

The influence of microorganisms on chlorophyll *a* degradation in the marine environment

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Abstract

This study investigated the action of natural microorganisms on the degradation (diagenesis) of chlorophyll *a* (Chl *a*). Unialgal cultures of the diatom *Chaetoceros gracilis* and the eustigmatophyte *Nannochloropsis oculata* were incubated with or without the addition of natural marine microorganisms in oxic (air) or anoxic (nitrogen sparge) conditions. Microorganisms were obtained from seawater recovered just above the sediment–water interface of a coastal lagoon of southeastern Florida. Incubations were over periods of 2, 4, 8 and 24 weeks. Pigments were analysed by high-performance liquid chromatography. The most rapid destruction of Chl *a* and its derivatives occurred in oxic conditions with added microorganisms. The next fastest rates of destruction were observed in oxic conditions without such additions. *N. oculata* and *C. gracilis* incubated under oxic conditions without added microorganisms were found to contain almost 50% or 25% of total chloropigments, respectively, as Chl *a* allomers after 24 weeks. Allomer formation is taken as a prelude to purpurin/chlorin formation and potential macrocyclic destruction. Pyropheophytin *a* and steryl chlorin (phorbide) esters were observed to form significantly under anoxic conditions, but only to a minor extent under oxic conditions, in cultures incubated with added microorganisms. The formation of the steryl chlorin esters was observed to occur in concert ($R^2 > 0.98$) with the disappearance of pyropheophorbide *a* and pyropheophytin *a*. The direct intervention of microorganisms in the course of diagenetic processing of Chl *a* is concluded. Thus, cellular senescence, grazing-induced cell disruption, physicochemical environment (O_2 and so on), and microbial processing all need to be considered in order to fully address chlorophyll digenesis.

The majority of chlorophyll *a* (Chl *a*) synthesized by phytoplankton undergoes degradation in the upper water layers, and only a small portion actually settles to the bottom. As with bulk organic matter in any aquatic environment, Chl *a* is decomposed under the influence of a variety of factors. Degradation processes start within phototrophic organisms during the senescence and death of the cells (e.g., references in Baker and Louda 1986; cf. Ziegler et al. 1988; Shioi et al. 1991; Spooner et al. 1994*a,b*; Louda et al. 1998, 2002). Other well-known factors responsible for

these processes include the physicochemical conditions in the environment (Leavitt and Carpenter 1990) or herbivores grazing on phytoplankton and detritus (Welschmeyer and Lorenzen 1985). The result of all these processes is that Chl *a* is either decomposed, with total destruction of the molecule to colorless compounds, or transformed without alteration of the macrocyclic nucleus (references in Baker and Louda 1986, 2002; see also Keely et al. 1990; Eckardt et al. 1992; Spooner et al. 1994*a,b*). In the latter case, retention of the macrocycle, a variety of different Chl *a* derivatives are formed. This second group of transformation products includes numerous compounds of which only a few are often dominant. For example, in the recent sediments of the Baltic Sea, these include chlorophyllide *a*, the allomer(s) and epimer of Chl *a*, pheophytin *a*, and pheophorbide *a*, as well as pyropheophytin *a* and chlorin steryl esters (Kowalewska 1997, 2005; Kowalewska et al. 2004). These compounds were also identified in the sediments of other aquatic basins (Furlong and Carpenter 1988; Leavitt and Carpenter 1990; Eckardt et al. 1992; Bianchi et al. 1993; Louda et al. 2000). The origin of these pigments begins within the material sinking through the water column. Degradation of pigments in sediments is often the result of a suite of long and complicated transformations that can be stimulated or inhibited by many factors, with the influence of oxic versus anoxic syndepositional and postdepositional conditions being quite prevalent (Baker and Louda 1986, 2002; Spooner et al. 1994*a,b*).

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Formation of Chl *a* derivatives during senescence and death of cells has previously been the subject of laboratory studies. Welschmeyer and Lorenzen (1985) observed that phytoplankton cell incubation in darkness yielded Chl *a* derivatives only in very small quantities. On that basis, they suggested the result of cellular senescence to be the decomposition of Chl *a* to colorless products. However, other authors have observed the formation of colored Chl *a* derivatives as the results of cellular senescence. Pigments such as chlorophyllides *a*, pheophytin *a*, pyropheophytin *a*, pheophorbide *a*, and pyropheophorbide *a* are commonly found (e.g., Baker and Louda 1986; Spooner et al. 1994b; Louda et al. 1998, 2002). According to these authors, these derivatives are formed as a result of reactions initiated by enzymes present in an algae cell, which are activated when the cellular metabolism shuts down and/or the cell is damaged. Pyropheophorbide *a* has been reported as an end product in senescent systems of both higher plants (Shioi et al. 1991) and microalgae (Ziegler et al. 1988; Louda et al. 1998, 2002). Moreover, it was stated that pheophorbide *a* observed in the decomposing algae is often or usually formed as a result of the demetallation of chlorophyllide *a* degradation rather than by phytol hydrolysis from pheophytin *a* (Ziegler et al. 1988). The type and amounts of the derivatives formed depend on the algal species, as enzyme activity in cells of different species likely varies. Species characterized by high chlorophyllase contents, notably diatoms (Jeffrey and Hallegraeff 1987), produce high amounts of chlorophyllides *a*, pheophorbide *a*, and pyropheophorbide *a* (Louda et al. 1998, 2002; references in Baker and Louda 2002). Chlorophyllide *a* has been suggested as a marker for senescent microalgae (Jeffrey and Hallegraeff 1987; Spooner et al. 1994b). However, it has also been shown that chlorophyllide *a* can indeed be generated during the extraction of fresh viable diatoms, and care must be taken in the interpretation of chlorophyllide equaling senescence (Louda et al. 1998, 2002).

The physicochemical conditions of seawater have a decisive influence on the decomposition of Chl *a*. A number of papers have been published concerning the influence of factors such as oxygen, light, and temperature on the selected pigments in algal cells and fecal pellets (Nelson 1993; Kowalewska and Szymczak 2001) as well as in sediments (Sun et al. 1993; Bianchi et al. 2000; Louda et al. 2000). All data reveal that pigments are decomposed more slowly under hypoxia/anoxia than in oxic conditions. Directly related to degradation rates are numerous findings that much higher pigment concentrations are in hypoxic anoxic versus oxic sediments (Sun et al. 1993; King 1995; Kowalewska 2005). Chl *a* diagenesis under oxic conditions proceeds with a drastic decrease of chloropigments in surface sediment layers (references in Baker and Louda 1986; Furlong and Carpenter 1988). Additionally, oxygen has a crucial effect on flora and fauna in the sea. Anoxia obviously eliminates macrofauna and aerobic microfauna, thereby reducing the mixing and processing of sediments by multicellular organisms (bioturbation), and inhibits the decomposition of Chl *a* (Bianchi et al. 2000). Light is also a factor of great importance, as it is the driving force of photooxidation processes in the cell and after its death

(Nelson 1993), especially in surface water layers (Welschmeyer and Lorenzen 1985).

Grazing of the heterotrophic organisms has a significant influence on Chl *a* decomposition. Many authors have focused on Chl *a* transformations by heterotrophs, concentrating mainly on the influence of zooplankton on Chl *a* derivative formation (Conover et al. 1986; Klein et al. 1986; Strom 1993; references in Baker and Louda 1986, 2002). Grazing leads to a decrease of Chl *a* with depth in seawater with the transformed material being excreted in the form of fecal pellets that settle rapidly to the bottom and may enrich the sediments with pigments (Welschmeyer and Lorenzen 1985; Head and Harris 1996). The dominant Chl *a* derivatives often reported as occurring in fecal pellets were pyropheophytin *a* and pyropheophorbide *a*. Gieskes et al. (1991) have shown that during zooplankton grazing, pheophorbides *a* are formed by the further decomposition (phytol hydrolysis) of pheophytin *a* and not via chlorophyllide *a*, as often occurs during cellular senescence (Ziegler et al. 1988; Louda et al. 1998, 2002). Pyropheophorbide *a* is routinely reported as a moderate to major product of grazing by copepods (Head et al. 1994; Head and Harris 1996; Hayashi et al. 2001). This is in contrast to salps (Tunicata, Thaliacea), which transform phytoplankton Chl *a* into the pheophytins *a* (King and Wakeham 1996; Hayashi et al. 2001).

Many observations indicated that a considerable percentage of Chl *a* is transformed to colorless products (Baker and Louda 1986; Conover et al. 1986; Klein et al. 1986; Barlow et al. 1988). That being said, the interplay of syndepositional and postdepositional oxygen fugacity plus the stage of organic diagenesis must always be taken into consideration.

Amounts of Chl *a* derivatives in fecal pellets depend on many factors, specifically the synergisms of species identity, the abundance of phytoplankton, and the relative proportions of phytoplankton to zooplankton (Gieskes et al. 1991; Claustre et al. 1992; McLeroy-Etheridge and McManus 1999). Penry and Frost (1991) observed that fecal pellets excreted by zooplankton grazing during the early phase of a phytoplankton bloom contained more nondecomposed pigments than did those of the late bloom phase. Another important influence is the size of zooplankton. Macrozooplankton grazing leads to higher amounts of chloropigments *a* being transferred to sediments since the larger fecal pellets settle more rapidly (Stoke's law). Fecal pellets excreted by microzooplankton are obviously smaller to nonconsolidated, and that organic matter will settle more slowly and will be held within the mixed layer longer, allowing photodegradation to become a major influence (Welschmeyer and Lorenzen 1985). Additionally, smaller fecal pellets appear to undergo enhanced recycling as a result of repeated ingestion/digestion (coprophagy) by other heterotrophic organisms (Lorenzen and Welschmeyer 1983; Strom 1993). Pigments in fecal pellets have been suggested as being less exposed to environmental physicochemical decomposition than are pigments retained within intact cells (Nelson, 1993).

The benthos, which is an important factor in the transformation processes of bulk organic matter, must also

play a role in Chl *a* derivative formation. On the basis of laboratory culture studies, it has been reported that the result of grazing by benthic organisms on the microphyto-benthos or detritus is the formation mainly of pheophorbides *a* (Bianchi et al. 1988, 2000; Abele-Oeschger and Theede 1991; Ingalls et al. 2000). Alternatively, other investigations revealed the preferential formation of pheophytin *a* (Abele-Oeschger and Theede 1991). Further, it has been stated that the rate of transformation of Chl *a* depends on the quality (species) and quantity of the food (Bianchi et al. 1988), similar to the zooplankton results covered here. Benthic species that are suspension feeders have been found to produce mainly pheophorbides *a* from pheophytins *a* (Szymczak-Zyła et al. 2006).

Microbial activity in aquatic basins is known to transform a considerable part of primary production in such a way that it significantly influences the carbon cycle (Valiela 1995). Because of the specific conditions within sediments, microorganisms are much more abundant than in the overlying waters. Based on that, one may propose that they must have a substantial influence on the transformation of Chl *a* in the marine sediments. To date, the literature contains only a few reports of the direct effects of microorganisms on the transformation/destruction of Chl *a*. The few papers on this subject (Spooner et al. 1994a, 1995; Afi et al. 1996; Chen et al. 2003) have not yet been able to elucidate the processes. Spooner et al. (1994a) observed the formation of certain Chl *a* derivatives, mainly the pheophorbides *a*, as a result of cell deterioration in the presence of aerobic bacteria. They state that the bacteria were not responsible for formation of these pigments and pointed to intracellular enzymes for the reactions involved.

Lacking from all these reports was formation of significant quantities of certain Chl *a* diagenesis products that are often quite abundant in recent sediments (e.g., pyropheophytin *a* and the steryl derivatives; cf. Eckardt et al. 1992; Kowalewska 1997). Some authors are of the opinion that steryl derivatives are formed mainly as a result of grazing of phytoplankton by zooplankton (King and Repeta 1991; Harradine et al. 1996; Chen et al. 2003). Roy and Poulet (1990) have suggested that it is the inquilant microorganisms that are responsible for formation of Chl *a* derivatives in zooplankton fecal pellets.

The aim of the present work was to determine which Chl *a* derivatives form under influence of microorganisms present in bottom waters, specifically those at the sediment–water interface, a site of potentially high microbial activity. In order to do this, a series of laboratory experiments were performed on cultures of unicellular algae with and without microorganism-laden seawater collected at the sediment–water interface from an inshore lagoonal environment and incubated in the dark under oxic or anoxic conditions.

Methods

Experiments were carried out at Florida Atlantic University (FAU), and the chloropigment analyses were performed at the Marine Pollution Laboratory of the Institute of Oceanology, Polish Academy of Sciences. Two

algal species have been used: a diatom of a genus abundant in the marine environment (*Chaetoceros gracilis*) and a eustigmatophyte (*Nannochloropsis oculata*). Both species were obtained from Harbor Branch Oceanographic Institution, Fort Pierce, Florida. The experiments consisted of incubation of the algae under controlled dark oxic or anoxic conditions with the amounts of Chl *a* and derivatives formed being monitored.

Experiments with microalgae—Identical experiments were performed for both algal species. An algal culture was centrifuged at low speed and then the medium decanted. Seawater to serve as a source of microorganisms (i.e., bacteria) was collected just above sediment from a tidally flushed inshore lagoon known as Lake Boca (26°22'N, 80°60'W) on the southeastern Florida coast. The chloropigment *a* content of the water sample was determined and found to be exceedingly low (e.g., <0.1 nmol mL⁻¹) to nonquantifiable. This seawater contained microorganisms but did not contain zooplankton, as revealed by microscopic examinations. Classification of the microbial community (culture techniques, cladistics, and so on) was not attempted. Algal cultures were prepared from concentrated unialgal culture material with added seawater and placed into vials. Vials for anoxic incubation received 40 mL, while those for oxic incubations received 50 mL. All experimental vials were adjusted back to original volumes such that initial Chl *a* concentrations were identical with the (*t*₀) stock culture. Control vials consisted of the pure unialgal culture without added (microorganisms) seawater. The “time zero” content of three controls were analyzed for chloropigment *a* type and concentration. The other vials were divided into two groups for incubation under different conditions, as follows: A = “anoxic,” vials sparged with nitrogen for several minutes and occlusively sealed, and B = “oxic,” wide-mouth vials left open to the air, mixed (swirling) by hand every week. This gave four groups being incubated, controls and experimental (added microorganisms), with each being kept in the dark at room (~24°C) temperature with (oxic) or without (anoxic) oxygen (i.e., air).

Two vials from each group were collected after each of 2-, 4-, 8-, and 24-week incubation periods. The contents of each vial were filtered onto a glass-fiber filter (Whatman GF/F, ≤0.7 μm) and kept at -20°C until extraction. A total of 70 samples were analyzed.

Pigment analyses—Analytical details have been described in greater detail elsewhere (Kowalewska 2005). Following is a brief description of the most salient features of the analytical scheme used.

The frozen filter was placed in a glass centrifuge tube, 15 mL of acetone were added, and the filter was ground with a glass rod. The tube was then placed in the ultrasonic bath, and after 2–3 min of sonication, the sample was centrifuged (10 min, ~2,500 rpm). Extraction was repeated until no more color could be detected in the extractant, usually no more than three times. The acetone extractions were pooled, placed in a separatory funnel, and diluted with water, and the pigments were transferred into benzene.

The benzene layer was collected, evaporated under a stream of argon, and kept at -20°C until analyzed by high-performance liquid chromatography (HPLC)-diode array detection (DAD). HPLC-DAD development and standardization is detailed elsewhere (Kowalewska 2005). Samples were dissolved in acetone and injected into the HPLC-DAD instrument (Knauer set), equipped with a diode array type detector (Chrom *a*-Scope), two pumps (Knauer, type 64), and a Merck Lichrospher 100RP18 end-capped column ($250 \times 4 \text{ mm}$, $5 \mu\text{m}$) with a Merck 100RP18 end-capped precolumn ($4 \text{ mm} \times 4 \mu\text{m}$) in an acetone–water gradient system. Spectra were collected over the range of 360–700 nm.

Results

The following pigments were identified and placed into three groups according to the extent of pigment alteration: precursor = Chl *a*; initial products = chlorophyllide *a*, Chl *a* allomers, and Chl *a'* (epimer), and pheophytin *a*; and secondary products = pheophorbide *a*-I (i.e., pheophorbide *a* free acid) and pheophorbide *a*-II (II = pyropheophorbide *a*), pyropheophytin *a*, and steryl chlorin esters. Therefore, initial products are defined here as those formed directly from Chl *a* as result of *a* single reaction (-Mg, -phytol, allomerization, epimerization), and the secondary products are defined as those derived from the initial products by additional reactions. These groups, in tables and figures, are then considered here as Chl *a* plus initial products as (ΣA) and the sum of secondary products as (ΣB).

In Figure 1b, the peak at about 5 min is pheophorbide-II (Kowalewska et al. 2004; Kowalewska 2005) or *a*3 (Vernet and Lorenzen 1987; cf. Baker and Louda 2002) of those earlier reports. The peak at about 3.5 min in Figure 1b, very small unless enlarged (not labeled), is pheophorbide-I, pheophorbide *a* free acid per se, as identified in previous reports from the Marine Pollution Laboratory IO PAS (Kowalewska et al. 2004; Kowalewska 2005). Presently, pheophorbide *a*-II of the Marine Pollution Laboratory chromatograms is shown to be pyropheophorbide *a* free acid. This was confirmed by analyses of selected duplicate samples using the FAU Organic Geochemistry Laboratory methodology (see Louda et al. 1998, 2000, 2002), including known pyropheophorbide *a* standardization (Louda, J. W. and P. Monghkonsri, unpubl. data). Chl *a* and derivatives isolated from the algae cultures and experimental incubations of (*C. gracilis* and *N. oculata*) are given as Tables 1 and 2, respectively.

C. gracilis—Figure 1 shows representative chromatograms for Chl *a* and Chl *a* derivatives for (Fig. 1a) the initial culture (t_0) of *C. gracilis*, (Fig. 1b) after 4 weeks, and (Fig. 1c) after 24 weeks of incubation with added microorganisms in anoxic conditions.

Figure 2 displays the data for the total chlorophyll plus derivatives yield (Fig. 2a) and the molar percentages (Fig. 2b) of the pigments isolated from *C. gracilis* in the original culture (t_0) and subsequent experimental conditions.

Chloropigment *a* concentration in the original stock culture was $3.35 \text{ nmol mL}^{-1}$ and represents the initial (t_0)

concentration for all experimental incubations with this species.

The greatest decrease of chloropigment *a* content was observed for samples incubated with the addition of microorganisms and incubated in oxic conditions. After 2 weeks of incubation under these conditions, the chloropigment *a* content was only $1.16 \text{ nmol mL}^{-1}$, and after 24 weeks, pigments were below detection limits ($<0.01 \text{ nmol mL}^{-1}$).

Considerable changes were also observed in the material incubated with microorganisms under anoxic conditions, but chloropigment *a* decrease was much slower. After 2 weeks of incubation, chloropigment concentration was $3.30 \text{ nmol mL}^{-1}$, $2.47 \text{ nmol mL}^{-1}$ after 4 weeks, $1.55 \text{ nmol mL}^{-1}$ after 8 weeks, and $0.49 \text{ nmol mL}^{-1}$ after 24 weeks. In the samples incubated without the addition of microorganisms, the changes in chloropigment *a* contents were much lower. In anoxic conditions, following a 24 week incubation, chloropigment *a* concentration decreased only slightly and was $2.71 \text{ nmol mL}^{-1}$. Whereas in oxic conditions, after the first 8 weeks, the chloropigment *a* content in the samples changed slightly, and only after 24 weeks was a distinct decrease, to about $1.80 \text{ nmol mL}^{-1}$, in the chloropigment *a* content observed.

The molar percentages of individual pigments *a* for each of the various incubation conditions (Fig. 2b) reveal the strong influence of both oxygen and, especially, microorganisms on the course of pigment alterations. The initial culture (t_0) of *C. gracilis* contained Chl *a* (73.7%), Chl *a* allomers (11.5%), Chl *a'* (4.0%), and pheophytin *a* (10.8%). Such a distribution is often found in fresh cultures containing actively growing and early senescent cells (cf. Louda et al. 1998, 2002). Pyropheophytin *a* and steryl chlorin esters did not occur in the starting material. Oxic and anoxic incubations without the addition of microorganisms lead to pheophytin *a* and pheophytin-*a'* as the main derivatives. The highest amounts of these derivatives occurred in anoxia. As the total molar content of Chl *a* plus derivatives decreased by about half after 24 weeks in the oxic case, it may be surmised that pigment was being lost by oxidatively destructive mechanisms, that is, loss of pigment to lower-molecular-weight colorless compounds (LMWCC; see Baker and Louda 2002; cf. Hendry et al. 1987; Matile et al. 1996). After 24 weeks in anoxia, pheophytin *a* content was $1.33 \text{ nmol mL}^{-1}$, representing about half (48.8%) of all chloropigments *a*. In the presence of oxygen, pheophytin *a* was only about 20% of all chloropigments *a* after 8 and 24 weeks of the experiment.

Under anoxic conditions without microorganisms, some pheophorbide-I (i.e., pheophorbide *a*) was formed and, after 24 weeks, represented 6.7% of the total chloropigments *a*. Incubations of *C. gracilis* in the presence of microorganisms led to large amounts of secondary diagenesis products being formed. These were not formed in the samples incubated without microorganisms.

Under anoxia with added microorganisms (Fig. 2b), the formation of large amounts of pheophorbide *a*-II (i.e., pyropheophorbide *a*), pyropheophytin *a*, and steryl derivatives was found. After 2 weeks of incubation, pyropheo-

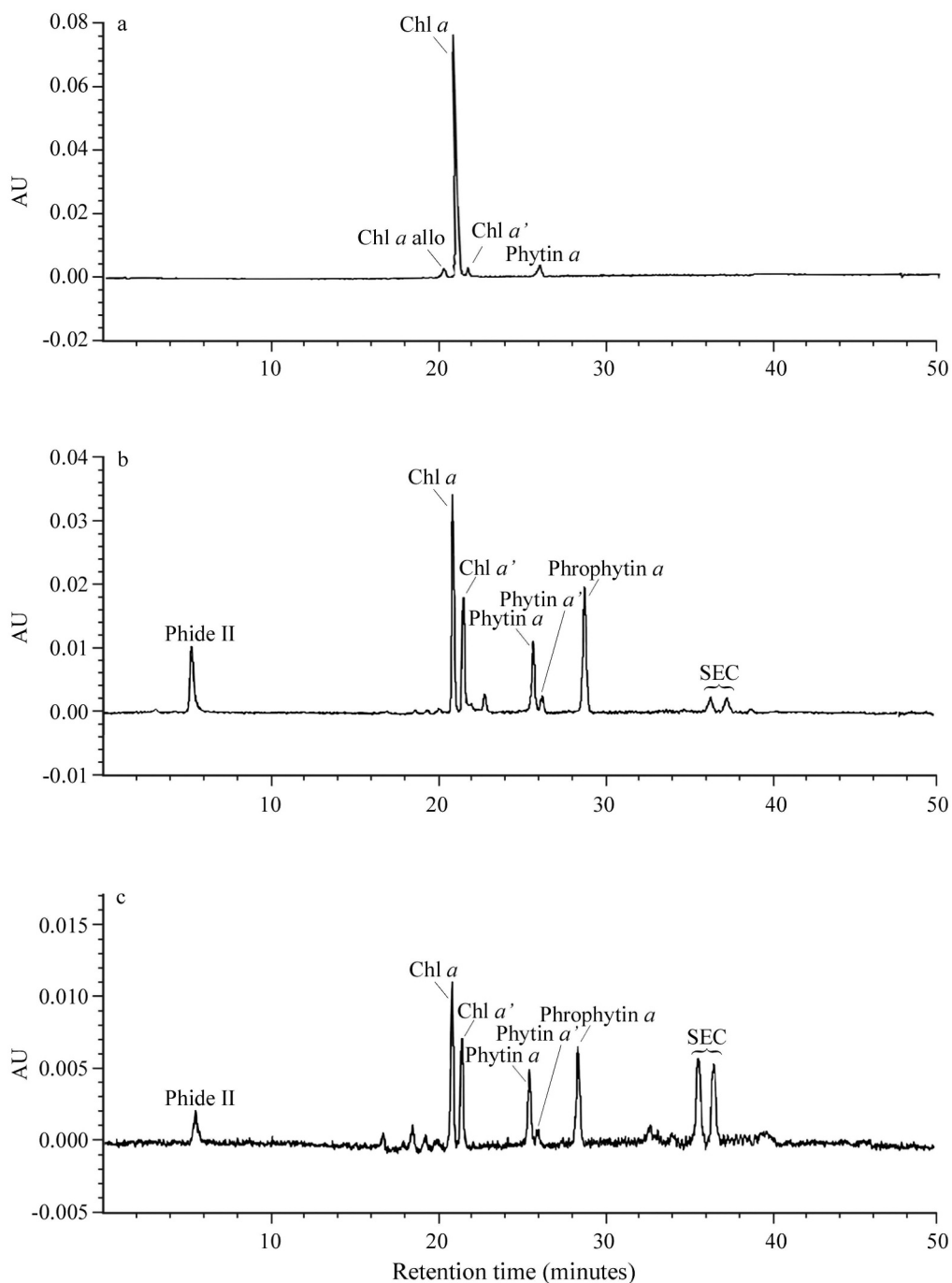


Fig. 1. Example of chromatograms ($\lambda = 660$ nm) for Chl *a* and Chl *a* derivatives in *C. gracilis* incubated with added microorganisms under anoxic conditions: (a) the initial culture (t_0); (b) after 4 weeks; and (c) after 24 weeks.

phorbide *a* content was $0.82 \text{ nmol mL}^{-1}$ (25.0% of the total chloropigments *a*), pyropheophytin *a* content was $1.04 \text{ nmol mL}^{-1}$ (31.7%), and the sum of steryl chlorin esters was $0.11 \text{ nmol mL}^{-1}$ (3.4%). In the next weeks, the pyropheophorbide *a* and pyropheophytin *a* content decreased, while the percentage of the steryl chlorin esters increased and after 24 weeks of incubation was 32.9% of the total chloropigments *a*. The change from pyropheophorbide *a* and pyropheophytin *a* to steryl chlorin esters was found to be quite direct when the sum of the pyro-

phopigments and the sum of steryl chlorin esters are individually plotted against the time of incubation (Fig. 3). The regression of each trend gave R^2 values in excess of 0.98, and the phenomenology indicates not only the esterification of pyropheophorbide *a* with sterols but either a transesterification exchange of phytol for sterol in pyropheophytin *a* or a concerted deesterification/re-esterification in the latter case. Examination of total chloropigments *a* (Fig. 2a) reveals that while the pyro-phopigments were being converted to steryl chlorin esters, total pigment

Table 1. Chlorophyll *a* and its major derivatives content (nmol mL⁻¹) in the algae cultures (*C. gracilis*) incubated under different conditions.*

Conditions	Time of incubation (weeks)	Chlide <i>a</i>	Chl <i>a</i> allo	Chl <i>a</i>	Chl <i>a'</i>	Phytin <i>a</i>	Phytin <i>a'</i>	Phides I	Phide II	Pyrophytin <i>a</i>	SCEs
—	0	<d.l.†	0.38 0.049	2.47 0.064	0.14 0.015	0.36 0.008	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
Nitrogen, darkness, room temperature	2	0.01 0.003	0.23 0.021	1.93 0.179	0.07 0.004	0.79 0.172	0.07 0.029	<d.l.	<d.l.	<d.l.	<d.l.
	4	<d.l.	0.24 0.018	1.38 0.118	0.15 0.011	0.94 0.115	0.03 0.009	0.05 0.024	—	<d.l.	<d.l.
	8	<d.l.	0.12 0.029	1.08 0.093	0.19 0.005	1.16 0.210	0.21 0.036	0.07 0.003	<d.l.	<d.l.	<d.l.
	24	<d.l.	0.05 0.019	0.83 0.046	0.11 0.015	1.33 0.152	0.21 0.005	0.18 0.037	<d.l.	<d.l.	<d.l.
Nitrogen, darkness, room temperature + microorganisms	2	<d.l.	0.04 0.027	0.54 0.177	0.06 0.005	0.58 0.054	0.10 0.019	<d.l.	0.82	1.04	0.11
	4	<d.l.	0.01 0.009	0.56 0.088	0.18 0.149	0.22 0.067	0.09 0.002	<d.l.	0.058	0.073	0.057
	8	<d.l.	0.01 0.000	0.29 0.096	0.12 0.053	0.13 0.045	0.06 0.019	0.02	0.67	0.59	0.15
	24	<d.l.	0.01 0.001	0.11 0.034	0.05 0.015	0.05 0.001	<d.l.	<d.l.	0.21	0.234	0.080
Air, darkness, room temperature	2	<d.l.	0.01 0.001	2.51 0.247	<d.l.	0.28 0.072	0.08 0.000	<d.l.	<d.l.	<d.l.	<d.l.
	4	<d.l.	0.05 0.005	2.10 0.093	<d.l.	0.43 0.041	0.08 0.017	<d.l.	<d.l.	<d.l.	<d.l.
	8	<d.l.	0.24 0.000	2.21 0.115	0.17 0.005	0.70 0.091	0.03 0.039	<d.l.	<d.l.	<d.l.	<d.l.
	24	<d.l.	0.43 0.020	0.90 0.004	0.14 0.031	0.33 0.120	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
Air, darkness, room temperature + microorganisms	2	<d.l.	0.02 0.006	0.28 0.026	<d.l.	0.22 0.025	0.04 0.004	<d.l.	0.36	0.18	0.04
	4	<d.l.	0.01 0.000	0.01 0.001	<d.l.	0.03 0.001	<d.l.	<d.l.	0.018	0.021	0.005
	8	<d.l.	<d.l.	0.01 0.001	<d.l.	0.01 0.001	<d.l.	<d.l.	0.07	0.01	0.01
	24	<d.l.	<d.l.	0.01 0.001	<d.l.	0.01 0.000	<d.l.	<d.l.	0.004	0.002	0.002
								0.02	0.01	<d.l.	
								0.005	0.000	—	
								<d.l.	<d.l.	<d.l.	

* Chlide *a*, chlorophyllide *a*; Chl *a* allo, chlorophyll *a* allomer; Chl *a'*, chlorophyll *a* epimer; Phytin *a*, pheophytin *a*; Phytin *a'*, pheophytin *a* epimer; Phides I, pheophorbides I; Phide II, pheophorbide II (pyropheophorbide *a*); Pyrophytin *a*, pyropheophytin *a*; SCEs, sum of steryl chlorins.

† <d.l., below detection limit.

Table 2. Chl *a* and its major derivatives content (nmol mL⁻¹) in the algae cultures (*N. occulata*) incubated under different conditions.*

Conditions	Time of incubation (weeks)	Chlide <i>a</i>	Chl <i>a</i> allo	Chl <i>a</i>	Chl <i>a'</i>	Phytin <i>a</i>	Phytin <i>a'</i>	Phides I	Phide II	Pyrophytin	
										<i>a</i>	SCEs
—	0	0.03	0.07	4.98	0.09	0.21	0.03	<d.l.†	<d.l.	<d.l.	<d.l.
Nitrogen, darkness, room temperature	SD	0.007	0.011	0.026	0.047	0.081	0.037	—	—	—	—
	2	0.20	0.23	4.10	0.30	0.46	0.01	<d.l.	<d.l.	<d.l.	<d.l.
	SD	0.043	0.024	0.083	0.048	0.090	0.002	—	—	—	—
	4	0.05	0.20	3.96	0.19	0.49	0.03	<d.l.	<d.l.	<d.l.	<d.l.
Nitrogen, darkness, room temperature + microorganisms	SD	0.027	0.063	0.203	0.005	0.067	0.004	—	—	—	—
	8	0.02	0.13	2.89	0.25	0.84	0.08	0.16	<d.l.	<d.l.	<d.l.
	SD	0.007	0.040	0.126	0.019	0.088	0.020	0.063	—	—	—
	24	<d.l.	0.14	1.90	0.36	1.05	0.20	0.36	<d.l.	<d.l.	<d.l.
Nitrogen, darkness, room temperature + microorganisms	SD	—	0.006	0.227	0.054	0.141	0.058	0.043	—	—	—
	2	0.12	0.15	4.06	0.21	0.13	<d.l.	<d.l.	<d.l.	<d.l.	0.06
	SD	0.010	0.010	0.072	0.061	0.008	—	—	—	—	0.011
	4	0.05	0.40	3.62	0.06	0.50	<d.l.	<d.l.	<d.l.	<d.l.	0.12
Air, darkness, room temperature	SD	0.004	0.049	0.067	0.005	0.029	<d.l.	<d.l.	<d.l.	<d.l.	0.006
	8	<d.l.	0.39	2.48	0.23	0.80	—	—	—	—	0.14
	SD	—	0.020	0.042	0.030	0.093	0.006	0.06	0.19	0.11	0.14
	24	<d.l.	0.02	0.50	0.14	1.10	0.31	0.36	0.046	0.002	0.032
Air, darkness, room temperature	SD	—	0.034	0.064	0.016	0.036	0.002	0.111	0.194	0.005	0.018
	2	<d.l.	0.08	4.12	0.04	0.18	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
	SD	—	0.014	0.197	0.007	0.041	—	—	—	—	—
	4	<d.l.	0.07	2.83	0.05	0.55	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
Air, darkness, room temperature + microorganisms	SD	—	0.011	0.219	0.017	0.622	—	—	—	—	—
	8	0.01	0.61	2.88	0.25	0.66	0.03	0.11	<d.l.	<d.l.	<d.l.
	SD	0.018	0.140	0.506	0.040	0.165	0.045	0.023	—	—	—
	24	<d.l.	1.17	1.04	0.14	0.18	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
Air, darkness, room temperature + microorganisms	SD	—	0.026	0.024	0.035	0.067	—	—	—	—	—
	2	<d.l.	0.03	1.49	0.05	0.25	0.04	0.01	0.08	0.07	<d.l.
	SD	—	0.010	0.203	0.013	0.033	0.005	0.015	0.007	0.007	<d.l.
	4	<d.l.	0.02	0.78	0.01	0.12	0.01	0.13	<d.l.	0.03	0.01
Air, darkness, room temperature + microorganisms	SD	—	0.012	0.120	0.001	0.014	0.004	0.004	—	0.003	0.002
	8	<d.l.	0.02	0.14	0.01	0.14	0.02	<d.l.	0.46	0.04	<d.l.
	SD	—	0.001	0.004	0.000	0.006	0.001	—	0.002	0.002	<d.l.
	24	<d.l.	<d.l.	<d.l.	<d.l.	0.02	<d.l.	<d.l.	0.01	<d.l.	<d.l.
	SD	—	—	—	—	0.001	—	0.001	0.001	—	—

* Chlide *a*, Chlorophyllide *a*; Chl *a* allo, chlorophyll *a* allomer; Chl *a*, chlorophyll *a*; Chl *a'*, chlorophyll *a* epimer; Phytin *a*, pheophytin *a*; Phytin *a'*, pheophytin *a* epimer; Phides I, pheophorbides I; Phide II, pheophorbide II (pyropheophorbide *a*); Pyrophytin *a*, pyropheophytin *a*; SCEs, sum of steryl chlorins.

† <d.l., below detection limit.

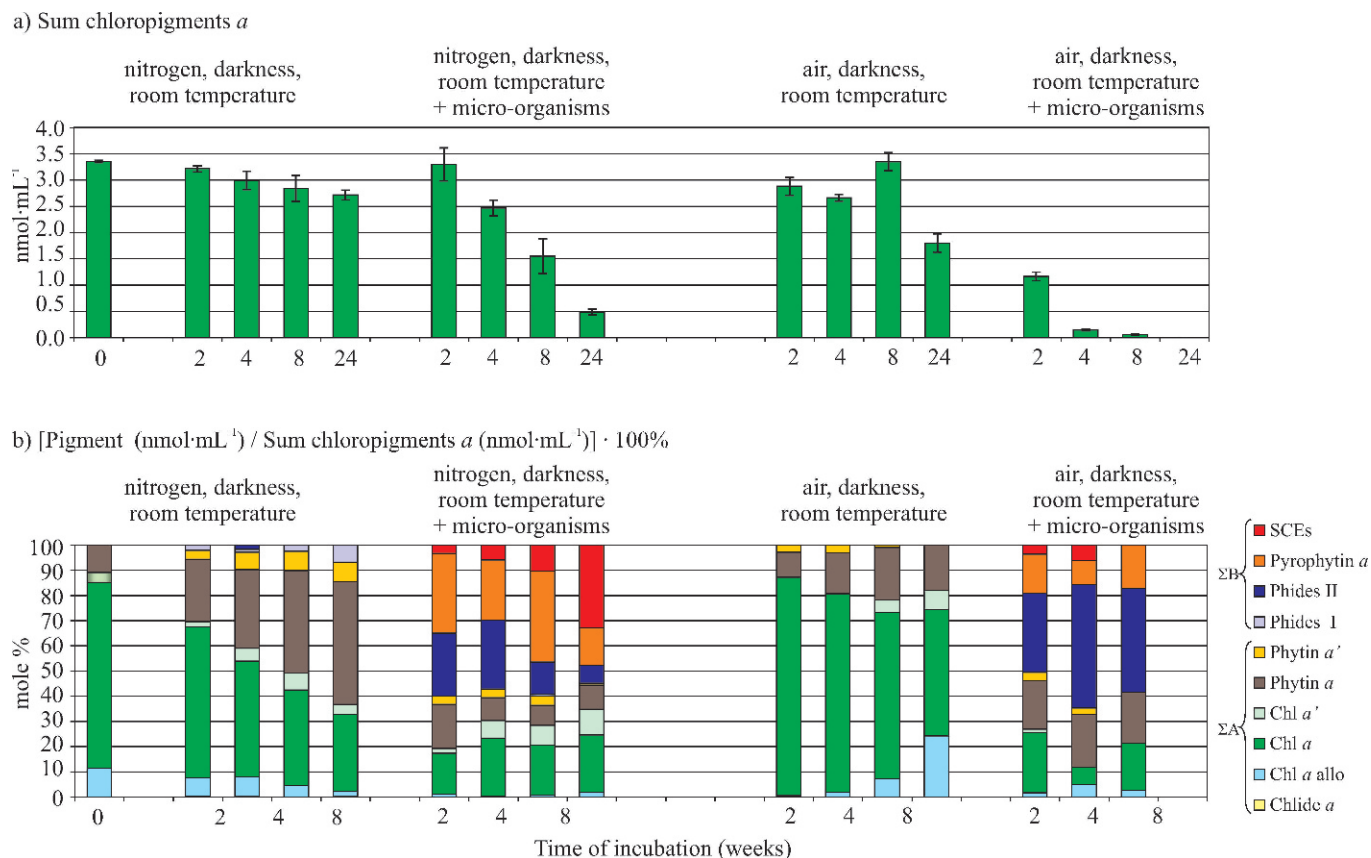


Fig. 2. Changes of chloropigments *a* in the algae *C. gracilis* incubated with and without microorganisms under oxic or anoxic conditions: (a) sum of chloropigments *a* (Σ Chl *a*) with standard deviation, $n = 2 \div 3$; (b) percentage of pigments in the sum of chloropigments *a* where ΣA is Chl *a* plus initial products and ΣB is the sum of secondary products.

yield decreased significantly. Thus, the idea that conversion of pyropheophorbide *a*—and now pyropheophytin *a*—to steryl chlorin esters acts to slow diagenetic destruction and more or less preserve the phorbide nucleus (cf. King and Repeta 1991; Talbot et al. 2000) appears valid.

In the oxic conditions with microorganisms added, the greatest percentage of the secondary products was pheo-

phorbide II (i.e., pyropheophorbide *a*). However, after 24 weeks, all Chl *a* derivatives had undergone total destruction (Fig. 2b).

N. oculata—Total chloropigment *a* concentration in initial culture was 5.4 nmol mL⁻¹ (Fig. 4a).

The greatest loss of total chloropigments *a* was in oxic conditions with added microorganisms, producing decreases after only 2 weeks to 2.01 nmol mL⁻¹ and after 24 weeks to 0.03 nmol mL⁻¹. Under oxic conditions without microorganisms, the decrease of chloropigment *a* amount was much more slower than in the presence of microorganisms and after 24 weeks 2.53 nmol mL⁻¹, or about half of the original complement remained.

Under anoxic conditions with and without microorganisms, total chloropigment *a* content was about 4 nmol mL⁻¹ after 24 weeks.

Chl *a* dominated and accounted for 92.3% the total chloropigments *a* (Fig. 4b) in the starting culture of *N. oculata*. The remaining chloropigments were chlorophyllide *a* (0.6%), Chl *a* allomers (1.2%), Chl *a*-epimer (1.6%), pheophytin *a* (3.8%), and pheophytin *a*-epimer (0.5%). Secondary products of diagenesis products of Chl *a* were not present in the initial culture (t_0).

As was the case with *C. gracilis*, the major Chl *a* derivative resulting from the incubation of *N. oculata*

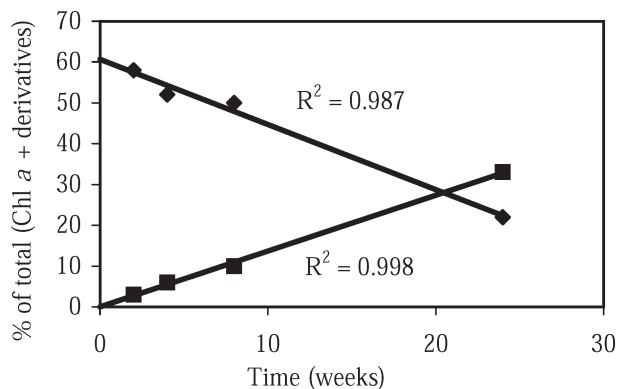


Fig. 3. Plot of the molar percentages of pyropheopigments *a* (sum of pyropheophorbide *a* plus pyropheophytin *a*: diamonds) and steryl chlorin esters (steryl phorbide esters: squares) versus time for *C. gracilis* incubated in anoxic conditions with added microorganisms.

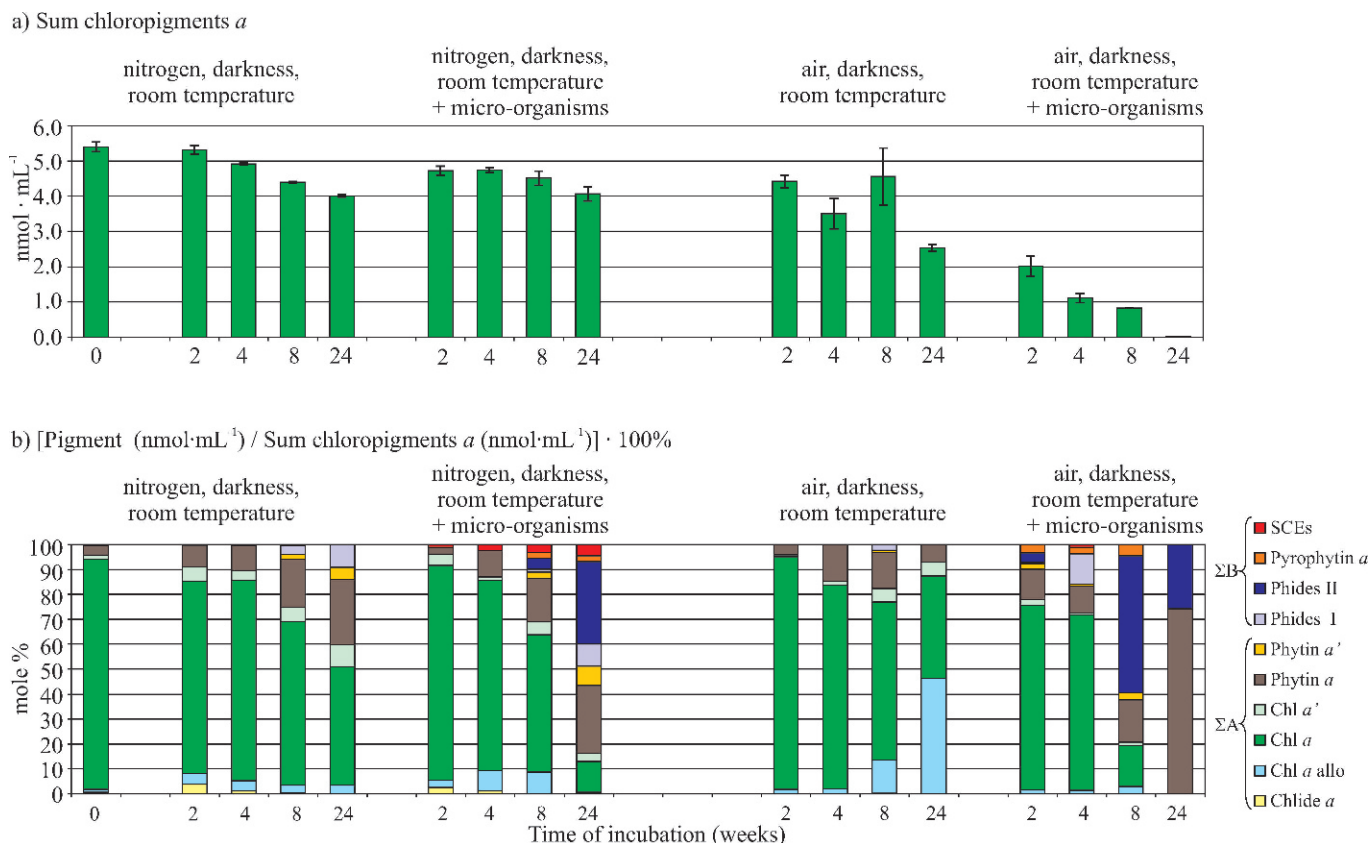


Fig. 4. Changes of chloropigments *a* in the algae *N. oculata* incubated with and without microorganisms under oxic or anoxic conditions: (a) sum of chloropigments *a* (Σ Chl *a*) with standard deviation, $n = 2 \div 3$; (b) percentage of pigments in the sum of chloropigments *a* where ΣA is chlorophyll *a* plus initial products and ΣB is the sum of secondary products.

without microorganisms was pheophytin *a*. Following incubation for 24 weeks under anoxic conditions (Fig. 4b), the pheophytin *a* percentage was 26.1% of the total chloropigments *a*. Smaller amounts of pheophorbide *a* (-I) were formed and amounted to about 9.1% after 24 weeks.

Under oxic conditions without microorganisms, the pheophytin *a* and pheophorbide *a* (-I) content was considerably lower, and Chl *a* allomers were formed. After 24 weeks of incubation, Chl *a* allomers formed just under 50% of the total chloropigments *a* (Fig. 4b). In *C. gracilis* (Fig. 2b), 24 weeks of oxic conditions without added microorganisms yielded about 25% Chl *a* allomers.

Both anoxic and oxic cultures with added microorganisms led to the formation of considerable amounts of derivatives of the secondary products (ΣB) category (Fig. 4b). The main component of secondary products was pyropheophorbide *a* (-II), which increased to 1.35 nmol mL⁻¹, or about 33.1% of the total chloropigments *a*. Pyropheophytin *a* and the steryl chlorin esters were also formed but in much lower quantities. After 24 weeks, these comprised 2.5% and 4.4% of the total chloropigments *a*, respectively. Additionally, oxic incubation with microorganisms led to the formation of pyropheophorbide *a* (-II), which, after 8 weeks, comprised 55.1% of the total chloropigments *a*. Chloropigments *a* of *N. oculata* cultures incubated under oxic conditions with

added microorganisms were nearly totally destroyed after 24 weeks.

Discussion

Total chloropigments *a* contents for both species (Figs. 2a, 4a) decreased much more rapidly with added microorganisms (Figures 2a, 4a) than without. Repeated-measure analysis of variance indicated significance differences between chloropigment *a* content in the cultures with and without added microorganisms. In the case of the anoxic incubation of the diatom *C. gracilis*, statistically significant differences ($p < 0.01$) between chloropigments *a* content in the control and the culture with microorganisms occurred after 8 weeks of incubation, while under oxic conditions this had already occurred after only 2 weeks.

In the case of the anoxic incubation of the picoplankton species *N. oculata*, degradation was considerably slower, and no distinct differences between chloropigments *a* content in the control and the culture with microorganisms were observed. However, under oxic conditions, significant differences in the extent of pigment degradation had occurred as early as 2 weeks into the overall incubation experiments.

During examination of the Chl *a* derivatives formed in different conditions, one may notice that both in the control cultures (without microorganisms) and in those with

microorganisms, significant changes have occurred, and these are somewhat different for each species. In the case of *C. gracilis*, the changes proceeded more quickly, while decomposition of pigments in cultures of *N. oculata* was much slower. These differences may be caused from different cell structure of diatoms and eustigmatophytes. In the control cultures, pheophytin *a* and its epimer, plus smaller amounts of pheophorbide *a* (-I), were formed under anoxia. However, under oxic conditions, the formation of considerable amounts of Chl *a* allomers was observed. This is in agreement with other reported studies on the influence of physicochemical factors on decomposition of Chl *a*, in cultures of diatom *Cyclotella meneghiniana*, and in the cyanobacteria *Anabaena variabilis* (Kowalewska and Szymczak 2001; cf. Spooner et al. 1994b; Louda et al. 1998).

In the cultures with microorganisms, Chl *a* derivatives were formed that did not occur in the control (i.e., no added microorganisms) experiments. These included secondary products (ΣB) of Chl *a*. Specifically, these products were pyropheophorbide *a* (-II), pyropheophytin *a*, and the steryl chlorin esters. The secondary products formed both under oxic and anoxic conditions but in different percentages, relative to total chloropigments *a* (Figs. 2b, 4b). The relative amounts of the secondary derivatives formed under oxic conditions were considerably smaller. Primarily pyropheophorbide *a* dominated with lower percentages of pyropheophytin *a* and steryl chlorin esters. This agrees well with the observations by Spooner et al. (1994a), in which mainly pheophorbides *a* resulted from the decomposition of the marine diatom *Thalassiosira weissflogii* by oxic bacteria. However, those authors also stated that bacteria were not responsible for formation of these pigments, as the same derivatives were formed as a result of decomposition of algae by the cellular enzymes. In the present study, in cultures incubated under anoxia with microorganisms, pyropheophytin *a* and steryl chlorin esters exhibited increased percentages within the total chloropigments *a*. This is a very interesting observation since it has been reported that the steryl derivatives are products of zooplankton grazing (Harradine et al. 1996; King and Wakeham 1996; Talbot et al. 2000). The formation of pyropheophytin *a* as well as small quantities of mesopyropheophorbides have previously been reported from anoxic incubation experiments (Spooner et al. 1995).

Here, we suggest that it is likely the inquilant microbiota of the zooplankton gut, rather than the grazers own digestive enzymes, that actually affects these changes. That is, it is well documented that intestinal bacteria perform a variety of esterifications, transesterifications, and complexation reactions with dietary and metabolic steroidal compounds. For example, human microflora are documented as transforming sulfolithochloate into four different esters (palmitoyl, palmitoleyl, stearyl, and oleyl) of isolithochloate (Kelsey et al. 1980), and carboxyl groups are esterified with cholate by saccharolytic *Bacteroides* spp. (Endenharder and Hammann 1985).

Statistical analysis confirmed that, in the case of cultures incubated with a microbial inoculum, the presence of oxygen exhibited a great influence on chloropigment *a* destruction in both *C. gracilis* and *N. oculata*.

Based on the present study, we conclude that microorganisms play a considerable role in the destruction and diagenetic pathways of Chl *a* cycling in the sea. Chl *a* destruction is much more rapid under oxic conditions. Under anoxic conditions, pyropheophytin *a* and steryl chlorin esters can become quite significant secondary products, whereas they are very minor products in the presence of oxygen. These observations provide an explanation of why the pyropheophytin *a* and steryl chlorin esters are abundant in anoxic marine sediments, such as in the Baltic (Kowalewska 2005) and Black (King and Repeta 1991; Pearce et al. 1993, 1998) seas. The activities of anaerobic to facultatively aerobic bacteria in both sediments and, by inference, grazer digestion processes (e.g., microzooplankton gut and fecal pellets) are concluded as leading to steryl chlorin ester formation.

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