Influence of selected abiotic factors on the decomposition of chlorophylls

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Abstract

The paper presents the results of experiments to determine the influence of selected physico-chemical factors – oxygen, visible light and temperature – on the decomposition of (1) chlorophylls *a*, *b* and *c*, chlorophyll *a* derivatives and *β*-carotene in acetone solution, and (2) chlorophyll *a* and *β*-carotene in axenic cultures of the blue-green algae *Anabaena variabilis*. The results indicate that both in acetone extracts and in blue-green algae cultures these pigments were most sensitive to light and oxygen; temperatures of up to 25° C had no marked influence on these compounds. Under anoxia in acetone solution, the stability towards light decreased in the order chlorophyll *a*, chlorophyll *b*, chlorophylls *c*. Chlorophyll *a*, moreover, was less stable than its derivatives – phaeophorbides, phaeophytins, pyrophaeophytins and steryl chlorins – but more stable than *β*-carotene, in the last case also in the blue-green algae cultures. Decomposition of all the pigments proceeded mainly via the breakdown of the porphyrin macrocycle, since the decomposition products were not detected in the VIS range. On the basis of these experiments one can state that while light and oxygen may have a decisive direct influence on the distribution of chlorophylls and *β*-carotene in sediments, in the natural environment, temperatures of up to 25° C may have very little immediate effect.

1. Introduction

Algal pigments began to attract the closer attention of environmental scientists in the 1980s. This resulted from the development of HPLC techniques, which enabled even very slight differences in the chemical structure of such pigments to be observed (Jeffrey et al. 1997). Most of this work was related to pigments in the water column. It is only recently that pigments in sediments have aroused the growing interest of hydrologists as possible markers of environmental conditions (Jeffrey et al. 1997). Even then, however, the relevant environments have usually been lacustrine rather than marine (Steinman et al. 1998). Geochemists have described – though only qualitatively – the occurrence of a great variety of chlorophyll derivatives in natural samples of diverse origin, mainly products of late diagenesis in old sediments (e.g. Prowse & Maxwell 1991, Harris et al. 1995). Quantitative studies have also been carried out to elucidate chlorophyll defunctionalisation during the senescence of axenic batch cultures of algae, and the fate of the pigments during the storage of algae and their extracts in the dark and at low temperatures (e.g. Jeffrey et al. 1997, Louda et al. 1998). Some work has also been done on the quantitative influence of oxygen, light and temperature on selected pigments in disrupted unicellular algal cells and faecal pellets (Nelson 1993), and in sediments (Sun et al. 1993). All these papers report on the possible formation of different derivatives and show that pigment transformation is a highly complex process influenced by diverse hypothetical factors. The authors of these articles have often reached contradictory conclusions.

As a result, despite all the effort to explain the occurrence of pigments in sediments, more questions have arisen than have been solved. This is principally because the analysis of pigments in sediments is very difficult: apart from their comparatively low concentrations in the organic matrix, a matrix richer than that in the water column, they may be accompanied by a great variety of pigment derivatives, most of which are unstable. Derivative formation can be initiated by a number of factors and proceeds not only during cell senescence and after their disruption in the water column, but also in the sediments, after the algal debris has settled (Gillan & Johns 1980, Méjanelle et al. 1995, King & Wakeham 1996). Although these factors are assumed to be mainly biotic ones like enzymatic action, microbial attack and the grazing of different organisms (Welschmeyer & Lorenzen 1985, Nelson 1993, Harradine et al. 1996), abiotic factors such as temperature, light and oxygen are likely to be involved, too (Sun et al. 1993). Moreover, pigment artefacts are actually formed in course of sampling and analysis (Wright et al. 1997).

All this means that interpretation of the pigment data for sediments is not easy. Even so, HPLC 'fingerprints' of pigment extracts from southern Baltic sediments display a far greater similarity than we had expected, containing as they do only a few pigments, most of which are tetrapyrroles (Kowalewska 1997). The essential differences between the chromatograms of the samples from various locations are in the relative proportions of the major tetrapyrroles and the amounts in which they occur. Contrary to the common view that chlorophylls decompose immediately after cell death, these compounds are abundant not only in recent but also in old Baltic sediments, particularly in those formed during anoxia (Kowalewska 1997, Kowalewska et al. 1999). One might suppose that these pigments are extracted from living cells occurring on the seafloor, but in the case of deep sediments, up to 6 m below the seafloor, this is a very remote possibility. The major chlorophyll derivatives in both recent and old Baltic sediments are chlorins; of the porphyrins, only phaeophorbides and their steryl derivatives have been found in substantial amounts. Therefore, in order to gain an understanding of the processes exerting a major impact on the occurrence of particular pigments in sediments, their production or relative stability towards different factors, an investigation of the effect of physico-chemical factors like oxygen, light and temperature on the transformation of chlorophylls seemed an interesting first step to take. To do this, acetone extracts of both parent pigments and their derivatives were subjected to decomposition under controlled conditions in the laboratory and then analysed by HPLC in order to follow this process quantitatively. Additionally, chlorophyll *a* and *β*-carotene, the pigment accompanying chlorophyll *a* in almost all the algae, were decomposed in batch experiments. In order to determine whether the conclusions drawn from these experiments are applicable to the natural environment, a second series of experiments with axenic cultures of the blue-green alga *Anabaena variabilis* isolated from the Baltic Sea was carried out.

2. Experimental

Chlorophylls *a* and *b*, as well as *β*-carotene, were isolated from the leaves of higher plants, chlorophylls *c* from axenic laboratory cultures of *Cyclotella meneghiniana* and from phytoplankton samples collected in the Szczecin Lagoon during a diatom bloom. Phaeophytin *a*, phaeophorbides, pyrophaeophytin *a* and steryl chlorins were isolated from acetone extracts of deep Baltic sediments. After extraction of the wet material with acetone, the pigments were re-extracted to benzene in the acetone:benzene:water 15:1:10 (v/v/v) system. The pigments were then isolated by preparative thin layer chromatography (TLC) using a CAMAG–Linomat IV applicator,

silica gel (Merck Kieselgel 60 plates) and the acetone:hexane 20:35 (v/v) system (Kowalewska 1997). The gel containing the separated pigments was scratched off the plate, extracted with acetone and centrifuged. The supernatant was decanted and evaporated to dryness under a stream of argon and the sample was stored in the deep-freeze until analysis.

The pigment extracts were dissolved in acetone and aliquots were transferred to glass vials and stored under different conditions: 1 – in air, in the natural daylight:night cycle, at room temperature (∼25° C), 2 – in argon, in the natural daylight:night cycle, at room temperature, 3 – in argon, in the natural daylight:night cycle, at room temperature, 4 – in argon, in the dark, at room temperature, 5 – in argon, in the dark, in the refrigerator (2–3° C). The vials stored in argon were purged with argon and tightly stoppered. Additionally, 0.5 ml samples were analysed to obtain the initial concentrations of the pigments.

A culture of *A. variabilis* in Kraft's medium (v = 100 ml) was centrifuged (10 min, ∼2500 rpm) and the supernatant decanted. 100 ml of distilled water was added to the residue. 2 ml of the algal culture thus prepared was transferred to each vial. Four vials were treated as reference samples and analysed immediately after preparation; the others were kept under the following conditions: A – in argon, in light (natural daylight:night cycle), room temperature (∼25° C); B – in air, in the dark, at room temperature; C – in argon, in the dark, at room temperature; D – in argon, in the dark, in the refrigerator (2–3° C). Each type of experiment was done in triplicate. After 1, 2, 4 and 8 weeks one sample from each set was treated as follows. The vial contents were centrifuged (10 min, ∼2500 rpm) and the water decanted. The separated algae were extracted with acetone (1.5 ml acetone, 1 h in a refrigerator), after which the acetone extract was separated by centrifugation. The residue was washed with 0.5 ml of acetone and centrifuged again. The combined acetone fractions were evaporated under a stream of argon and kept in a deep-freeze until analysis, though for no longer than three days.

The light intensity during the pigment and algae experiments was measured with a phytophotometer (Sonopan, FF–01) at 09:00, 12:00 and 15:00 hrs. Pigment decomposition was followed with a high-performance liquid chromatography set (HPLC–DAD(FL) Knauer, Germany), a Chroma-Scope diode-array and Shimadzu RF–551 fluorescence detectors in the previously described acetone-water gradient system (Kowalewska 1997). A Merck Lichrospher 100 RP–18e column (250 × 4 mm, 5 μm) was used

with the same pre-column. The reproducibility of HPLC pigment determination for the same sample in two separate injections was better than 1%; in two separate experiments it was better than 5%.

3. Results and discussion

The decomposition of chlorophylls *a*, *b* and *c* under different conditions is presented in Fig. 1. In all three cases we observe a similar first-order reaction with respect to time of the particular chlorophylls to the factors applied. Oxygen and light have a very distinct impact on the decomposition of all the chlorophylls. This phenomenon is known (e.g. Nelson 1993). Unlike Nelson's experiments, however, where an artificial, high-intensity source of light was applied ($\sim$300 μE m^{-2} s^{-1}), the samples in the present work were irradiated by natural daylight. But this parameter is variable, so it was difficult to compare the rate of decomposition in the two separate experiments; moreover, the rate of decomposition itself varied during the experiment. The most unusual aspect is the only slight influence of temperature on the decomposition rate of all three compounds. Samples kept in a refrigerator and at room temperature decomposed at almost the same rate, regardless of whether oxygen was present or not (Fig. 1a). A similar observation was reported by Sun et al. (1993), though only for anoxia. Therefore, the distinct influence of temperature on the decomposition of chlorophyll *a* in sediments reported by Sun et al. (1993) must be due entirely to biotic factors, i.e. enzymatic and microbial activity.

To compare the relative stability of chlorophylls *a*, *b* and *c*, the isolated pigments were exposed to deterioration simultaneously, to ensure exactly the same conditions. The results of two such experiments under low and high light intensity and under argon are shown in Fig. 2. The stability of these compounds decreased in the order chlorophyll *a*, chlorophyll *b*, chlorophylls *c*. This is in agreement with the results obtained for Baltic sediments (Kowalewska 1997, Kowalewska et al. 1999), where chlorophylls *c* were usually much less abundant than chlorophyll *a*, though diatoms do make up a considerable share of the total phytoplankton biomass (Kowalewska et al. 1996 and the references therein). The comparison of the stabilities of chlorophyll *a* and β-carotene, a pigment accompanying chlorophyll *a* in almost all plant cells, also seems interesting (Fig. 3). The results indicate that β-carotene is more sensitive than chlorophyll *a* to oxygen and light. Depending on the light intensity, all the pigments decomposed during a period of no more than one week.

It is difficult to compare these data with the literature reports. For example, Gillan & Johns (1980) found that chlorophyll *a* was more stable towards light and oxygen than were chlorophyllides *c* (most probably

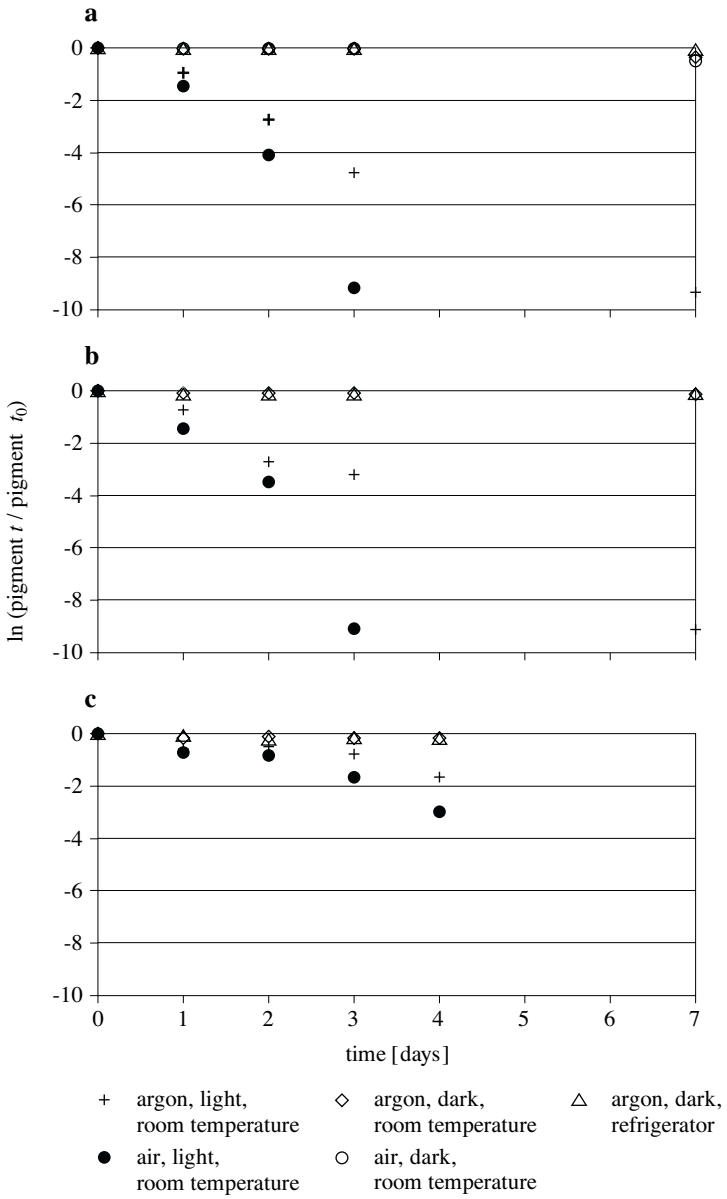

Fig. 1. Decomposition of chlorophylls: chl *a*, light intensity 2–210, mean ∼40 μE m^{-2} s^{-1} (a), chl *b* (2–21, mean ∼9 μE m^{-2} s^{-1}) (b), chls *c* (1–26, mean ∼9 μE m^{-2} s^{-1}) (c)

chlorophylls *c*). This investigation was by TLC in extracts of algal cultures isolated from intertidal sandy sediments, but the chloroform – methanol solvent used for isolating the pigments in that work must surely have contributed to chlorophyll decomposition. Klein et al. (1986) reported

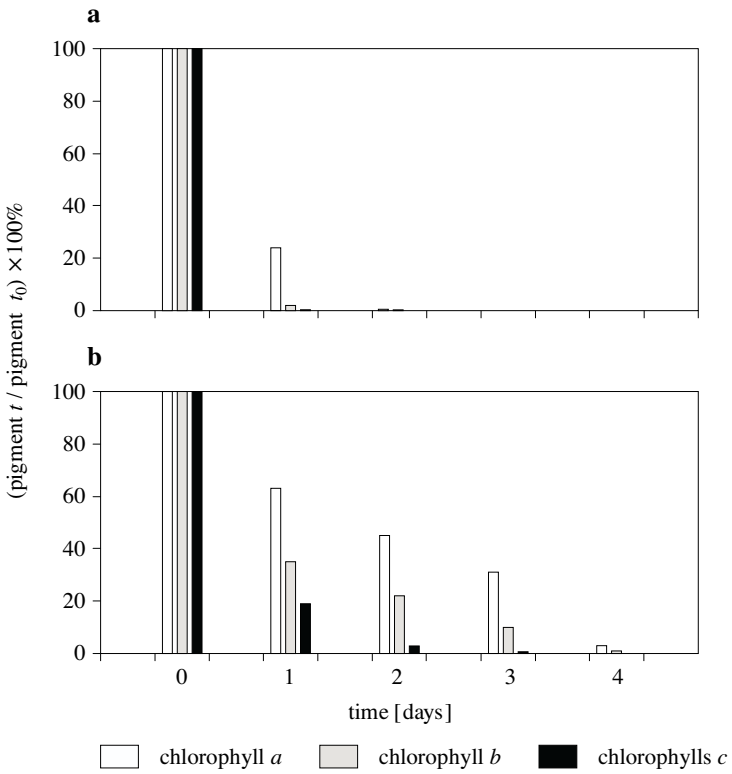

Fig. 2. Relative stability of chlorophylls *a*, *b* and *c* in argon, under different light regimes: 11–320, mean 120 μE m^{-2} s^{-1} (a), 3–22, mean 15 μE m^{-2} s^{-1} (b)

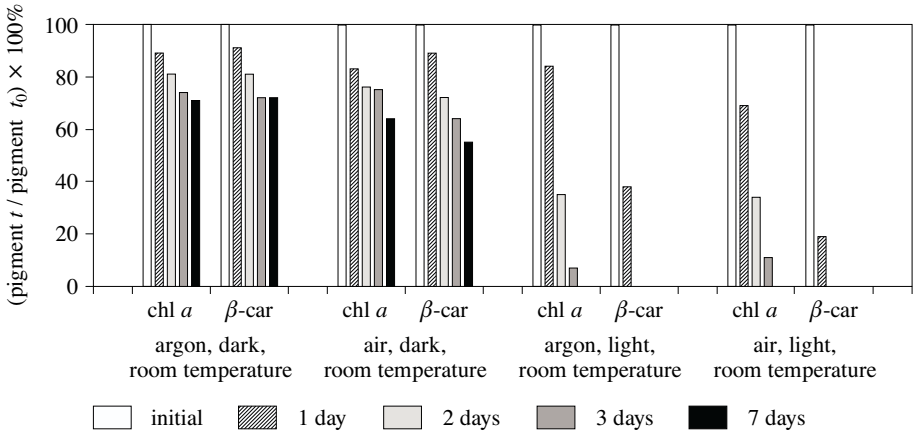

Fig. 3. Relative stability of chlorophyll *a* and β-carotene; light intensity 1–26, mean ~8 μE m^{-2} s^{-1}

a higher decomposition rate for chlorophylls *c* than chlorophyll *a*, though on the basis of laboratory experiments (Nelson 1993), other authors are of the opinion that chlorophylls *c* are more stable than chlorophyll *a* in the environment. The author of the last paper also reported the greater stability of *β*-carotene than of chlorophyll *a* extracted with 100% acetone; in 90% acetone he reports the opposite relationship.

HPLC chromatograms of extracts of Baltic bottom sediments reveal the presence of several pigment derivatives, but the most abundant tetrapyrroles are chlorophyll *a* derivatives: phaeophorbides, phaeophytin *a*, pyrophaeophytin *a* and steryl chlorins (Fig. 4). It should be underlined here that the quality of separation of modern HPLC sets is so high that even slight differences in chemical structure result in the appearance of a new peak in the chromatogram. Good examples here are the epimers (e.g. chl *a*′, phaeo′), which differ from the parent pigment solely in the opposite position in space of the hydrogen at C13. The experiments show that the main derivatives are more stable than chlorophyll *a*, but like the parent pigment, they are most sensitive to light and oxygen (Fig. 5). Klein et al. (1986) observed that phaeophytin *a* was in some degree more stable than chlorophyll *a* to the influence of light.

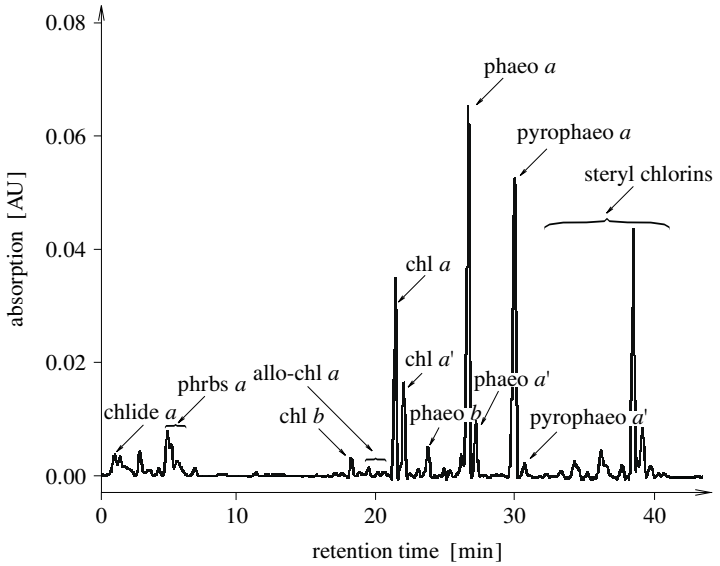

Fig. 4. HPLC chromatogram of an extract of Baltic sediment; wavelength $\lambda = 660$ nm

Taking the example of chlorophyll *a*, one sees that during decomposition in the presence of oxygen, only allo-compounds (oxygenated derivatives)

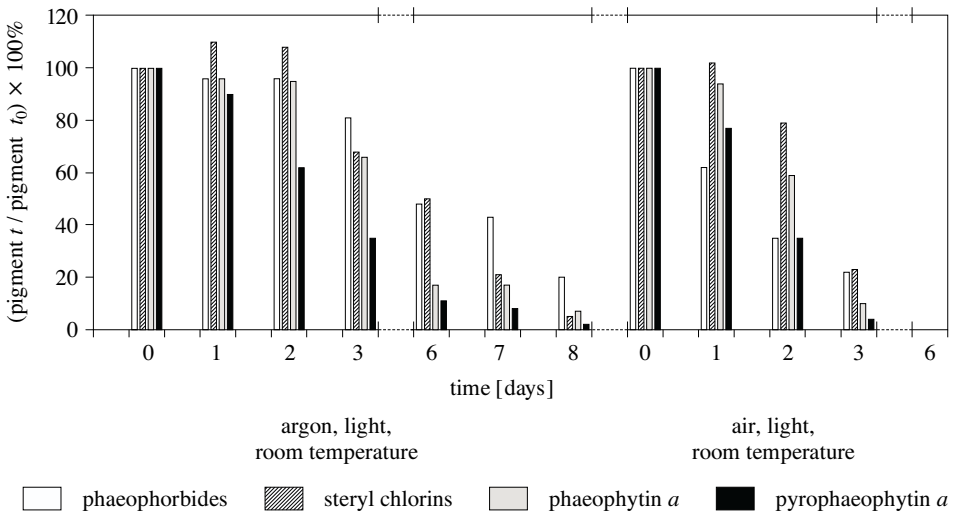

Fig. 5. Decomposition of chlorophyll *a* derivatives: phaeophorbides, phaeophytin *a*, pyrophaeophytin *a* and steryl chlorins; light intensity 5–70, mean 24 μE m^{-2} s^{-1}

of all the possible transformation products were formed and then only in small quantities (Fig. 6c). Moreover, under all the conditions applied chlorophyll *a* decomposed without any visible increase in the other major derivatives reported in the bottom sediment extracts (Figs. 6a, b). The experiments showed that decomposition of the other pigments studied under the conditions described also proceeded to colourless products. These results are in agreement with the observations of some other authors, e.g. the decomposition of chlorophyll *a* in sandy sediments (Gillan & Johns 1980). All this suggests that physical and chemical conditions have no direct influence on the formation of the major chlorophyll *a* derivatives in natural sediments, although they can affect the quantities in which the derivatives occur there, since they are responsible for the decomposition of those derivatives. Similarly, physical and chemical factors, especially light and oxygen, are bound to have a decisive impact on the distribution of the parent pigments (chlorophylls and β-carotene) in sediments.

In the axenic cultures of *A. variabilis* kept in a nutrient-depleted medium (distilled water) two contradictory effects were observed: on the one hand an increase in chlorophyll *a* and β-carotene due to the growth of the cultures, since at the beginning of the experiment these were living cultures of algae, and on the other, the decomposition with time of pigments in senescent and dying cells (Fig. 7). This is one of the reasons why pigment decomposition in the cultures proceeded much more slowly than in acetone solution. The overall decomposition rate of chlorophyll *a* and β-carotene

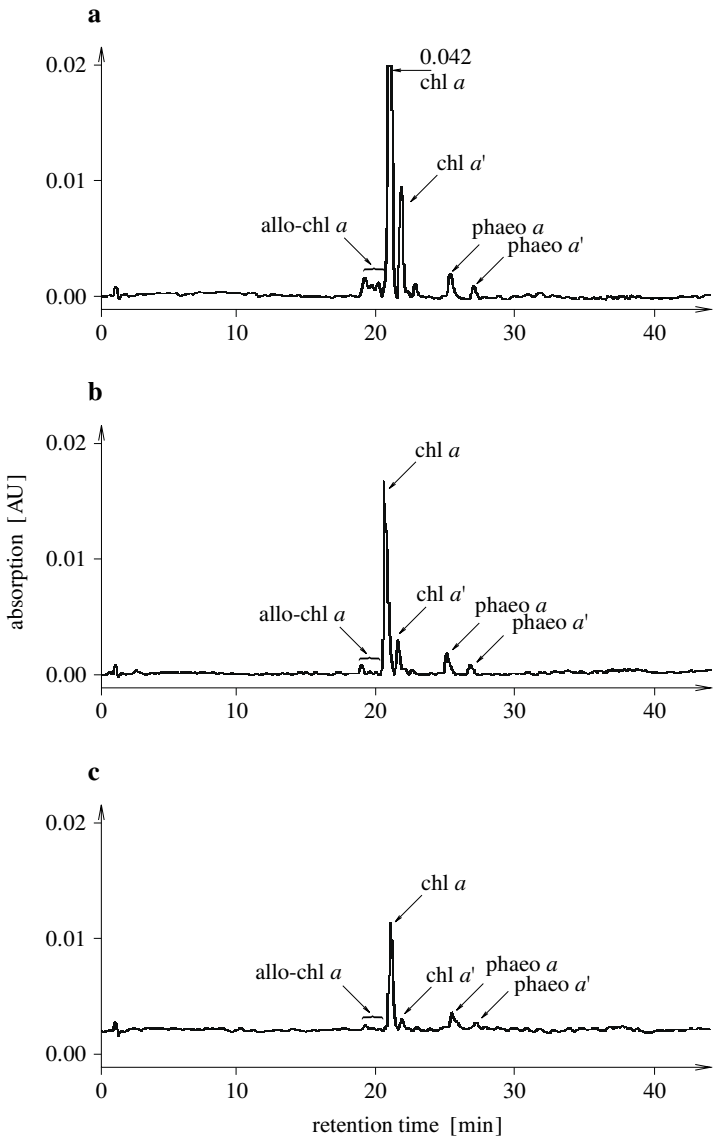

Fig. 6. HPLC chromatograms of chlorophyll *a* decomposition in light: initial solution chromatogram (a), in argon, light, room temperature (after 1 day) (b), air, light, room temperature (after 1 day) (c)

was the highest in air and in the dark (Fig. 7b). In light and in the presence of argon, the rate of culture growth was higher than that of its deterioration (Fig. 7a). In the dark and in the presence of argon both at room temperature and in the refrigerator, the chlorophyll *a* content remained constant, but β-carotene decreased visibly at room temperature after 8 weeks (Figs. 7c, d).

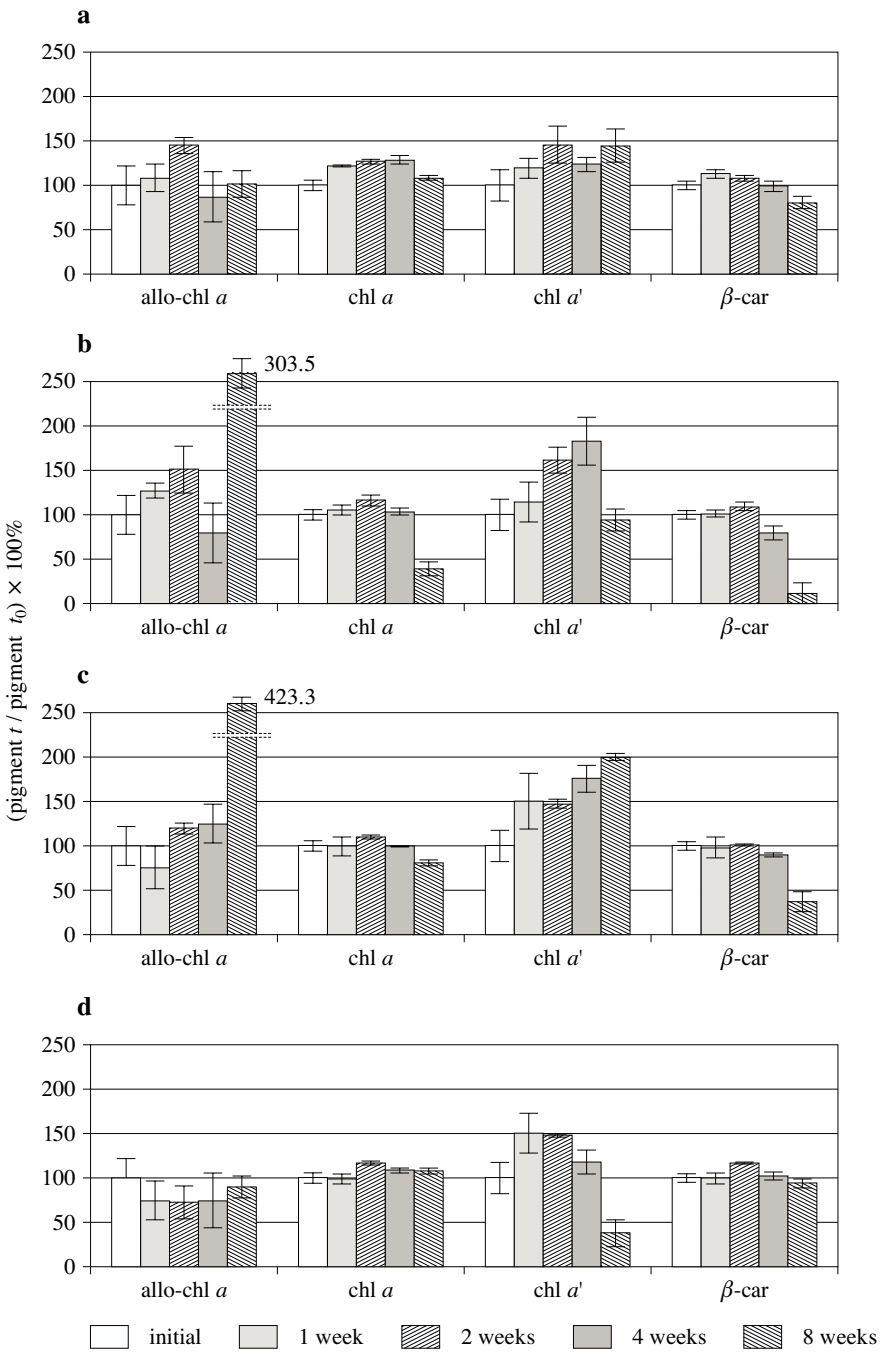

Fig. 7. Pigment content in the cultures of blue-green algae *A. variabilis*: argon, light (9–60, mean 18 μE m^{-2} s^{-1}), room temperature (a); air, dark, room temperature (b); argon, dark, room temperature (c); argon, dark, refrigerator (d)

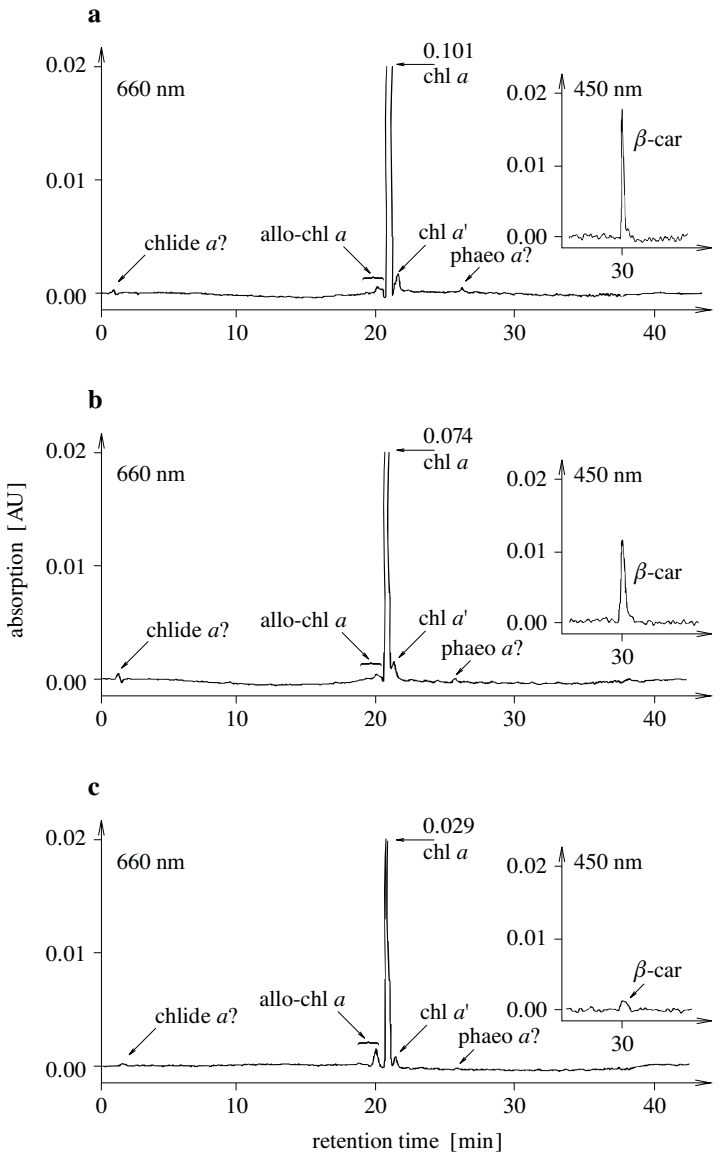

Fig. 8. HPLC chromatograms of chlorophyll *a* and β-carotene decomposition in cultures of the blue-green algae *Anabaena variabilis*: initial pigment chromatogram (a); argon, light, room temperature, 8 weeks (b); air, dark, room temperature, 8 weeks (c)

Very small amounts of allo- and epi- (chl a') chlorophyll *a* transformation products were formed (Figs. 7, 8). Trace amounts of derivatives eluting at the retention times of chlorophyllide *a* and phaeophytin *a* were formed as well. Unfortunately, these amounts were too small for their spectra

to be identified. As in the acetone pigment extracts, the decomposition of chlorophyll *a* and *β*-carotene proceeded with the formation of colourless products (Fig. 8).

4. Conclusions

- The pigments studied were most sensitive to light and oxygen; temperatures up to 25° C had no great direct effect on these compounds.
- In anoxic acetone solutions chlorophyll *a* displayed the greatest stability; chlorophyll *b* was the next stable, and the least stable were chlorophylls *c*.
- Chlorophyll *a* was less stable than its derivatives – phaeophorbides, phaeophytins, pyrophaeophytins and steryl chlorins, but more stable than *β*-carotene, in the last case both in acetone solution and in the algal cultures.
- Under the given conditions, decomposition of all the chlorophylls as well as their derivatives proceeded to colourless products.
- Light and oxygen both in the water column and during post – deposition may have a decisive direct influence on the distribution of chlorophylls, chlorophyll *a* derivatives and *β*-carotene in sediments.

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