

## Laboratory cultures of calcifying biomicrospheres generate ooids - A contribution to the origin of oolites

Ulrike BREHM<sup>1</sup>

Katarzyna A. PALINSKA<sup>2</sup>

Wolfgang E. KRUMBEIN<sup>2</sup>

**Abstract:** The *in vitro* production of ooid-like structures as possible precursors of oolites has been observed in laboratory cultures of spherical microbial communities isolated from the Wadden Sea (North Sea). The microbial spherulites consist of aggregated benthic diatoms (*Navicula perminuta*) enveloped by layers of filamentous cyanobacteria of the genus *Phormidium* and a halo-like biofilm of heterotrophic bacteria. The development of the structures takes several months and these configurations appear to be stable, before they calcify. The precipitation starts on the surface of the spheres as clouds of small scattered crystals, which later increase in size and aggregate to form hollow spheres around the microbial assemblage. Here we report for the first time carbonate precipitation in defined spherical microbial communities. .

**Key Words:** Carbonate precipitate; cyanobacteria; diatoms; ooids; spherulites; microbial association.

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**Zusammenfassung: Ooidbildung durch carbonatfällende Biomikrosphären - Laboruntersuchungen zur Entstehung von Oolithen.**- Die *in vitro* Entstehung von ooidähnlichen Strukturen als möglichen Vorläufern der Oolithe, konnte in sphärischen mikrobiellen Gemeinschaften im Labor beobachtet und dokumentiert werden. Die Biomikrosphären wurden aus dem Wattenmeer (Nordsee) isoliert. Die Sphären bestehen aus einer Ansammlung von benthischen Diatomeen (*Navicula perminuta*), die mit mehreren Lagen fädiger Cyanobakterien der Art *Phormidium* und einer äußeren Hülle von heterotrophen Bakterien umgeben sind. Die Strukturentwicklung zieht sich über mehrere Monate hin und scheint stabil zu sein, ehe die Calcifizierung eintritt. Die Kristallisierung beginnt auf der Oberfläche der Sphären in Form von vereinzelt kleinen Kristallansammlungen, die sich später vergrößern, zusammenwachsen und damit eine Hohlkugel um die mikrobielle Gemeinschaft bilden. Wir berichten hier zum ersten Mal über die Carbonatausfällung in sphärischen mikrobiellen Gemeinschaften.

**Stichworte:** Carbonatausfällung; Cyanobakterien; Diatomeen; Ooide; Sphären; mikrobielle Gemeinschaft.

**Résumé : Production d'ooïdes à partir de cultures en laboratoire de biomicrosphères se calcifiant - Une contribution à la genèse des oolithes.**- La production *in vitro* de structures semblables aux ooïdes a été observée à partir de cultures d'associations microbiennes sphériques récoltées en mer de Wadden (Mer du Nord). Les sphérules microbiennes sont des agrégats de diatomées benthiques (*Navicula perminuta*) entourés de couches de cyanobactéries filamenteuses du genre *Phormidium* et d'une auréole de bactéries hétérotrophes. Le développement de ces structures prend plusieurs mois et ces organisations sont stables avant qu'elles ne se calcifient. La calcification débute à la surface des sphères sous forme de nuages de petits cristaux isolés qui vont ensuite se développer et s'agglomérer pour constituer des sphères creuses autour des associations microbiennes. Il s'agit de la première observation de calcifications au sein de communautés microbiennes de forme sphérique.

**Mots-Clefs :** Calcification ; cyanobactéries ; diatomées ; ooïdes ; sphérulites ; association microbienne.

### Introduction

Precipitation of calcium carbonate is widespread in microbial communities forming biofilms and microbial mats. The laminated structure of these communities consists of layers of carbonate which

outlast the microbial colony that produced them. Fossilized remains of these communities in which particles of other sediment are also included are known as stromatolites. They have a long fossil record since early Proterozoic and still flourish in particular in the reefs of the Bahamas and Australia (e.g. VISSCHER *et alii*,

<sup>1</sup> Carl von Ossietzky Universität Oldenburg, Institute for Chemistry and Biology of the Marine Environment, Arbeitsgruppe Geomikrobiologie, PO Box 2503, D 26111 Oldenburg (Germany)

[Ulrike.Brehm@uni-oldenburg.de](mailto:Ulrike.Brehm@uni-oldenburg.de)

<sup>2</sup> Carl von Ossietzky Universität Oldenburg, Institute for Chemistry and Biology of the Marine Environment, Arbeitsgruppe Geomikrobiologie, PO Box 2503, D 26111 Oldenburg (Germany)

[http://paleopolis.rediris.es/cg/CG2004\\_L03/](http://paleopolis.rediris.es/cg/CG2004_L03/)

2002).

The typical stromatolitic structure is laminated. Each lamina represents a horizon of former microbial biofilm or mat (KALKOWSKY, 1908). Associated mineral particles (precipitates and detrital grains) are overgrown and sometimes entirely coated by microbial assemblages (RIDING and AWRAMIK, 2000). Small (mm size), spherical to oval concentrically laminated carbonate bodies or aggregates, which form in shallow tropical seas are called ooids and are known to become consolidated into rocks called oolitic limestones (oolith, Rogenstein; KALKOWSKY, 1908). The genesis of ooid grains is still enigmatic. The alternative explanations are confronted along the lines of predominantly abiotic vs. biogenic origin of ooid grains and the associated carbonate precipitates.



**Figure 1:** Microbial biosphere. Assemblage of filamentous cyanobacteria, heterotrophic bacteria and diatoms within a sphere. Diameter of the sphere 166  $\mu\text{m}$ .

The principal biochemical processes that have been recognized to affect the degree of carbonate saturation and therefore may cause carbonate precipitation include:

- environmental carbon depletion by autotrophs during photosynthesis (and possibly chemolithotrophy),
- deamination of amino acids in the course of bacterial proteolysis,
- anaerobic bacterial dissimilatory sulfate reduction. All result in an increase in pH and/or alkalinity, which promote carbonate precipitation (BROWNE *et alii*, 2000).

In this study we show the microbially induced formation of ooids in the laboratory.

### Materials and methods

Filamentous cyanobacteria of the genus *Phormidium*, diatoms (*Navicula perminuta*) and heterotrophic bacteria were isolated from Wadden Sea (North Sea) microbial mats. Species identification was based on both

morphological features and the sequencing of the 16Sr RNA gene fragment (data not shown).

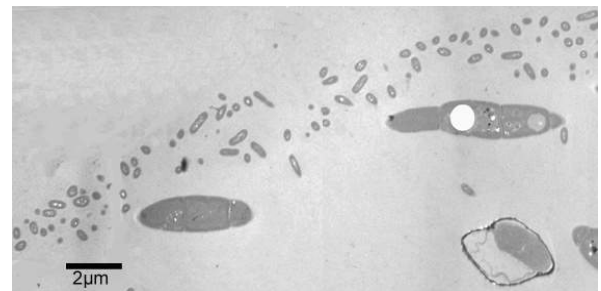
The isolates were grown on artificial seawater medium ASNIII solidified with 1% of Bacto Agar, prepared according to RIPPKA *et alii* (1979). The Petri Dishes were maintained at 18°C, and 120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Osram tungsten light tubes) and with a light/dark cycle of 12/12 h. For the control experiments, the same medium and conditions of incubation were used, without organisms.

The cultures used in our experiments were not axenic. We worked on microbial community consisting of cyanobacteria, diatoms, and bacteria. However, filamentous cyanobacteria were always the dominant species regulating the development of the spheres.

In order to accelerate bacterial activity the experiments were set up with signal substance BHL (Butyroyl-Homoserinlacton) in a final concentration of 10 mM (BREHM *et alii*, 2003).

Light microscopy was performed on an inverted microscope (ZEISS Axiovert).

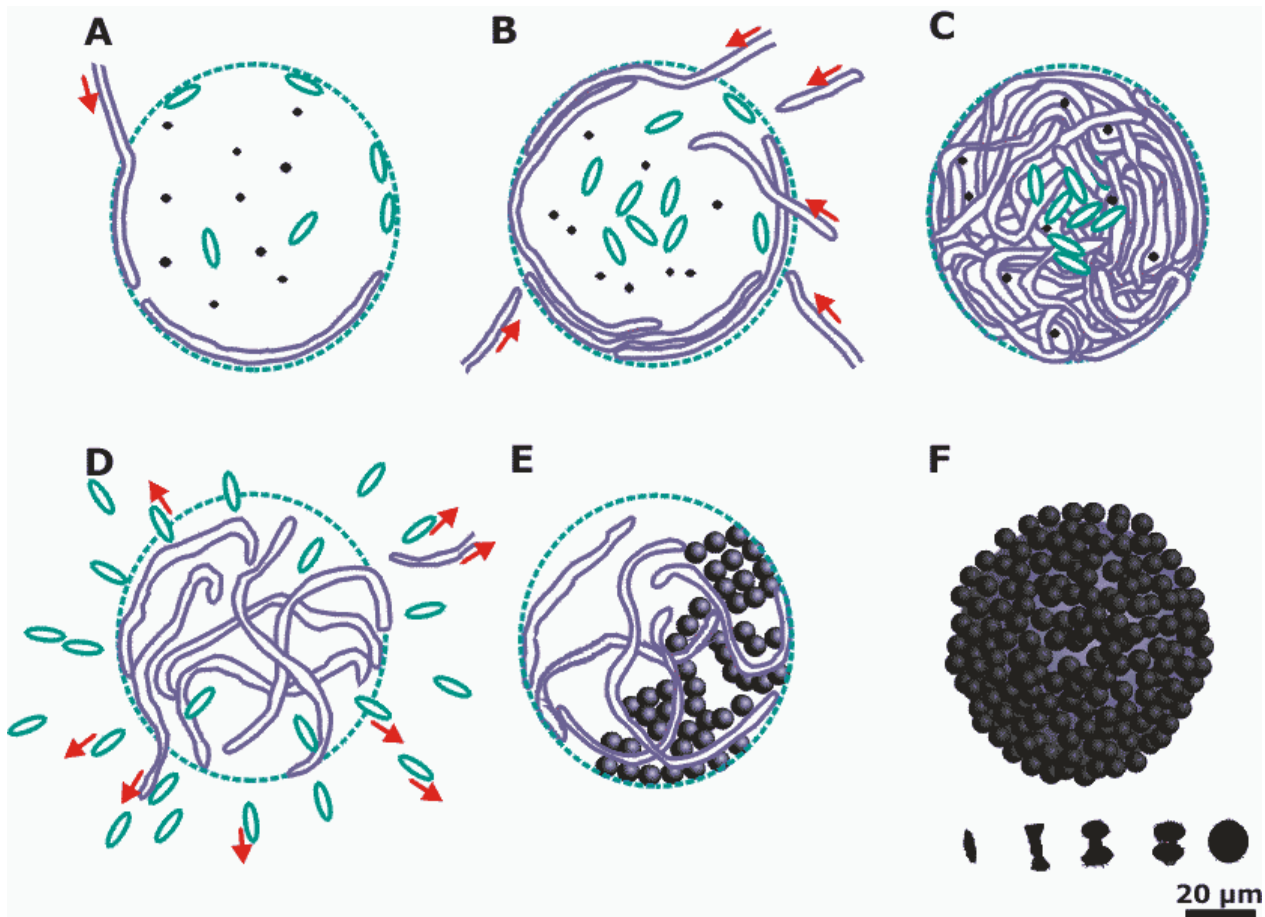
Samples for TEM were prepared as described previously (PALINSKA and KRUMBEIN, 2000).



**Figure 2:** Transmission electron microscopy of the outer part of the sphere. The envelope consisting of heterotrophic bacteria and their excreted products form the surface of the sphere. Below the envelope two filaments of *Phormidium* sp. and one diatom (lower right side) are documented. The space between organisms is filled by EPS (Extracellular Polymeric Substances).

### Results

Distinct spherical structures (Fig. 1) developed in culture by aggregation of cells of filamentous cyanobacteria (*Phormidium* sp.), heterotrophic bacteria and benthic diatoms (*Navicula perminuta*), persist for an extended time and may suggest a symbiotic relationship (BREHM *et alii*, 2003). Biomicrospheres isolated from a microbial mat of the Wadden Sea (German Bight) have now been cultured and systematically transferred in the laboratory for more than four years (BREHM *et alii*, 2003). Interestingly, the same type of biomicrospheres has also been repeatedly observed and isolated from fresh, microbial mat samples.



**Figure 3:** Schematic biomicrosphere development stages. Cyanobacteria are represented as long filaments, diatoms as rice-shaped grains, and heterotrophic bacteria as small dots. Five different stages of carbonate crystallization are shown at the right, lower edge. The final stage of the carbonate crystallization appears in E and F. A-B Initial stage; Cyanobacteria passing through the spherical, bacterial envelope and encompassing the diatoms. C Densely packed biomicrosphere with diatoms concentrated in the centre and cyanobacteria surrounding them. D Late stage of the biomicrosphere. Diatoms and some cyanobacteria abandon the sphere. E-F Carbonate precipitation following the emplacement of cyanobacteria. F Calcified biomicrosphere.

Invariably after a cultivation period of two to three weeks a community of one cyanobacterium species (*Phormidium* sp.), several well defined heterotrophic species of bacteria and a diatom (*Navicula perminuta*) created biomicrospheres 40-400 μm in diameter. Under laboratory conditions the first step in the formation of biomicrospheres is the appearance of a thin 1-3 μm thick envelope. This spherical envelope is always observed and documented using light- and transmission electron microscopy (Fig. 2) and is probably produced by the heterotrophic bacteria. When the spheres appear they are recognized by filamentous cyanobacteria that rapidly approach and forcefully penetrate into them (see Video file). All trichomes arrange themselves in the shape of a thin spherical film inside the biomicrosphere. *N. perminuta* eventually sneaks in with the *Phormidium* trichomes and by massive multiplication fill the whole interior of the sphere (frustules and EPS). After twelve weeks the peripheral part of the cyanobacterial coating of the sphere turn "sclerotic", i.e. tiny scleres or sclera form a layer at or near the outer surface.

The spheres promote calcification in the surface layers and ultimately produce ooid-like hollow carbonate structures (Fig. 3 A-F). In control runs without microorganisms no carbonate precipitation was observed.

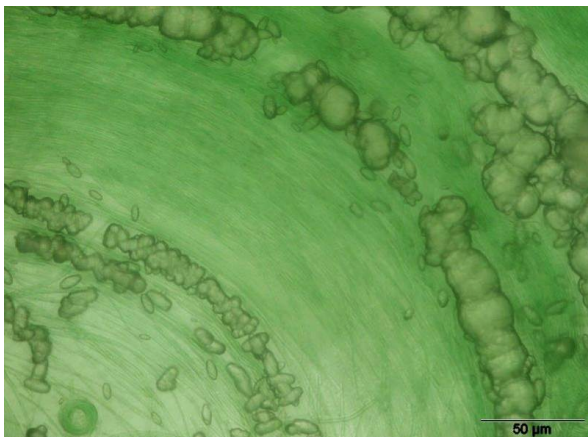
The calcium carbonate precipitates in many forms: microscopic carbonate needles, wheat seed-shaped grains, small rods, dumbbells, simple balls and joined balls (Fig. 4). Fractal growth influences not only the size of the fractals but also the inclination of the next generation (KRUMBEIN, 1983; BUSCH *et alii*, 1999). All stages from sticks to balls can exist at the same time. The solids merge mutually and build shells and complex structures. In the laboratory these precipitates of carbonate are closely connected with the appearance of structured cyanobacterial assemblages (Fig. 5-6). After two or three months of cultivation the spheres appear as multilayered circular assemblages in which several belts of carbonates are precipitated (Fig. 7).



**Figure 4:** Different stages of carbonate deposition in biomicrospheres. In the background filaments of cyanobacteria are visible.

### Discussion

The term "oolite" was introduced and defined by BRUECKMANN (1721) using material collected a few kilometers away from the type locality where the terms "stromatolite" and "oid" were introduced and defined more than 175 years later by KALKOWSKY (1908). Interestingly BRUECKMANN (1721), KALKOWSKY (1908) and LUDWIG and THEOBALD (1852) suggested identical conditions of formation for ooids and stromatolites. However, ooids would often be washed out of stromatolitic microbial mats and deposited elsewhere. The formation of a typical ooid is connected with a nucleation center, which can be of biotic or abiotic origin (KÜHL *et alii*, 2003). Here we supply laboratory evidence that the aforementioned authors correctly analyzed the situation despite the vast literature on calm water benthic stromatolites contrasted with agitated water planktonic ooids and oolites. The formation of microbial mat derived ooids and oolites has never before been demonstrated in laboratory experiments.

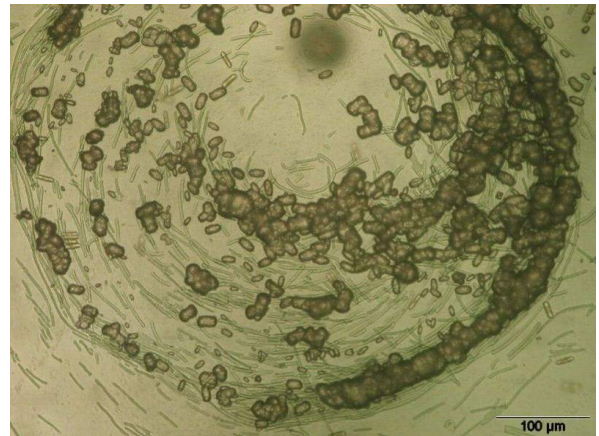


**Figure 5:** Calcium carbonate precipitate, composed of numerous spherically arranged layers of small calcite crystals. Intergrown crystals are connected to cyanobacterial filaments.

Our studies were focused on carbonate precipitation in spherical microbial communities

(Fig. 1): assemblages of cyanobacteria (*Phormidium* sp.), heterotrophic bacteria and diatoms (*Navicula perminuta*) (BREHM, 2001; BREHM *et alii*, 2003). Precipitation occurs where masses of bacteria are enclosed and concentrated in spherical envelopes.

The envelopes are permeable only for cyanobacteria; other organisms cannot penetrate them. Within the sphere diatoms accumulate in the centre and cyanobacteria surround them. In this way a lamination is established, comparable to the lamination of common benthic biofilms. A similar phenomenon of microspheres formation has already been reported by Fox *et alii* (1959). They demonstrated that when placed in water certain proteins spontaneously self-organize into structures, known as microspheres, that resemble primitive cells and proposed that microspheres might represent a significant early stage in precellular evolution.



**Figure 6:** Concentric layers of calcium carbonate following the cyanobacterial orientation.

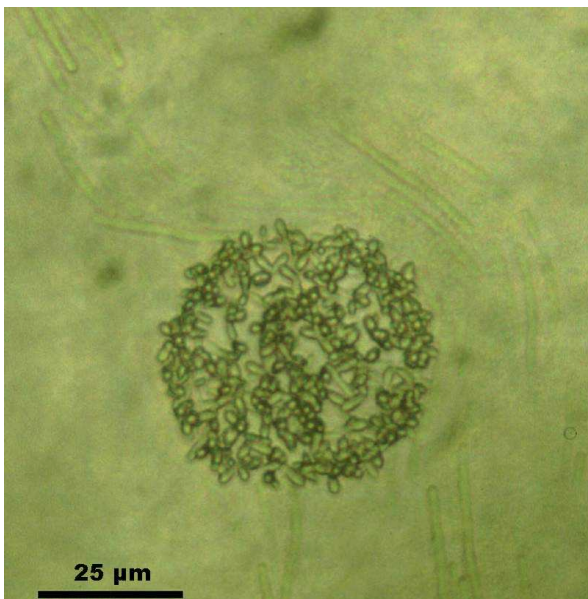
Calcium carbonates precipitate in the laminations formed by cyanobacteria and associated heterotrophs following the form of the organism's organization: in stromatolites as horizontal laminations and in the biomicrospheres studied here as concentric layers. The geometry of the biofilm determines the shape and size of the carbonate layer. Subsequently a calcisphere or spherulite, composed of numerous small calcite crystals will form; the first step in the development of an ooid (Fig. 8).

This structure is comparable to that of fossil oolites and suggests a common genesis for calcispheres and oolites.



**Figure 7:** Late stage of calcification. Two coalescent spheres of carbonate grains.

Oolites are always built up in several carbonate layers in which each layer represents a separate population of organisms. These fossilized concentric oolitic layers are preserved in carbonate rocks. The main processes in the development of the aggregates studied in the laboratory are limited to diffusion and/or cluster-cluster mechanisms. The aggregates range from 10 to 40 μm in diameter. In sparsely populated areas sparsely disseminated carbonates precipitate. They may be the result of several discrete chemical reactions.

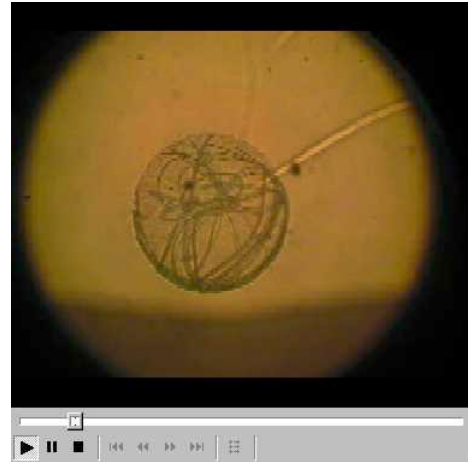


**Figure 8:** Spherulitic surface of the biomicrosphere. Numerous, discrete calcite crystals are visible.

About three months elapse before the first carbonates crystallize in the Petri dishes. The calcification of the biomicrospheres was always observed to start on the spherulitic surface in the form of numerous discrete calcite crystals. Ongoing precipitation leads to the covering of the surface by carbonates. At the same time our control runs without microorganisms never showed any precipitation.

Our investigations indicate that the building

of oolites is biologically induced and external nuclei are not necessary to create spherulites. The microorganisms create the basis for their structures in complete independence.



**Video File:** Cyanobacterial movement inside a biomicrosphere. The video file can be downloaded from:

- <[http://paleopolis.rediris.es/cg/CG2004\\_L03/CG2004\\_L03\\_Video.wmv](http://paleopolis.rediris.es/cg/CG2004_L03/CG2004_L03_Video.wmv)> (= 762 KB),
- <[http://paleopolis.rediris.es/cg/CG2004\\_L03/CG2004\\_L03\\_Video.mpg](http://paleopolis.rediris.es/cg/CG2004_L03/CG2004_L03_Video.mpg)> (= 8,606 KB), or
- <[http://paleopolis.rediris.es/cg/CG2004\\_L03/CG2004\\_L03\\_Video.avi](http://paleopolis.rediris.es/cg/CG2004_L03/CG2004_L03_Video.avi)> (= 4,774 KB).

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