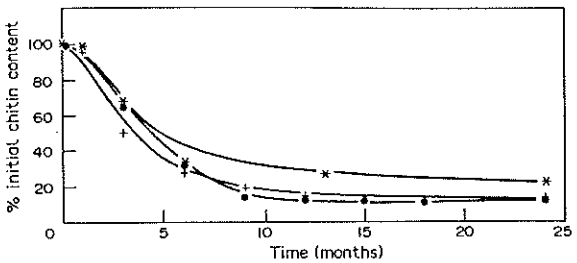

FIG. 7. MOLLUSC SHELL CHITIN BIODEGRADATION (*IN SITU* AEROBIC EXPERIMENT). —●— Calvi exp. setting; — + — Britain; — * — Virgin Isl. Mean of triplicate estimations in three experimental settings.

other biodegradation phenomena already studied [10, 12]. After the fast step of biodegradation, the slowing down of the process corresponds to the lowering of the concentration level of readily available growth substrates. The remaining organic compounds are mainly of a refractory type or engaged in stable structural bonds (scleroproteins of the chitinoproteic complex) and thus are much less accessible.

Chitin biodegradation processes in anoxic marine sediments

Anoxic conditions occur to a rather limited extent in the ocean. But although they cover only 10% of the sea-floor, anoxic superficial sediments underlie most of the shallow highly productive areas of the world oceans [23]. These sediments accumulate more than 90% of the total organic matter buried in sediments annually, so processes of anaerobic decomposition are very important to determine to what extent some compounds, chitinous ones in this case, "survive" the surface biotic layers of rapid mineralization and are buried long term. An important difference between aerobic and anaerobic decomposers is that most aerobic microorganisms can oxidize a wide range of substrates to CO_2. Individual anaerobic microorganisms metabolize a rather restricted range of molecules, often incompletely. However, anaerobic communities can be very efficient in decomposing relatively refractory molecules [24]. Most organic polymers can be decomposed by aquatic anaerobic communities.

Anaerobic biodegradation of skeletal substrates is mainly due to bacteria, diatoms and fungi [13, 14, 24, 25]. Despite the fact that few forms of decomposers are common to both aerobic and anaerobic conditions, biodegradation patterns are very similar to those in oxic conditions. The same colonization curve is observed, culminating at the same densities of borers. The results of estimates of enzymatic activity (Table 2) show that, in the case of mollusc shells, there is no significant difference between hydrolytic levels. This is not true for other samples where anaerobic hydrolytic processes appear lower. This was interpreted as a result of high organic content [24]. As far as chitin is concerned, the kinetics of biodegradation processes appear essentially similar, with comparable rate constants [13]. The slowing down of the process after a fast biodegradation step is also observed. The composition of these refractory compounds may be somewhat different in the two experimental conditions, as shown by the results of transfer experiments (Fig. 8).

Material (mother of pearl) was laid down in oxic sedimentary layers for six months. After this period, a part of the experimental material was transferred to anoxic sedimentary conditions, other samples remaining in the same environment for another six-month period. After a rapid decrease of the organic content during the first six months (70% degradation), the extraction of the organic compounds slowed down as normal in the refractory phase (speed constant $K_{aer} = 0.91$). The material then

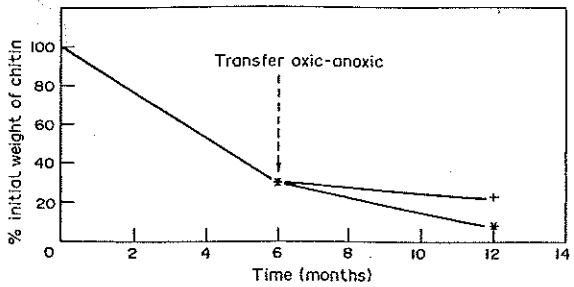

FIG. 8. FATE OF REFRACTORY COMPOUNDS (RESULTS OF TRANSFER EXPERIMENTS). —•— oxic degradation; — + — oxic degradation; — * — anoxic degradation.

transferred to anaerobic conditions, is weathered three times faster than that remaining in aerobic conditions (Kana = 3.00) [12].

This can be interpreted as adaptation of the anaerobic microbiocenoses to biodegradation of less labile compounds remaining after aerobic degradation activity. The cumulative effect of both processes (aerobic and anaerobic weathering) results in optimization of the recycling of the chitinous compounds of skeletal substrates.

Conclusions

Chitin produced in the marine environment is unlikely to accumulate to a large extent or to be preserved in sediments. From the open water compartment down to the anoxic sedimentary layers, we have followed the fate of chitinous compounds and showed that, at each level, the efficiency of chitinoclastic microorganisms is high. The specialized features of some of them (microborer mode of life in sediments, adaptation of anaerobic decomposers to more or less refractory compounds, etc.) leads to an optimization of the recycling processes of chitin. This explains why chitin does not accumulate to a large extent in most marine sediments.

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