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THE PIGMENT COMPOSITION OF *PHAEOCYSTIS ANTARCTICA* (HAPTOPHYCEAE) UNDER VARIOUS CONDITIONS OF LIGHT, TEMPERATURE, SALINITY, AND IRON[1]

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The pigment composition of *Phaeocystis antarctica* was monitored under various conditions of light, temperature, salinity, and iron. 19′-Hexanoyloxyfucoxanthin (Hex-fuco) always constituted the major light-harvesting pigment, with remarkably stable ratios of Hex-fuco-to-chl *a* under the various environmental conditions. Increased pigment-to-chl *a* ratios at low irradiance confirmed the light-harvesting function of Fucoxanthin (Fuco), 19′-Hexanoyloxy-4-ketofucoxanthin (Hex-kfuco), 19′-butanoyloxyfucoxanthin (But-fuco), and chl *c2* and *c3*. Increased pigment-to-chl *a* ratios at high irradiance, low iron concentrations, and to a lesser extent at high salinity confirmed the photoprotective function of diadinoxanthin, diatoxanthin, and ß,ß-carotene. Pigment ratios were not always according to expectations. The consistent increase in But-fuco/chl at high temperature, high salinity, and low iron suggests a role in photoprotection rather than in light harvesting. Low Hex-kfuco/chl ratios at high salinity were consistent with a role as light harvester, but the high ratios at high temperature were not, leaving the function of Hex-kfuco enigmatic. Dedicated experiments were performed to test whether or not the light-harvesting pigment Fuco could be converted into its structural relative Hex-fuco, and vice versa, in response to exposure to light shifts. Rapid conversions could not be confirmed, but long-term conversions cannot be excluded. New pigment ratios are proposed for chemotaxonomic applications. The ratios will improve pigment-based diagnosis of algal species in waters dominated by *P. antarctica*.

Key index words: **19′-Hexanoyloxyfucoxanthin; CHEMTAX; fucoxanthin; HPLC; irradiance; *Phaeocystis*; pigments; salinity; temperature**

Phaeocystis antarctica is a dominant microalgal species in the Southern Ocean and a key player in biogeochemical cycles (Verity et al. 2007). *P. antarctica* belongs to a genus that contains at least six species and over 12 strains (Medlin and Zingone 2007). The genus is characterized by a complex life cycle, including various life forms like flagellated cells as well as colonial forms that can vary in size and shape (Rousseau et al. 2007). These various life forms have been subject of a number of studies, with an emphasis on trophic relations (see Rousseau et al. 2007 and refs. therein). It is especially the colonial life form that escapes predation and that can develop into large algal blooms (Schoemann et al. 2005). Such blooms are mainly formed by the three species *Phaeocystis pouchetii, Phaeocystis globosa*, and *P. antarctica* (Schoemann et al. 2005). These species have overlapping temperature limits (Baumann et al. 1994, Schoemann et al. 2005), with *P. antarctica* dominating Antarctic waters. *P. pouchetii* is restricted to the Arctic waters, whereas *P. globosa* has a bi-polar distribution but is more abundant in temperate waters.

In the Southern Ocean, *P. antarctica* forms large blooms along the ice-edge and the continental margins (Schoemann et al. 2005). In spring, the alga also occurs in high concentrations in the upper layers of sea ice (Kennedy et al. 2012). Algal blooms may sequester large amounts of CO_2, and therewith *P. antarctica* plays an important role in the global carbon cycle (Schoemann et al. 2005). In addition, *P. antarctica* is the major producer of the climate active gas dimethyl sulfide in the Southern Hemisphere (Stefels 2000, Stefels et al. 2007). The role of the alga in biogeochemical cycles is particularly well studied in the Ross Sea, where large blooms of *P. antarctica* precede the spring diatom bloom (Arrigo et al. 1999, Smith et al. 2006).

In oceanography, the presence of algal species is often determined by pigment fingerprinting. The method is based on the use of so-called marker pigments that are assigned to specific algal species. The development of the program CHEMTAX (e.g.,

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Wright et al. 1996) further promoted the use of this technique. The application of CHEMTAX depends on reliable pigment signatures. Over the years, several studies have addressed the pigment composition of *P. antarctica*, *P. pouchetii*, and *P. globosa* (e.g., Wright and Jeffrey 1987, Vaulot et al. 1994, Zapata et al. 2004, Schoemann et al. 2005, Kropuenske et al. 2009). All species contain chl c_2, c_2-monogalactosyl diacylglyceride ester and chl c_3, 19′-butanoyloxyfucoxanthin (But-fuco), fucoxanthin (Fuco), 19′-hexanoyloxy-4-ketofucoxanthin (Hex-kfuco), 19′-hexanoyloxyfucoxanthin (Hex-fuco), diadinoxathin, and diatoxanthin (dt). For taxonomic purposes, Hex-fuco is generally used as the marker pigment for *Phaeocystis*, where the cellular Hex-fuco -content varies between the various *Phaeocystis* species. The ratio of Hex-fuco-to-chl *a* is generally higher in *P. antarctica* compared to *P. pouchetii*, with low values to nil in *P. globosa*. Moreover, very low Hex-fuco-to-chl ratios were recorded in some strains of *P. antarctica* (Zapata et al. 2004). Besides Hex-fuco, Fuco can be assigned to the presence of *Phaeocystis*, although the Fuco-content is more subject to variation. Within the species, both the Fuco and Hex-fuco-content are dependent on environmental conditions (Van Leeuwe and Stefels 1998, Moisan and Mitchell 1999, Schluter et al. 2000, Schoemann et al. 2005). In the extreme, in *P. antarctica*, Fuco was found to be absent under iron-limited conditions (Van Leeuwe and Stefels 1998).

Few studies exist that provide detailed information on the pigment signature of *P. antarctica* under varying environmental conditions. Pigments play a role in algal physiology through regulation of the cell's energy supply, and as membrane stabilizers. Light as the primary source of energy is the most important controlling factor of pigment synthesis. Whereas acclimation to low light will require the up-regulation of light-harvesting pigments, high-light acclimation will induce the synthesis of photoprotective pigments (Falkowski and LaRoche 1991). In polar regions, the algal pigment composition is furthermore controlled by extremes in salinity, temperature, and iron availability (Boyd 2002, Thomas and Dieckmann 2002, DiTullio et al. 2007). The latter conditions commonly create physiological stress, with ensuing consequences for pigment synthesis. Firstly, stress may induce the down-regulation of the light-harvesting capacity to avoid radical formation. Secondly, physiological acclimation of the thylakoid membranes may involve adaptation of pigment components as well (Lepetit et al. 2012). An increase in salinity and a decrease in temperature are known to result in a lower degree of saturation of membrane lipids (Morgan-Kiss et al. 2006), which can induce an increase in the content of photoprotective pigments (Havaux 1998).

The potential conversion of Fuco into Hex-fuco and vice versa also is relevant for chemotaxonomy. Van Leeuwe and Stefels (1998) were the first to discuss this conversion process and hypothesized that in response to exposure to high irradiance, the light-harvesting pigment Fuco could be converted into its structural relative Hex-fuco. This hypothesis has received support from various studies (e.g., Stolte et al. 2000), but is so far not resolved.

The role of Fuco as an intermediate between diadinoxanthin (dd), Hex-kfuco, But-fuco, and Hex-fuco (Fig. 1; after Lohr 2011) can be explored in more detail with the aid of various inhibitors. Dithiothreitol (DTT) is an inhibitor that blocks the conversion of diadino- into diatoxanthin (Fig. 1). If the interconversion of Fucos has a function in energy regulation (as hypothesized by Van Leeuwe and Stefels 1998), blocking xanthophyll cycling as it occurs in *P. antarctica* under conditions of high light (e.g., Van Leeuwe and Stefels 2007), might induce the conversion of Fuco into Hex-fuco. Alternatively, norflurazon (NF) can be applied as an inhibitor of de novo carotenoid synthesis (Fig. 1). If Fuco and Hex-fuco are interconvertible, exposure to low light in the presence of NF might stimulate the conversion of Hex-fuco into the light harvester Fuco. Exposure to high light might stimulate a reverse response.

The present paper covers an extensive study on the pigment composition of *P. antarctica*, cultured under a range of environmental conditions. Besides light and iron, which are dominant factors in the open ocean (Boyd 2002), this study describes the pigment composition in relation to temperature and salinity; dominant factors in sea ice (Kirst and Wiencke 1995, Thomas and Dieckmann 2002). We discuss the function of pigments both from a physiological perspective and as a biochemical marker. A table with pigment ratios is provided, based on data from this study and from literature. This table can

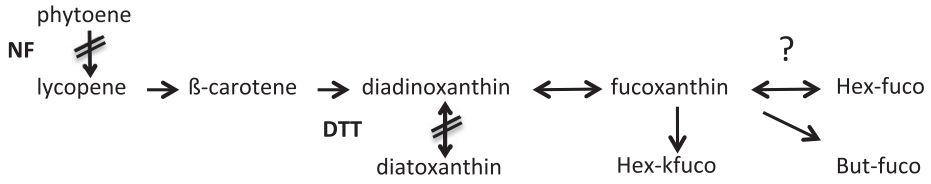

FIG. 1. A simplified model of fucoxanthin synthesis (after Lohr 2011). Carotenoid synthesis is blocked by norflurazon (NF) at the level of phytoene desaturation. Dithiothreitol (DTT) inhibits the de-epoxidation of diadinoxanthin to diatoxanthin (But-fuco = 19′-butanoyloxyfucoxanthin, Hex-fuco = 19′-hexanoyloxyfucoxanthin, Hex-kfuco = 19′-hexanoyloxy-4-ketofucoxanthin).

be used as input for CHEMTAX analysis of *P. antarctica*-dominated systems. This paper provides new insight in the physiological role of pigments in *P. antarctica*, and supports a more accurate pigment-based species identification of *Phaeocystis*-dominated Antarctic waters.

MATERIALS AND METHODS

Culture conditions. *Phaeocystis antarctica* cf. Karsten (CCMP 1871; nonaxenic) was cultured in 500 mL erlenmeyer flasks. In a series of experiments, the cultures were exposed to various environmental conditions. The growth form of *P. antarctica* was studied by microscopy. All experiments were carried out with two cultures. From these cultures, samples for pigment analyses were taken two to four times during exponential growth (cell densities ranging between 5,000 and 350,000 cells $\cdot$ mL^{-1}). The algae were grown on f/10 medium (140 µM NO_3, 7.5 µM PO_4; salinity ~35; based on Admiraal and Werner 1983) at 4°C (except for the temperature experiments). The same light source (Philips MHN-TD, Eindhoven, the Netherlands) was used for all experiments; the light regime applied was 18:6 light:dark. Light (photosynthetically active radiation, PAR) was measured inside the culture flasks (except under iron-limited conditions) with a spherical sensor (Biospherical Instruments Inc. QSL-100, San Diego, CA, USA). The algae were kept in suspension by daily manual shaking of the cultures. Culture experiments were carried out to study acclimation of the pigment composition to different environmental factors. Light shift experiments were performed to test whether or not Fuco could be converted into Hex-fuco and vice versa.

Culture experiments. *Light*: *P. antarctica* was exposed to a range of light intensities by placing the cultures at increasing distance from the light source. Light intensities applied were 1, 25, 50, 100, and 200 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. Cultures were acclimated to each of the light conditions for 3–4 weeks before starting the experiment. Samples for pigment analyses were taken four times during exponential growth.

Temperature: *P. antarctica* was cultured at −0.5°C, 1°C, 2.5°C, 4°C, and 5.5°C at 100 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. Cultures were acclimated to each temperature for 1 week before starting a new experiment. Samples for pigment analyses were taken twice during exponential growth.

Salinity: *P. antarctica* was cultured at a salinity range of 16, 24, 32, 40, 48 at 100 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. Cultures were acclimated to each salinity for 1–2 months. Samples for pigment analyses were taken twice during exponential growth.

Iron-light: Cultures of *P. antarctica* were grown in 500 mL acid-cleaned polycarbonate erlenmeyer flasks in a clean room. Medium was prepared as described by Morel et al. (1979). Chelexed, nutrient-poor seawater originating from the Atlantic sector of the Southern Ocean (salinity ~35) was enriched with 140 µM NO_3, 7.5 µM PO_4 and 60 µM Si. All cultures were spiked with 10 µM EDTA. At the start of the experiment, iron concentrations (measured by solvent extraction followed by atomic absorption spectrophotometry; Nolting and de Jong 1994) in the iron-limited cultures were 15 nM. Iron-rich cultures were enriched with $FeCl_3$ to a final concentration of 10 µM iron. Both iron-enriched (Fe^+) and iron-limited (Fe−) cultures were exposed to three different light intensities: 50, 100, and 200 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. Before starting the experiments, cultures were stressed by iron deficiency and adapted to light conditions for several weeks. Samples for pigment analyses were taken four times during exponential growth. Iron limitation was established by means of fluorescence analyses, with significant decreases of the maximum quantum yield of photosynthesis (data not shown). In addition, iron limitation was confirmed by comparison of pigment ratios of the study in 1998 (Van Leeuwe and Stefels 1998).

Shift experiments. *Short-term light shift*: Cells that were acclimated to 25 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} were exposed for 3 h to 400 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. To this end, duplicate cultures were split up in two 250 mL Erlenmeyer flasks. To one flask, DTT (100 µM final concentration) was added, one was left untreated; all flasks were exposed to the light shift. Pigments were measured before the shift and after 3 h of exposure.

Long-term light shift: Cells that were acclimated to either 25 or 100 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} were transferred to 100 and 25 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}, respectively, where they were left for 2 d. Before exposing them to the light shift, cultures were split in two flasks: to one flask, NF (10 µM final concentration) was added and one was left untreated. Samples for pigment analyses were taken before and after the 2 d of photoacclimation.

Pigment analysis. Samples for pigment analysis were filtered gently (<15 KPa) over Whatman GF/F filters and subsequently snap-frozen in liquid nitrogen and stored at −80°C until analysis. Before extraction in 90% acetone, filters were freeze-dried at −55°C during 48 h (Van Leeuwe et al. 2006, Pasquet et al. 2011). Pigments were analyzed by high-performance liquid chromatography on a Waters system equipped with a photodiode array (Van Heukelem and Thomas 2001, Van Leeuwe et al. 2006). A Waters DeltaPak reversed-phase column (C18, fully end-capped) was used. Pigment standards were obtained from DHI Water Quality Institute (Horsholm, Denmark). At the time the "shift" and the "iron-light" experiments were performed, 19′-hexanoyloxy-4-ketofucoxanthin and chl c_2-monogalactosyl diacylglyceride ester were not yet recognized as such and therefore not recorded. Unfortunately, information on those pigments was lost with the original data.

Changes in pigment composition in relation to environmental conditions were tested for significance by means of one-way ANOVA with repeated measures (one-way for single factor analyses and two-way for interactions; significance level at $P < 0.05$). Results of the shift experiments were tested for significance by paired *t*-tests (significance level at $P < 0.05$).

RESULTS

P. antarctica contained chl *a*, chl c_2 and c_3, chl c_2-monogalactosyl diacylglyceride ester (Chl c2-MGDG), But-fuco, Hex-kfuco (formerly known as 4-keto-19′-hexanoyloxyfucoxanthin), Hex-fuco, Fuco, dt, dd, and ß,ß-carotene. The growth form varied with environmental conditions. Whereas at low PAR, single cells were dominant, with increasing light, cells clustered into larger colonies. Similarly, iron-limited cells mainly consisted of single cells; the iron-replete cultures consisted mainly of colonies. Changes in temperature did not affect the growth form; the cultures consisted of a mixture of single cells and colonies. This mixture was also observed over the range in salinities. At 48, cells became distorted in shape.

Light. Hex-fuco was the major light-harvesting pigment. Hex-fuco/chl did not vary with light, showing an overall average of 0.54 (Fig. 2A). The ratios of Fuco/chl, Hex-kfuco/chl, and But-fuco/chl decreased with increasing irradiance (Fig. 2, B–D).

Fuco/chl was greater than double Hex-kfuco/chl and But-fuco/chl, with maxima of 0.16, 0.07, and 0.07, respectively.

The chls *c* decreased slightly with increasing light (Fig. 2, E and F), with the strongest decline in Chl c_3/chl.

Increasing light intensities resulted in an increase in the ratios of the photoprotective pigments dd plus dt and ß,ß-carotene to chl *a* (Fig. 2, G and H).

Temperature. Hex-fuco/chl slightly decreased with increasing temperature, with a minimum of 0.46 at 5.5°C (Fig. 2A). Fuco/chl increased with temperature until a maximum of 0.13 at 2.5°C, after which the ratio declined (Fig. 2B). Hex-kfuco/chl steadily increased with increasing temperature (Fig. 2C). But-fuco/chl only responded with a strong increase at 5.5°C (Fig. 2D).

Chl c_2 and c_3 responded oppositely to temperature (Fig. 2, E and F). At 2.5°C, Chl c_3/chl had a minimum of 0.12, whereas Chl c_2/chl showed a maximum value of 0.43. Chl c_2-MGDG increased with increasing temperature (Fig. 2F).

Dt + dd/chl significantly increased at the temperature extremes (Fig. 2G), with an average value of 0.21. ß,ß-carotene/chl increased slightly, but significantly at −0.5°C (Fig. 2H).

Salinity. Hex-fuco/chl was not significantly affected by salinity and averaged 0.63 (Fig. 2A). The ratios of Fuco/chl and Hex-kfuco/chl both decreased with increasing salinity. Maximum values of 0.07 and 0.04 respectively were obtained at 16 (Fig. 2, B and C). But-fuco/chl increased with increasing salinity to a maximum of 0.06 at 48 (Fig. 2D).

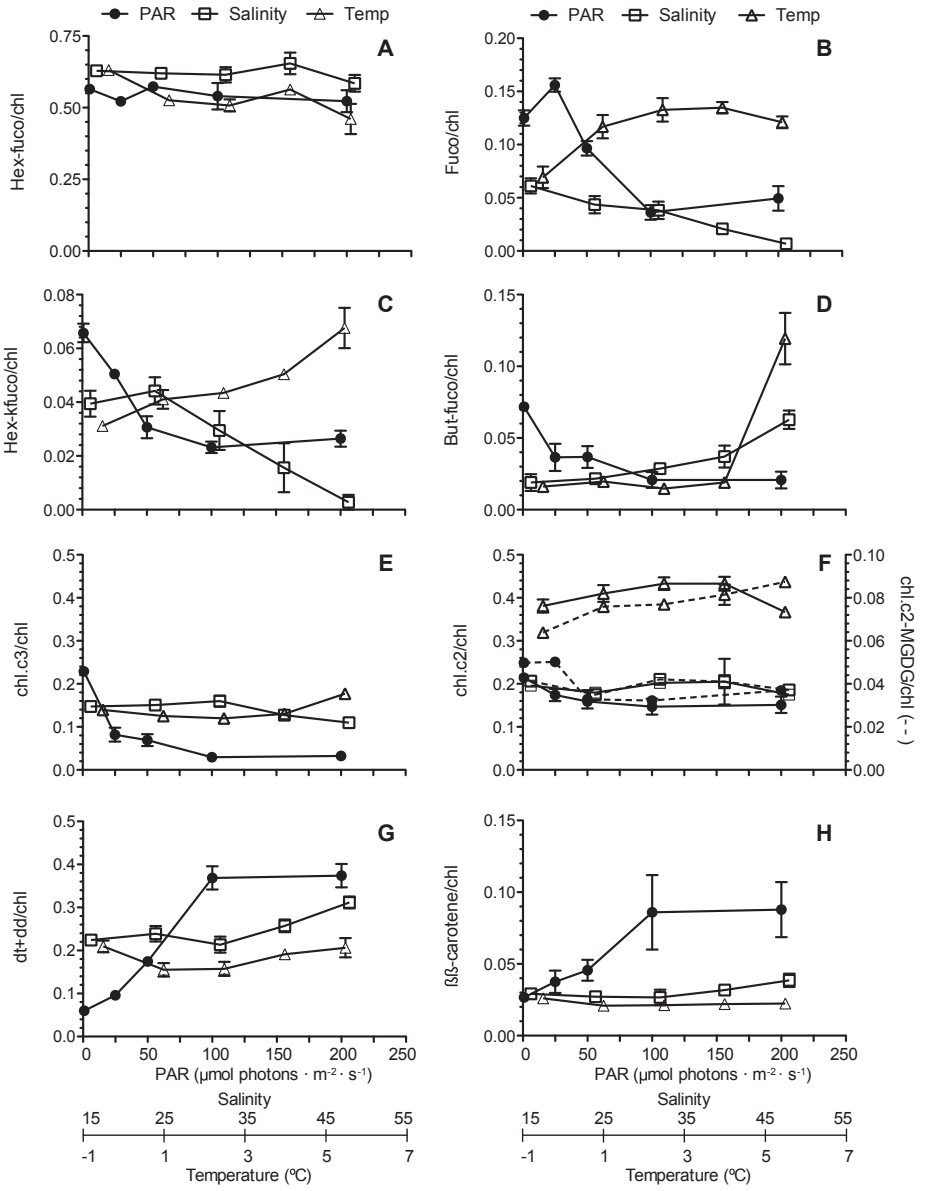

FIG. 2. Pigment ratios (±SEM; sometimes within the symbol) in *Phaeocystis antarctica* cultured under a range of light intensities. A. Fuco/chl, Hex-kfuco/chl and But-fuco; B. Hex-fuco/chl, dt+dd/chl and ß-ß carotene/chl; C. Chl c_2/chl and Chl c_3/chl; for abbreviations see text. At 200 µmol photons. m-2. s-1, hex-kfuco was not resolved.

The chl *c* hardly responded to changes in salinity (Fig. 2, E and F). The ratios of the photoprotective pigments dd plus dt and ß,ß-carotene to chl *a* increased with increasing salinity (Fig. 2, G and H).

Iron-light. Hex-fuco/chl was highest in Fe^- cells (Fig. 3A), with a maximum of 0.62 at 50 µmol photons · m^{-2} · s^{-1} under iron limitation. Fuco/chl was higher in Fe^+ than in Fe^- cells, with a maximum ratio of 0.08 at 50 µmol photons · m^{-2} · s^{-1} (Fig. 3B). The ratio decreased with increasing light. At 200 µmol photons · m^{-2} · s^{-1}, Fuco could not be detected in either the Fe^+ or the Fe^- cells. The interaction between iron and light was not significant. But-fuco/chl was also highest in Fe^- cells (Fig. 3C). In Fe^+ cells, But-fuco could not be detected at 200 µmol photons · m^{-2} · s^{-1}. Compared to Fe^+ cells, Fe^- cells also showed higher ratios of dt + dd/chl (Fig. 3D). The ratio increased with irradiance. Chl c_2/chl increased under iron limitation, whereas Chl c_3/chl was not significantly affected (Fig. 3, E and F).

Shift experiments. An upward shift in light intensity resulted in a ca. 30% decrease in chl *a* after 3 h (Fig. 4A). The light shift did not result in any significant changes within the pool of Fuco pigments. The addition of DTT suppressed the increase in dt/dd, and led to a 25% decrease in Fuco/chl and 20% decrease in Hexa/chl.

On a timescale of days rather than hours, an upward shift in irradiance resulted in stronger changes within the Fuco pigment pool (Fig. 4B), with a 70% decrease in Fuco/chl and minor increases in But-fuco/chl and Hex-fuco/chl. The addition of NF inhibited chl synthesis, and suppressed the decrease in Fuco/chl. But-fuco/chl and Hex-fuco/chl increased after NF addition. The dt + dd/chl ratio almost doubled under control conditions, whereas the NF treatment suppressed this increase.

The downward shift resulted in a doubling of Fuco/chl (Fig. 4C). The addition of NF suppressed chl synthesis and inhibited the increase in Fuco/chl. But-fuco/chl and Hex-fuco/chl slightly increased after NF addition. The ratio of dt + dd/chl decreased over the course of 2 days. The decrease was further enhanced by NF addition.

DISCUSSION

The experimental data presented here have been collected over a number of years, using the same *P. antarctica* strain. Although basic conditions (e.g., light source, temperature) were always the same, pigment ratios under control conditions varied slightly over the course of time, which sometimes led to unexplained shifts between experiments: e.g., relatively high Fuco/chl and Chl

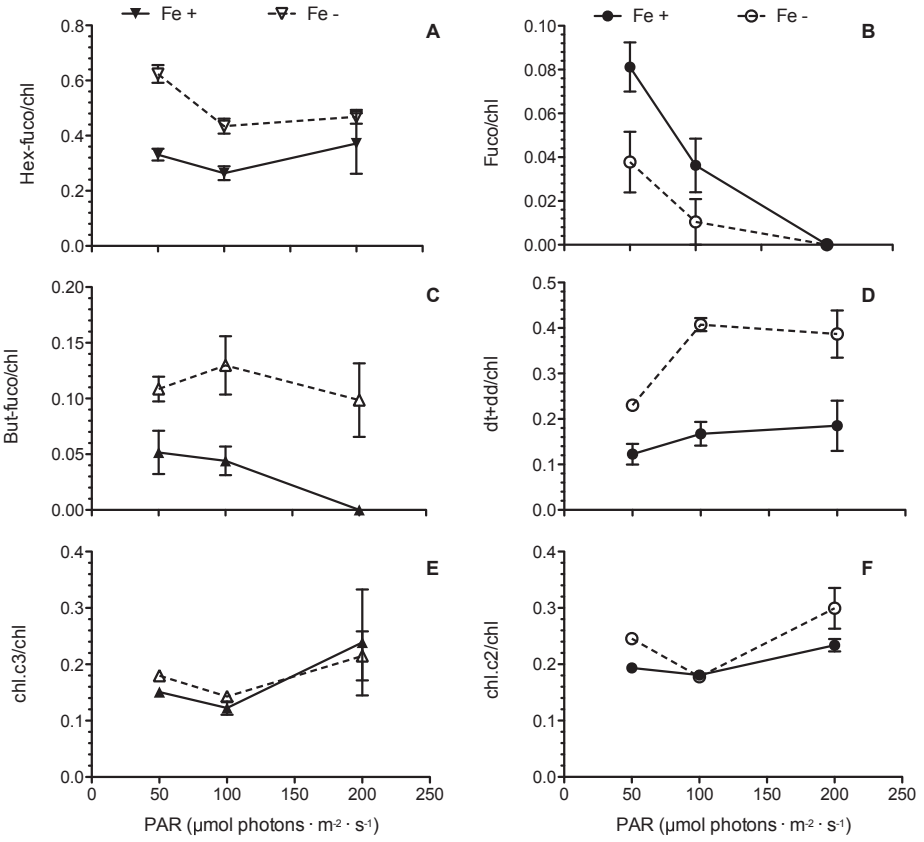

FIG. 3. Pigment ratios (±SEM) in *Phaeocystis antarctica* cultured under three different light intensities, with (Fe+) and without (Fe-) iron enrichment. A. Fuco/chl and Hex-fuco/chl; B. But-fuco/chl and dt+dd/chl; C. Chl c_2/chl and Chl c_3/chl; for abbreviations see text. ß-ß carotene was not resolved in these experiments.

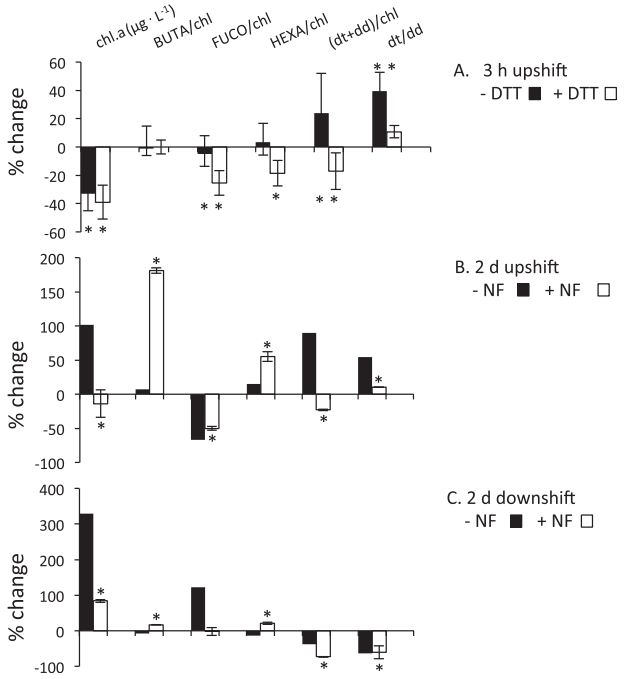

FIG. 4. Changes in pigment ratios in *Phaeocystis antarctica* expressed in percentage of initial ratios (±SD), at the end of the shift experiments. (A) Short-term light shift (upshift: 25 to >400 µmol), dt/dd depicted are divided by 10; (B) Long-term light shift (upshift: 25 to >100 µmol), dt/dd depicted are divided 10; (C) Long-term light shift (downshift: 100 to >25 µmol). *Significantly different from t0 by paired *t*-test ($P < 0.05$).

c_2/chl ratios in the temperature experiments and relatively low Hex-fuco/chl ratios in the iron-light experiments. Nevertheless, the strongest—and consistent—variations were recorded in response to the various experimental conditions. We will discuss these variations on the basis of their physiological role in algae.

Light harvesting. Under all conditions, Hex-fuco constituted the major light-harvesting pigment in *P. antarctica*. The Hex-fuco/chl ratio was not affected by light, indicating a tight coupling with chl *a* content. Under saturating light conditions, the levels of Hex-fuco were ten times higher than for Fuco, Hex-kfuco, and But-fuco. The contribution of these other Fuco pigments as light-harvesting pigments becomes relevant only under low-light conditions, when together they made up almost half of the Fuco pigment pool. Their flexible response to light, make these pigments important in the adaptation to low-light conditions.

Chlorophyll c_2 and c_3 also are light-harvesting pigments (Porra et al. 2011), and part of the Fucochl a/c protein complex. In *P. antarctica*, the chl *c* are important components as they make up 20%–50% of the accessory pool of light-harvesting pigments. Chl c_3, and to a lesser extent Chl c_2, were especially responsive to low-light conditions. A light-harvesting function has also been suggested for Chl c_2-MGDG (Rodriguez et al. 2006). In our experiments, Chl c_2-MGDG to chl *a* ratios were low and showed a minor response to light. These results neither support nor contradict a role in light harvesting, which leaves the function of this pigment enigmatic.

Energy dissipation. Under conditions when more energy is available than can be utilized in photosynthesis, the cells need to dissipate excess energy. Such conditions are not restricted to high-light conditions, but can also be the result of impaired functioning of the photosystems due to salinity and temperature extremes or to iron limitation.

Increased salt concentrations lead to malfunctioning of photosystems and decreased electron transport by a loss in proteins in the thylakoid membrane (Mehta et al. 2010). This results in the production of harmful oxygen radicals that need to be scavenged to prevent photodamage. Our experiments indeed showed that *P. antarctica* adapts to high salinities by a decrease in the light-harvesting pigments Fuco and Hex-kfuco and a simultaneous increase in the photoprotective pigments diato- and dd and ß,ß-carotene. In addition, an increase in But-fuco/chl was observed. In contrast, low salinities did not appear to affect pigment ratios significantly. Apparently, *P. antarctica* belongs to the group of species that tolerate reduced salinity better than high salinity (Bates and Cota 1986).

Photo-oxidative stress is also experienced at low temperatures, when hampered electron transport and cell metabolism may result in energy excess (Davison 1991). As for high salinity, these conditions led to reductions in the light-harvesting pigments Fuco and Hex-kfuco and modest increases of photoprotective pigments diato- and dd and ß,ß-carotene. These findings are consistent with a field study on a *P. antarctica*-dominated community by Kennedy et al. (2012) who ascribed a decrease in photosynthetic activity with decreasing temperatures and high salinity (>100) to photo-oxidative stress. High temperatures, on the other hand, may disturb cell metabolism by changes in cell structures, denaturation of proteins, and inactivation of enzymes (Kottmeier and Sullivan 1988, Davison 1991). This also leads to photo-oxidative stress with expected corresponding reductions in the Fuco/chl and chl c_2/chl ratios and increases in dd+dt/chl ratios. However, in contrast to the earlier stress conditions, increases in Hex-kfuco/chl, But-fuco/chl, chl c_3/chl, and chl c_2-MGDG/chl ratios were observed.

Iron limitation is another factor that may induce photo-oxidative stress. In the ocean, iron and light often interact together in growth limitation (van Leeuwe et al. 2000, Boyd 2002). *P. antarctica* responded to iron limitation with a decrease in the light-harvesting pigment Fuco, confirming previous studies (Van Leeuwe and Stefels 1998, 2007, DiTullio et al. 2007). Simultaneously, the irradiance levels at which photoinhibition occurs appear lower for

Fe^- than for Fe^+ cells. In Fe^- cells, the dt + dd/chl ratio increases at lower light intensity and shows a stronger response than in Fe^+ cells. These results confirm the study by Alderkamp et al. (2012a) who describes that the xanthophyll cycle in *P. antarctica* is highly effective under conditions of iron limitation.

Some of the above-described responses in pigment ratios were contra expectations. Especially the role of But-fuco is enigmatic. But-fuco is often ascribed a light-harvesting role (e.g., Rodriguez et al. 2006). In our experiments, the increased But-fuco/chl ratio at low PAR may confirm such a role. However, the increase in But-fuco/chl with increasing salinity, elevated temperature, and decreasing Fe suggests a role in photoprotection rather than in light harvesting. These data confirm the previous findings by Van Leeuwe and Stefels (1998). Also Schluter et al. (2000) and Alami et al. (2012) suggested a role for But-fuco in photoprotection.

Membrane stabilization. Temperature and salinity are factors that not only control photosynthetic activity and associated pigment synthesis via pathways of energy flow but also these factors directly affect the structure of photosynthetic membranes. In microalgae, a decrease in temperature coincides with an increase in the content of unsaturated fatty acids to maintain the flexibility of the membranes (Nishida and Murata 1996, Dalsgaard et al. 2003, Morgan-Kiss et al. 2006). A similar change in saturation of membrane lipids is observed with increasing salinity (Morgan-Kiss et al. 2006). Within the thylakoid membrane, pigments, proteins, and lipids are tightly connected (Mock and Kroon 2002, Schaller et al. 2011, Lepetit et al. 2012). An increase in unsaturated fatty acids may be beneficial for membrane fluidity, yet it also leaves the membranes more susceptible to photo-oxidation. In response to these changes in lipid content, microalgae will increase the content of photoprotective pigments. Indeed, we observed increased contributions of dt + dd and ß,ß-carotene. These compounds not only act as photoprotective pigments but are also known to function as a membrane stabilizer under stress conditions (Havaux 1998, Strzalka et al. 2003).

The function of Hex-kfuco is still far from clear. It is the most complex molecule of the Fuco family (Egeland et al. 2000). Whether or not this has consequences for its embedding in the thylakoid membranes is yet unknown. Its precise location in the cell might vary with species (Lohr 2011). It is suggested that ketocarotenoids play a photoprotective role in the thylakoid membranes (Jin et al. 2006). In our experiments, Hex-kfuco resembled a typical light harvester, except for its increase at high temperatures. Our results agree with the field study by Cook et al. (2011). In their paper describing two morphotypes of *Emiliania huxleyi*, Hex-kfuco was only present in the morphotype that was characteristic for coastal areas. We speculate that this may be the result of physiological adaptation to the prevailing conditions of low salinity and low-light availability in coastal zones.

Alternatively, the increased Hex-kfuco/chl ratio at elevated temperatures may indicate a photoprotective function. Rodriguez et al. (2006) recorded a higher ratio of Hex-kfuco/chl in *P. globosa* under high compared to low irradiance, which also suggests a photoprotective function. In addition, Zapata et al. (2004) found strong variations in the ratio of Hex-kfuco-to-chl *a* in *P. antarctica* under similar light conditions, indicating that the ratio is potentially strain dependent.

Conversion processes. In previous experiments, iron limitation induced synthesis of Hex-fuco at the cost of Fuco, which led to the hypothesis that the pigments are interconvertible (Van Leeuwe and Stefels 1998). The DTT-addition experiments did not support this hypothesis. The short-term light upshift that was applied in this experiment induced bleaching, as reflected in the decrease in chl *a*. But, a shift within the pool of Fuco pigments was not observed; the decrease in Fuco/chl after DTT addition did not coincide with an increase in Hexa-fuco/chl (Fig. 4). In fact, all pigment ratios to chl decreased after DTT addition, suggesting rapid degradation of pigments in the antenna system.

The long-term NF-addition experiments, designed to induce conversion under conditions that required Fuco syntheses (downward light shift) or that would promote the conversion of Fuco into Hex-fuco (upward shift), provided a complex picture in which potential interconversions of several xanthophyll pigments may have taken place. Although NF specifically inhibits carotenoid synthesis, it appeared that chl synthesis was also inhibited over the course of 2 days, which may have indicated reduced growth. The upward light shift coincided with a strong increase in dt/dd. This increase was suppressed after NF addition, which likely resulted in photo-oxidative stress, with subsequent negative effects on overall growth. The simultaneous increase in But-fuco/chl and Hex-fuco/chl may be an expression of energy excess and can at least partly be explained by the reduction in Fuco/chl and confirms But-fuco's potential role as a photoprotective pigment. In addition, the reduction in dd + dt may have fueled the production of But-fuco and Hex-fuco through Fuco (Fig. 1). Also following a downward light shift, NF addition led to a reduction in chl *a* production, possibly as the result of suppressed energy supply by carotenoids. Since all Fucos-to-chl ratios stayed more or less constant, their pools must have increased at a similar rate as chl *a*. We suggest they were derived from the dd and dt pool.

From these experiments, we conclude that a rapid interconversion of Fuco into Hex-fuco does not occur. On timescales of days, however, interconversions within the xanthophyll pigment pool cannot be excluded. Upshift experiments resulted in strong

TABLE 1. Pigment-to-chl *a* ratios for *Phaeocystis antarctica* under various environmental conditions. Ratios of this study are compared to literature vales. For abbreviations, see text.

	Source[h]	PAR (μmol photons · m^{-2} · s^{-1})	chl.c3	chl.c2	c2 MGDG	But-fuco	Fuco	Hex-kfuco	Hex-fuco	Diadino	Diato	ß,ß-Car
Temp (°C)												
−0.5	This study		0.139	0.381	0.064	0.016	0.069	0.031	0.631	0.124	0.086	0.026
2.5	This study		0.120	0.433	0.077	0.015	0.133	0.043	0.508	0.107	0.051	0.021
5.5	This study		0.177	0.367	0.087	0.119	0.121	0.068	0.460	0.106	0.101	0.022
Salinity												
16	This study		0.150	0.201	0.041	0.014	0.068	0.043	0.636	0.144	0.080	0.029
32	This study		0.160	0.202	0.042	0.029	0.038	0.029	0.615	0.148	0.066	0.027
48	This study		0.110	0.175	0.039	0.063	0.007	0.003	0.585	0.137	0.175	0.039
Fe												
Fe−	1	25	0.257	0.457	–	0.172	0.525	–	0.789	0.332	0.017	–
	1	100	0.134	0.329	–	0.225	0.209	–	0.778	0.628	0.075	–
	2	20	0.270	–	–	–	0.010	–	1.100	–	–	–
	3	0–400D[a]	0.116	0.197	–	0.013	0.011	0.021	0.734	0.214	0.017	0.016
	4	0–250D[a]	0.290	–	–	0.036	–	–	1.014	–	–	–
	5[b]		0.130	–	–	–	0.010	–	1.210	–	–	–
	6[b]		–	–	–	–	0.138	–	0.761	–	–	–
	7[b]		0.309	0.212	–	–	0.008	–	1.490	–	–	–
	8[b]		0.372	–	–	0.001	0.004	–	0.376	–	–	–
	This study		0.143	0.178	–	0.130	0.010	–	0.435	0.183	0.226	–
	Mean		0.225	0.275	–	0.096	0.103	0.021	0.869	0.339	0.084	0.016
Fe+	1	25	0.322	0.420	–	–	0.723	–	0.018	0.170	–	–
	1	100	0.219	0.345	–	0.036	0.582	–	0.109	0.275	0.017	–
	2	20	0.130	–	–	–	0.080	–	0.560	–	–	–
	3	0–400D[a]	0.091	0.202	–	0.009	0.017	0.015	0.344	0.057	0.025	0.014
	4	0–250D[a]	0.230	–	–	0.055	0.060	–	0.724	–	–	–
	5[b]		0.340	–	–	–	0.130	–	0.430	–	–	–
	9[b]		0.141	0.144	0.054	0.099	0.011	–	0.916	–	–	–
	7[b]		0.228	0.579	–	–	0.106	–	0.336	–	–	–
	8[b]		0.171	–	–	0.017	0.057	–	0.294	–	–	–
	This study		0.122	0.181	–	0.044	0.036	–	0.264	0.081	0.089	–
	Mean		0.199	0.312	0.054	0.043	0.180	0.015	0.400	0.146	0.044	0.014
PAR[g]												
LL	10	14, 37	0.488	0.321	0.003	–	0.019	–	0.629	–	–	–
	1	25	0.322	0.420	–	–	0.723	–	0.018	0.170	–	–
	2	20	0.130	–	–	–	0.080	–	0.560	–	–	–
	11	5	–	–	–	–	–	–	–	0.035	0.010	–
	12[c]	40	0.118	0.196	0.047	0.070	0.186	0.029	0.393	–	–	–
	13[d]		0.423	–	–	0.688	1.032	–	0.900	0.113	–	–
	This study		0.143	0.187	0.050	0.048	0.146	0.058	0.536	0.067	0.017	0.034
	Mean[e]		0.240	0.281	0.033	0.059	0.231	0.044	0.427	0.091	0.014	0.034
ML	14	100	0.150	0.170	–	0.010	0.030	–	0.550	0.115	0.052	0.011
	1	125	0.219	0.345	–	0.036	0.582	–	0.109	0.275	0.017	–
	10	84	0.555	0.245	0.010	–	0.010	–	0.265	–	–	–
	11	65, 125	–	–	–	–	–	–	–	0.023	0.025	–
	13[d]		0.215	–	–	0.655	0.577	–	0.668	0.085	–	–
	This study		0.065	0.152	0.033	0.031	0.071	0.027	0.555	0.140	0.142	0.068
	Mean[e]		0.247	0.228	0.022	0.026	0.173	0.027	0.370	0.138	0.059	0.040
HL	15[f]	150	–	–	–	–	0.245	–	0.117	–	–	–
	10	151–542	0.348	0.228	0.017	–	0.019	–	0.330	–	–	–
	3	0–400D[a]	0.091	0.202	–	0.009	0.017	0.015	0.344	0.057	0.025	0.014
	4	0–250D[a]	0.232	–	–	0.055	0.060	–	0.724	0.147	0.006	0.022
	13[d]		0.262	0.199	–	0.263	0.374	–	0.644	0.150	–	–
	This study		0.046	0.151	0.037	0.030	0.055	0.026	0.523	0.173	0.201	0.088
	Mean[e]		0.179	0.194	0.027	0.031	0.038	0.021	0.401	0.126	0.077	0.041

[a]Sinusoidal light regime with minimum and maximum light intensities indicated.
[b]Optimized ratios for field sites dominated by *P. antarctica*.
[c]Average of various different strains.
[d]Field ratios.
[e]Mean of all values except # 13.
[f]Colonies only.
[g]LL: low light (<50 μmol); ML: medium light (50–150 μmol); HL: high light (>150 μmol).
[h]Average values of published data. Source: 1. Van Leeuwe and Stefels 1998, 2. DiTullio et al. 2007, 3. Van Leeuwe and Stefels 2007, 4. Alderkamp et al. 2012a, 5. Wright et al. 2010, 6. Mills et al. 2012, 7. Gibberd et al. 2013; 8. Alderkamp et al. 2012b, 9. Rodriguez et al. 2002, 10. Moisan and Mitchell 1999, 11. Kropuenske et al. 2009, 12. Zapata et al. 2004, 13. Higgins et al. 2011, 14. Vaulot et al. 1994, 15. Buma et al. 1991.

increases of But-fuco/chl, which confirms a photoprotective role for this pigment. A similar function for Hex-fuco cannot be excluded.

Single cells versus colonies. Colonies were the dominant life form under more favorable growth conditions (high light, iron replete, moderate-high salinity), whereas single cells prevailed under iron limitation and low light. Previously, it was already noted that colonies are the life form that display higher maximum growth rates (Baumann et al. 1994, Kennedy et al. 2012). Hex-fuco was not significantly affected by the various growth conditions, suggesting a decoupling with the life form. These findings are consistent with a study by Buma et al. (1991), who also showed that the ratio of Hex-fuco-to-chl *a* was not affected by the life form. It is noteworthy that in none of these studies Fuco and Hex-fuco responded in parallel: a decrease in Fuco/chl does not necessarily coincide with an increase in Hex-fuco and *vice versa*. This is consistent with the results from the shift experiments as discussed above: there is limited evidence for rapid conversion processes between the two Fuco pigments in response to growth conditions.

Chemotaxonomy. Our experiments show that the pigment concentrations in *P. antarctica* change with environmental conditions. For chemotaxonomy as applied by CHEMTAX (e.g., Wright et al. 1996), we therefore propose a new set of pigment ratios for *P. antarctica*-dominated algal communities, under a range of environmental conditions (Table 1). The table is based on literature values and this study. Literature values include data from culture experiments as well as field data from *P. antarctica*-dominated systems. For comparison, we also included the field ratios tabulated by Higgins et al. (2011) for Haptophytes (defined as HAPTO-8), which currently sets the standard for CHEMTAX applications. The application of the ratios published by Higgins has restrictions. These authors presented a limited set of pigment ratios and combined the results from various regions and a variety of *Phaeocystis* species; also those of e.g., *P. globosa*, a species that does not contain Hex-fuco (Antajan et al. 2004). This reduces the potential for a chemotaxonomic characterization in specific areas. Hence, we here propose a *P. antarctica*-specific set of ratios, that includes additional pigment ratios and a wider range of environmental conditions.

The different data sets in Table 1 show similar patterns, which correspond to the findings in our experiments (e.g., highest values for Fuco at low light and under iron-replete conditions). Relative to the mean values, Higgins et al. (2011) calculated significantly higher ratios for all Fucos to chl *a*. Our study showed relatively low values for Chl c_3/chl (Table 1). Overall, Hex-fuco/chl appears to be quite stable, which validates its usefulness as a marker pigment, although iron limitation led to a doubling of the ratio. Environmental conditions leading to photo-oxidative stress resulted in strong changes in the ratios of most other pigments to chl *a*. Hence, for use within CHEMTAX, input ratios of these pigments must be adjusted to the predominant environmental conditions, especially the light climate.

Creating a seeding matrix and defining bins for CHEMTAX requires prior knowledge on dominant species and environmental conditions (see Kozlowski et al. 2011 and refs therein). The ratios provided in this paper will help to improve pigment-based diagnosis in systems that are dominated by *P. antarctica* and may be a guide in setting up input ratios for CHEMTAX with changing environmental conditions in space or time.

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