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Alderkamp, Anne-Carlijn

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Chapter 1

Carbohydrate production by phytoplankton and degradation in the marine microbial food web – The cycling of chrysolaminaran

THE MARINE CARBON CYCLE

Two-thirds of the earth's surface is covered by water, and approximately half of the global primary production is produced in the marine ecosystem. The most important process responsible for primary production is oxygenic photosynthesis by phytoplankton (Hedges 1992). Phytoplankton are microscopic plants, mostly algae, which live suspended in the water column. All plants use light, to convert carbon dioxide and water to organic compounds and oxygen in the photosynthesis process. The organic carbon produced by photosynthesis in the sea is respired in the marine food web (Figure 1). In the linear food chain carbon and energy is channelled through algae, to herbivores and on to higher trophic levels. In all these processes dissolved organic carbon (DOC) is released. DOC is respired by heterotrophic prokaryotes and stored as biomass that can re-enter the linear food chain after grazing by protozoans via the "microbial loop" (Azam et al. 1983). The prokaryotes play an important role in the marine carbon cycle, since an estimated 50% of marine primary production is channelled via the microbial loop (Azam 1998). Amongst the prokaryotes, bacteria are the most important consumers of DOC in the marine system (Williams 2000). Archaea have been detected in virtually all marine systems (Olsen 1994; Stein and Simon 1996) and may play a significant role in the carbon cycle in the deep sea (Karner et al. 2001; Herndl et al. 2005). At present, however, their role in the global carbon cycle remains largely unknown.

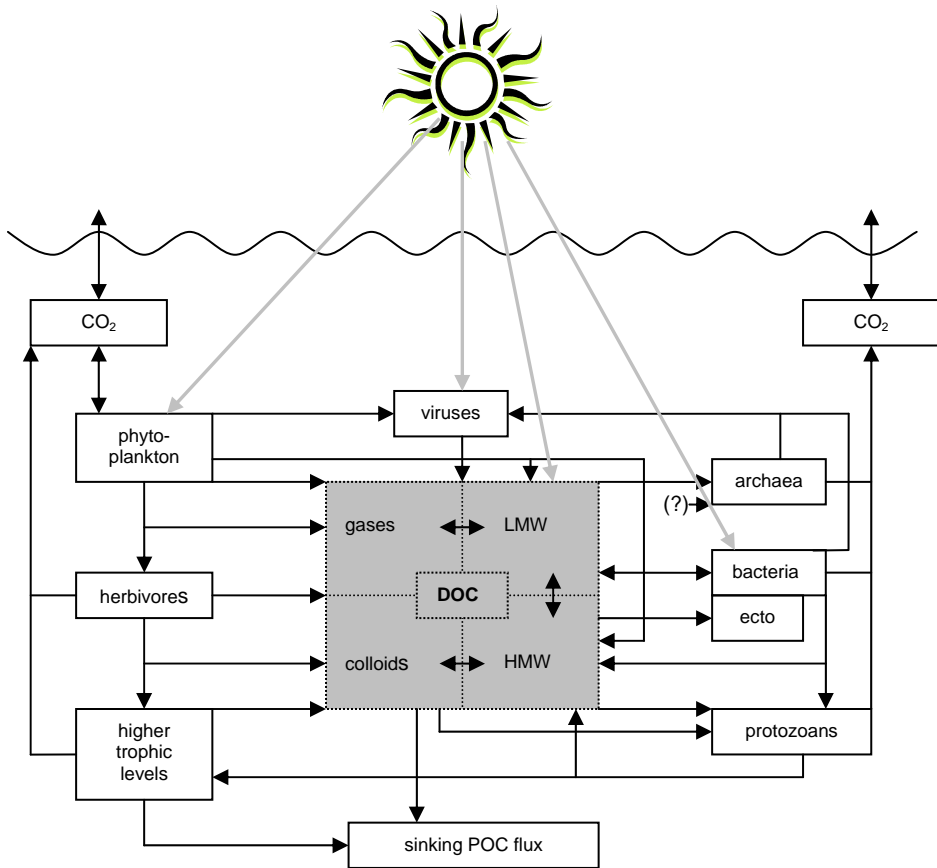


Figure 1. Schematic representation of the marine microbial food web. On the left the “linear food chain” is depicted, where energy and carbon from photosynthesis in phytoplankton is channeled through herbivorous grazers, onto higher trophic levels. On the right the “microbial loop” is depicted, in which dissolved organic carbon (DOC) is respired by prokaryotes and stored as biomass that can re-enter the classic food chain via the protozoans. Although archaea are detected in virtually all marine systems, their role in the marine carbon cycle remains largely unknown. High-molecular-weight (HMW) DOC is cleaved by either “free” extracellular enzymes, or cell-attached ectoenzymes (“ecto”) prior to uptake by bacteria. Energy from the sun fuels phototrophic phytoplankton and prokaryotes. Excessive irradiance and especially the UV component may, however, cause damage to phytoplankton, prokaryotes and viruses. In addition irradiance may alter properties of DOC. At the bottom the flux of sinking particulate organic carbon (sinking POC flux) is depicted. In this way carbon sinks to the deep waters and may eventually become buried in sediments, whereby it is withdrawn from the carbon cycle for prolonged times (adapted from Karl 1994; Delong and Karl 2005).

CARBOHYDRATE PRODUCTION BY PHYTOPLANKTON

The cycling of carbohydrates is a key process in the marine carbon cycle because of the abundance and omnipresence of carbohydrates in the marine ecosystem. Carbohydrates form an important fraction of the organic carbon produced by phytoplankton (Biddanda and Benner 1997; Biersmith and Benner 1998). They are major constituents of marine particles and sediments (Cowie and Hedges 1984), and marine DOC (Benner et al. 1992). Different types of carbohydrates with different functions are produced by phytoplankton. Two major pools are extracellular and storage carbohydrates.

Extracellular carbohydrates are typically large heteropolymers and may form a mucous layer around the algal cells (Guillard and Helleburst 1971; Hoagland et al. 1993). In addition, phytoplankton release a part of the primary production directly as DOC (Nagata 2000; Teira et al. 2001; 2003), a significant part of which typically consists of carbohydrates (Biddanda and Benner 1997; Biersmith and Benner 1998; Aluwihare and Repeta 1999). Excess photosynthesis, e.g. under high light conditions or nutrient limitation, may enhance release of extracellular carbohydrates (e.g. Staats et al. 2000). In this case the contribution of extracellular carbohydrates to the phytoplankton carbon will depend on the nutrient status and light conditions of the algae.

Storage carbohydrates are produced in the light and serve as an internal energy and carbon reserve. At low light conditions, or at night, this reserve can be used for maintaining cell metabolism and protein synthesis (Cuhel et al. 1984; Lancelot and Mathot 1985; Granum and Mykkestad 2001). In addition, excess photosynthesis may lead to accumulation of storage carbohydrates (Mykkestad and Haug 1972; Mykkestad 1988; Janse et al. 1996b). Therefore, the contribution of storage carbohydrates to cellular carbon is highly variable, depending on the time of the day, the light intensity and the nutrient status of the cell.

The storage carbohydrates of most algae are α - or β -glucans. Chrysolaminaran is the most abundant type of storage carbohydrate in marine phytoplankton (Figure 2). It is a β -1,3-D-glucan with some branching at position 6 and/or 2, typically containing 20-30 glucose residues, corresponding to approximately 4 kDa in size. It is also known as leucosin or laminarin. The latter name refers to the type of glucan found in some macroalgae (e.g. the Phaeophyta), with the same structure but containing mannitol end-groups (Meeuse 1962; Craigie 1974; Mykkestad 1978; Painter 1983; Janse et al. 1996b). In diatoms chrysolaminaran is located intracellular in vacuoles (Chiovitti et al. 2004). Because of its presence in phytoplankton all over the world, chrysolaminaran is one of the most abundant carbohydrates on earth.

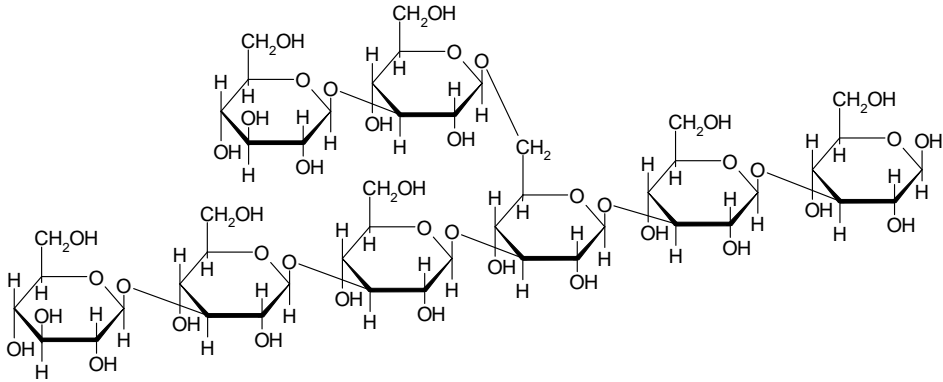


Figure 2. Structure of part of a chrysolaminaran molecule, a β -1,3-D-glucose backbone with a β -1,6 branch.

CARBOHYDRATE DEGRADATION BY PROKARYOTES

The carbon of phytoplankton becomes available for prokaryotes as DOC after sloppy feeding and egestion by zooplankton (Strom et al. 1997; Møller et al. 2003), or cell lysis (Van Boekel et al. 1992; Brussaard et al. 1995b; 2005), e.g. as a result of viral infection (Fuhrman 1999; Suttle 2005). Although carbohydrates are usually considered to be labile substrates for prokaryotes, the high concentration of carbohydrates in marine DOC (Benner et al. 1992), marine particles and sediments (Cowie and Hedges 1984), and sediment pore waters (Arnosti and Holmer 1999) demonstrates carbohydrates are not always easily metabolized. Whether a carbohydrate is utilized by a certain prokaryote largely depends on 1) the chemical structure of the carbohydrate and 2) the biochemical capacities of the prokaryote.

1) The chemical structure of carbohydrates displays a remarkable diversity, a consequence of the wide variety of naturally occurring monosaccharides and the different glycosidic bonds. For example two different hexoses can form 16 different disaccharides; three different hexoses can form 384 different trisaccharides. There are three levels of structural diversity. The primary structure is determined by the type of monosaccharide and their linkage. This leads to a secondary structure in the shape of the polymers (e.g. β -1,3-linked glucans form helices). Polymers pack into structures that are held together predominantly by hydrogen bonds, determining a tertiary structure (e.g. a loose hydrogel, or a tightly packed network structure like cellulose). Since the enzymes involved in degradation of polymers are selective with respect to their substrate (Arnosti and Repeta 1994; Warren 1996), this diversity requires a similar enzyme diversity. The secondary and tertiary structure may lead to inaccessibility of glycosidic bonds. Therefore, simple, linear polymers consisting of just one monosaccharide, such as storage glucans, are generally believed to be more labile than heteropolymers with complex tertiary structures, such as extracellular mucopolysaccharides or cell wall constituents (Warren 1996).

Due to the large diversity and low concentrations of carbohydrates in the marine environment, the possibilities of analyzing the structural complexity in marine samples are extremely limited. In addition, the properties of carbohydrates released as DOC into the marine environment are not static, but influenced by abiotic factors. Polymers assemble spontaneously, forming the matrix of hydrogels, supported by ionic interactions such as calcium bridges. In this way soluble polymers end up in particles capable of sedimentation (Chin et al. 1998; Verdugo et al. 2004) (Figure 1). In addition, it alters the secondary and tertiary structure of the carbohydrates. Assembly may, however, be inhibited by ultraviolet (UV) radiation. Moreover, UV radiation may disperse assembled gels and even cleave polymers (Orellana and Verdugo 2003) (Figure 1).

2) The biochemical capacities of the prokaryotes largely depend on the properties of their enzyme systems. Carbohydrates are degraded by glycoside hydrolases; enzymes hydrolyzing the glucosidic bond between two monosaccharides. Since prokaryotes can only transport small molecules (< 600 Da) across their cell membrane (Weiss et al. 1991), larger molecules have to be cleaved by extracellular enzymes prior to uptake. These enzymes may be “ectoenzymes” (Figure 1) that are attached to the prokaryotic cell, either to the cell wall or embedded in exopolymer layers, or “free enzymes” that are released into the environment. Free enzymes may be actively excreted by cells, but may also have been liberated after cell lysis, e.g. as a result of autolysis, viral lysis, or sloppy feeding (Chróst 1991). Ectoenzymes are most abundant in the marine environment (Bochdansky et al. 1995). Very little is known about properties of enzymes of marine prokaryotes, largely due to low amounts of biomass and slow *in-situ* degradation rates of relevant substrates. Therefore, enzyme characteristics in the marine environment have mainly been determined using small substrate proxies, consisting of a monomer such as glucose linked to a fluorophore such as methylumbelliferyl (MUF), whose fluorescence increases upon hydrolysis (e.g. Martinez et al. 1996; Arrieta and Herndl 2002). While these small substrate proxies are easy to apply and the use of fluorophores ensures the sensitivity that is required detecting low activities in marine samples, these substrates lack the structural properties of macromolecules in solution (Warren 1996).

AIM OF THE THESIS

Given the difficulty of direct structural analysis of marine carbohydrates and their slow degradation rates *in-situ*, I chose the approach to study the cycling of a well-characterized, relatively simple carbohydrate: chrysolaminaran. Studying the cycling of chrysolaminaran enabled me to circumvent the structural diversity of carbohydrates occurring in the marine ecosystem, yet the structural complexity to mimic complex macromolecules in solution is still present. Therefore, some of the principles found by studying this relatively simple carbohydrate may help to elucidate factors hampering rapid carbohydrate degradation by prokaryotes and better understand carbohydrate cycling in the marine ecosystem. Moreover, chrysolaminaran is one of the most abundant carbohydrates in the marine system, yet little is known about factors controlling its production and degradation. This thesis follows key

elements of the chrysolaminaran cycle, specifically its production by phytoplankton and subsequent degradation by prokaryotes.

The main research questions are:

- **How is the production of chrysolaminaran by phytoplankton influenced by nutrient concentrations and irradiance levels?**
- **What are the characteristics of bacterial enzyme systems involved in degradation of chrysolaminaran?**
- **What is the influence of a massive release of carbohydrates on the abundance and activity of prokaryotes?**

Some of the principles found by studying this relatively simple carbohydrate provide further insights in the carbohydrate production and degradation patterns observed in marine ecosystems.

OUTLINE OF THE THESIS

The production of chrysolaminaran was studied in the phytoplankton groups *Phaeocystis* and diatoms. Both groups are cosmopolitan algae that often dominate the phytoplankton of temperate and polar waters (Nelson et al. 1995; Schoemann et al. 2005). Chrysolaminaran is the principal storage glucan of both groups (Paulsen and Myklestad 1978; Painter 1983; Janse et al. 1996b). Diatoms are extremely ubiquitous both in the marine ecosystem and in fresh water. They may be planktonic, benthic, or growing on sea ice or other organisms, such as seaweeds, animals (from crustaceans to whales). Photosynthesis by marine diatoms generates as much as 40% of the annual carbon production in the sea (Nelson et al 1995).

Phaeocystis has a polymorphic life cycle, comprising various stages of single cells of 3-9 µm in diameter and a colonial stage with cells embedded in a mucous layer usually reaching several mm (Rousseau et al. 1994). This mucous layer consists predominantly of extracellular carbohydrates (Guillard and Helleburst 1971; Solomon et al. 2003), that form a thin, yet strong, gel, surrounding an aqueous lumen (Van Rijssel et al. 1997; Hamm et al. 1999). Colonial *Phaeocystis* forms massive blooms in temperate and polar waters (Lancelot et al. 1987; Baumann et al. 1994; Mathot et al. 2000; Schoemann et al. 2005). During these blooms organic matter is produced that is rich in carbohydrates (Veldhuis et al. 1986a; Fernández et al. 1992; Janse et al. 1996a) comprising both extracellular mucopolysaccharides and chrysolaminaran.

In **chapter 2**, a new method is described to separate mucopolysaccharides and chrysolaminaran in organic matter derived from *Phaeocystis* colonies. This method was applied during a *Phaeocystis* bloom in a mesocosm experiment near Bergen, Norway. In several experiments, the effect of light intensity and nutrient conditions on the production of both chrysolaminaran and mucopolysaccharides was determined.

The light climate that phytoplankton residing in the water column experience can be highly variable due to wind induced vertical mixing. Thus, phytoplankton may be exposed to short

periods of excessive high light levels, including UV radiation. This requires specific acclimation to find a balance between protection against photodamage at high-light levels and optimal photosynthesis conditions for sufficient energy for growth at low-light levels (Neale et al. 2003). In **chapter 3** the production of storage carbohydrates under excessive light conditions were investigated in an Antarctic and a temperate diatom. This was part of a laboratory study into the effect of acclimation to high-light conditions on tolerance to short periods of excessive light conditions.

The characteristics of enzyme systems of selected marine bacteria involved in degradation of chrysolaminaran are described in **chapter 4**. After a bloom of *Phaeocystis* in the Dutch coastal North Sea several bacterial strains capable of utilizing laminarin as a sole carbon source were isolated. The laminarin degradation system of three strains of *Vibrio* was studied in detail in physiological experiments in the laboratory.

In **chapter 5** the dynamics in abundance and activity of prokaryotes was determined in the coastal North Sea during a spring and summer season, thus when primary production was dominated by diatoms and *Phaeocystis*. A combination of Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization with microautoradiography (MICRO-CARD-FISH) was applied to combine quantitative detection of specific phylogenetic groups with their group-specific activity. The results are discussed in relation with the release of carbohydrates produced in a *Phaeocystis* bloom.

Finally, in **chapter 6** the current state of the knowledge on the production of carbohydrates by *Phaeocystis* and their degradation in the microbial food web is reviewed, including the results from the previous chapters.

