Carbon fixation and carbonic anhydrase activity in *Haslea ostrearia* (Bacillariophyceae) in relation to growth irradiance

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Abstract

The metabolic pathway of primary carbon fixation was studied in a peculiar pennate marine diatom, *Haslea ostrearia* (Bory) Simonsen, which synthesizes and accumulates a blue pigment known as "marennine". Cells were cultured in a semi-continuous mode under saturating [350 μmol(photon) m^{-2} s^{-1}] or non-saturating [25 μmol(photon) m^{-2} s^{-1}] irradiance producing "blue" (BC) and "green" (GC) cells, characterized by high and low marennine accumulation, respectively. Growth, pigment contents (chlorophyll *a* and marennine), ^{14}C accumulation in the metabolites, and the carbonic anhydrase (CA) activity of the cells were determined during the exponential growth phase. Growth rate and marennine content were closely linked to irradiance during growth: higher irradiance increased both growth rate and marennine content. On the other hand, the Chl *a* concentration was lower under saturating irradiance. The distribution between the Calvin-Benson (C_3) and β-carboxylation (C_4) pathways was very different depending on the irradiance during growth. Metabolites of the C_3 cycle contained about 70 % of the total fixed radioactivity after 60 s of incorporation into cells cultured under the non-saturating irradiance (GC), but only 47 % under saturating irradiance (BC). At the same time, carbon fixation by β-carboxylation was 24 % in GC *versus* about 41 % in BC, becoming equal to that in the C_3 fixation pathway in the latter. Internal CA activity remained constant, but the periplasmic CA activity was higher under low than high irradiance.

Additional key words: ^{14}C; C_3 and C_4 plants; chlorophyll; metabolic pathways.

Introduction

The marine diatom, *Haslea ostrearia* (Bory) Simonsen, regularly colonizes oyster-ponds in French Atlantic coastal region (Rincé 1978, Robert 1983), and synthesizes a water-soluble blue pigment "marennine". This pigment accumulates in vesicles at both ends of the cell (Nassiri *et al.* 1998), and is then released into the seawater. It produces greening of the oyster gills, a phenomenon exploited in French Atlantic coastal regions as it adds economic value to the oysters.

When cultured in the laboratory under low irradiance [about 20 μmol(photon) m^{-2} s^{-1}], *H. ostrearia* cells contain almost no marennine, and are known as "green" cells, GC (Shubert *et al.* 1995), as distinct from "blue" cells (BC) that contain very high amounts of marennine when cultured under higher irradiance (Rech 2004). Tremblin and Robert (1996) have shown that photosynthesis (oxygen exchange) in *H. ostrearia* is related to the blueing of the cells. These authors also investigated the overall inorganic carbon fixation during batch growth and marennine accumulation, and showed that the highest carbon fixation rate occurred when cells had not yet accumulated the blue pigment. This carbon fixation rate decreased concomitantly with the accumulation of marennine. Since the overall determination of carbon fixation did not make it possible to differentiate between the relative contributions of the C_3 and C_4 pathways, the activities of three related carboxylases, ribulose bisphosphate carboxylase-oxygenase (RuBPCO), phospho*enol*-pyruvate carboxylase (PEPC), and phospho*enol*pyruvate carboxykinase (PEPCK), were measured during batch growth and marennine accumulation. This has shown that the activity of RuBPCO fell below that of PEPCK as the marennine concentration increased in the cells. Contrary to Reinfelder *et al.* (2000) in *Thalassiosira weissflogii*, no PEPC activity was detected in *H. ostrearia*. A hypothesis that the C_4-like metabolism plays a significant role (linked to the physiological stage of the cells) in the carbon budget of BC of *H. ostrearia* has been proposed (Tremblin and Robert 2001). On the other hand, the existence and importance of the C_4-like metabolism with

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significant fixation of carbon by β-carboxylation in marine microalgae and especially in diatoms has been a subject of controversy for over a quarter of century (Beardall *et al.* 1976, Morris 1980, Mortain-Bertrand *et al.* 1987, Beardall 1989, Glover 1989, Zimba *et al.* 1990, Descolas-Gros and Oriol 1992, Raven 1997, Reinfelder *et al.* 2000, 2004, Riebesell 2000, Johnston *et al.* 2001, Raven and Beardall 2003, Granum *et al.* 2005). However, this C_4-like pathway does seem to play a role in the carbon-concentrating mechanism (CCM) in the marine diatom *T. weissflogii* when it is acclimated to low CO_2 concentrations (Reinfelder *et al.* 2004). Recently, Granum *et al.* (2005) confirmed the existence of a C_4-like pathway in *T. pseudonana*, in which the expression of the PEPCK gene is slightly up-regulated at low CO_2 concentrations.

In a different way, CO_2 is the substrate of RuBPCO but, in the upper layer of the oceans, HCO_3^- ions predominate, and CO_2 represents only about 1 % of the total dissolved carbon (Riley and Chester 1971). Some photosynthetic organisms have developed CO_2-concentrating mechanisms that allow them to overcome this potentially limiting shortage of CO_2. Two such mechanisms are the direct uptake of HCO_3^- and the conversion of HCO_3^- into CO_2 before absorption. Carbonic anhydrase (CA) is essential for the reversible HCO_3^--CO_2 conversion both in the cell and in the periplasm (Graham *et al.* 1984, Badger and Price 1992, 1994, Raven 1995, Raven and Beardall 2003). CA plays an important role in photosynthesis by supplying either CO_2 to RuBPCO or HCO_3^- to PEPC. Other cellular carboxylation and decarboxylation reactions also require CA (Raven 1995).

Our work was therefore undertaken in order to investigate the relationship between external and internal CA activity, and the relative contributions of the C_3 and C_4 pathways in BC and GC of *H. ostrearia* grown under saturating and un-saturating irradiances, respectively.

Materials and methods

Algal culture: *Haslea ostrearia* (Bory) Simonsen was obtained from the microalgae culture collection of the "Centre de Ressources Biologiques" (Nantes University, France), and had been isolated from "Baie de Bourgneuf" oyster-ponds. The diatoms were grown in Provasoli 33 % (v/v) medium (Robert 1983) in 500 cm^3 glass Erlenmeyer flasks, with a 14/10 h light/dark photoperiod and under different irradiances [25, 75, 100, 350 µmol(photon) m^{-2} s^{-1}]. In the ^{14}C fixation and CA activity experiments, only the low irradiance of 25 µmol(photon) m^{-2} s^{-1}, and the high irradiance of 350 µmol(photon) m^{-2} s^{-1} were used to obtain GC and BC, respectively. Irradiation was provided by an *Osram HQI-T* source (400 W) fitted with a neutral density filter to adjust irradiance that was measured using a *Walz US-SQS 4π* probe (*Walz*, Germany) connected to a *Li-Cor 189* quantameter (*Li-Cor Instruments*, USA). Cultures were maintained in an exponential growth phase at 15±1 °C by diluting with fresh medium every three days.

Growth rate and pigment analysis: Cell density was estimated using a *Nageotte* hematocytometer. Photosynthetic pigments were extracted in dimethylformamide (DMF), and the total chlorophyll (Chl) *a* content was determined spectrophotometrically according to Speziale *et al.* (1984).

For quantitative marennine analysis cells were collected by centrifuging (5 min, 900×*g*). Three cm^3 of hypo-osmotic phosphate buffer (pH 8) were added to the algal pellet, and then stirred for 30 min, before being centrifuged again for 15 min at 10 000×*g*. The absorbance of the supernatant at 669 nm was measured *versus* fresh phosphate buffer, and the marennine concentration was calculated according to Robert *et al.* (2002).

CA activity: BC or GC were concentrated by centrifuging (5 min, 900×*g*), with gradual acceleration and deceleration. They were re-suspended in 3 cm^3 veronal–HCl buffer 0.02 M (pH 8.3) enriched with NaCl 0.48 M, with a final concentration of 1.6 to 2.0×10^6 cells cm^{-3}. The CA activity was determined by measuring the time required for the pH to fall 0.4 units between pH 8.3 and 7.9 in the cell suspension after adding 3 cm^3 of CO_2-saturated water containing NaCl 0.48 M, using a fast-response pH electrode (*N 5800 A*, *Schött Geräte*, Germany) coupled to a temperature compensating pH-meter (*P-907*, *Consort Instruments*, Belgium). The temperature during the reaction was maintained at 2 °C. Enzyme activity was calculated according to Haglund *et al.* (1992). One Enzyme Unit (E.U.) was defined as $(T_0/T) - 1$, where T_0 and T represent the times required for the pH to drop 0.4 units without and with algal samples, respectively. The total CA activity (CA_{tot}) was measured on crude extracts of cells homogenized in veronal buffer (as previously) and in liquid nitrogen. External (periplasmic) CA (CA_{ext}) activity was determined using intact cells. Internal CA (CA_{int}) activity was calculated as the difference between total and external activities. The values obtained were then expressed in terms of the cell density (E.U. 10^{-6} cells).

Short-term ^{14}C fixation (for times ranging from 0 to 60 s) was studied using $NaH^{14}CO_3$ solution (*Amersham Bioscience*, UK) with a specific activity of 2.22 GBq $mmol^{-1}$. For each fixation time tested, a 10 cm^3 sample of BC or GC containing 1.65×10^6 cells cm^{-3} was exposed to saturating irradiance at 15±1 °C, and continuously stirred before and after adding 3.7 MBq of $NaH^{14}CO_3$ for the required fixation times (5, 10, 20, 30, 40, 50, and 60 s). Within seconds, the sample was filtered over a *GF-F*

filter (*Whatman*, USA) and the filter was dropped in liquid nitrogen to stop the metabolic reactions. The primary labelled metabolites were extracted with 50 % (v/v) ethanol-water in the presence of *Dowex 50* (H^+ form), NaF, and KBH_4. Further separation and detection protocols were then performed as described previously (Schürmann 1969, Coudret *et al.* 1992, Tremblin *et al.* 1993). Two-ways separation was performed by high voltage electrophoresis (750 V) and thin layer chromatography (cellulose *MN300*, *Macherey-Nagel*, Germany). Autoradiography of the plates was then performed using *Biomax MR-1* film (*Kodak-Eastmann*, USA). The radioactivity (^{14}C) that had been incorporated into the metabolites was measured by liquid scintillation counting (*Wallac 1410* counter, *ECG Instrument*, *Perkin-Elmer*, USA) after identifying the compounds by autoradiography using the metabolite maps of Mortain-Bertrand (1987). Each spot was carefully scraped off, placed in a scintillation vial, and counted after adding 1 cm^3 of distilled water and 9 cm^3 of scintillation cocktail (*Quicksafe A*, *Zinsser Analytics*, Germany). The radioactivity reading was corrected for quenching using the external standard ratio method. Results are expressed as the radioactivity [Bq] incorporated per 10^6 cells, or as the percentage of the radioactivity of the total soluble organic matter. Each experiment was carried out in triplicate. The radioactivity of the ethanol-insoluble fraction was determined and compared to the radioactivity of the soluble fraction. In these short-term pulses (60 s maximum), products contained in the insoluble organic matter were ignored as they accounted for less than 0.5 % of the total radioactivity.

Statistical analysis: All data were analyzed using *MVSP 3.13* statistical analysis software (*Kovach Computing*, U.K.). For each parameter in the tables, results significantly not different at $p \leq 0.05$ share the same identification superscripted letter.

Results

Cultured in semi-continuous mode under different irradiances [from 25 to 350 µmol(photon) m^{-2} s^{-1}] the growth rate of *H. ostrearia* was only significantly different under the highest photon flux density [350 µmol(photon) m^{-2} s^{-1}], when the cellular concentration of total Chl *a* decreased (Table 1). The marennine content increased significantly with irradiance. However, GC [obtained under 25 µmol(photon) m^{-2} s^{-1}], that usually do not display any visible accumulation of blue pigment at either end of the cells under photon-microscopic observation, contained a significant quantity of marennine (Table 1), which must therefore have been distributed among the cell.

Table 1. Growth rate (µ), and chlorophyll (Chl) *a* and marennine contents in *Haslea ostrearia* cultured in semi-continuous mode related to irradiance [25, 75, 100, 350 µmol(photon) m^{-2} s^{-1}]. Means ± SE (n = 3). For each column, values that are not significantly different ($p \leq 0.05$) carry the same superscripted letter.

Irradiance [µmol(photon) m^{-2} s^{-1}]	µ [j^{-1}]	Chl *a* [µg 10^{-6} cells]	Marennine [µg 10^{-6} cells]
25	0.34 ± 0.03 ^{b}	8.92 ± 0.86 ^{a}	12.34 ± 1.63 ^{d}
75	0.38 ± 0.05 ^{b}	7.45 ± 0.23 ^{b}	18.52 ± 2.41 ^{c}
100	0.30 ± 0.02 ^{b}	3.51 ± 0.37 ^{c}	24.92 ± 3.58 ^{b}
350	0.52 ± 0.08 ^{a}	3.33 ± 0.25 ^{c}	33.79 ± 9.07 ^{a}

The total radioactivity incorporated in the form of soluble organic matter and the relative distribution of ^{14}C in cellular metabolites as a function of pulse time in GC and BC are shown in Figs. 1 and 2, respectively. The total carbon uptake observed in BC (Fig. 1) was slightly lower than in the GC. For the 5- and 10-s pulses, the values obtained (Fig. 2) were almost zero, making the values unusable. The major finding was that the main products of carbon fixation in GC after 20-s radioactive pulses were 3-phosphoglyceric acid (PGA), corresponding to about 39 %, and dihydroxyacetone phosphate (DHAP), corresponding to about 16 %, while in BC the main product was malate (53 %). Most of the radioactivity was transferred into phosphorylated sugars and sucrose in the GC. Radioactivities incorporated into aspartate and glutamate were similar in BC and GC. On the contrary, radioactivity in alanine was significantly higher in BC than in GC. Some of the metabolites of photorespiration (glycine, glycerate, serine) became slightly labelled during the pulse (always accounting for less than 10 % of the total radioactivity). The distribution of the radioactivity among the metabolites of the Calvin-Benson (C_3), β-carboxylation (C_4), and photorespiration pathway during 60 s of ^{14}C pulses is summarized in Table 2. In BC, ^{14}C was preferentially accumulated in the metabolites of β-carboxylation during the first 40 s of the pulse; then (50 and 60 s of pulse) the C_3 and C_4 pathways became equivalent. In cells cultured under un-saturating irradiance the C_3 pathway was always dominant. No significant changes were observed in the photorespiration metabolites, the contents of which were low under all conditions.

CA_{tot} activity was significantly higher in GC than in BC (Table 3). CA_{int} activity was equivalent in both cell types, but a significant decrease in CA_{ext} was observed in BC.

Discussion

The maximum growth rate was linked to the highest irradiance during growth (Table 1). The blue marennine content increased with irradiance whereas the content of Chl *a* in the cell fell (Table 1). A similar pattern was previously reported for the long-term photoacclimation of *H. ostrearia* to high irradiance (Mouget *et al.* 1999). A decrease in contents of photosynthetic pigments, such as Chl *a*, is frequently described in diatoms, phytoplankton, and other algae in high-irradiance acclimation (Cunningham *et al.* 1992, Geider *et al.* 1997, Melis *et al.* 1999).

In *H. ostrearia*, as in other diatoms (Beardall *et al.* 1976, Mortain-Bertrand *et al.* 1987, Descolas-Gros and Oriol 1992, Reinfelder *et al.* 2000, 2004), similar to the C_3 pathway *via* RuBPCO, there is also a complementary carbon-acquisition C_4-like pathway mechanism *via* other carboxylases, such as PEPC, PEPCK, or pyruvate carboxylase. The kinetics of the metabolic pathways of ^{14}C fixation in the BC and GC of *H. ostrearia* show that part of each metabolic pathway varied depending on the culture irradiance. In BC the ^{14}C incorporation into the metabolites of the C_4-like pathway was greater than that into the metabolites of the C_3 pathway. In contrast, in the GC, the C_3 pathway was always dominant (Table 2) with higher ^{14}C incorporation in sucrose and lower in glycerol (data not presented). Those metabolites are not commonly found in diatoms but they have been previously observed during short-term pattern of ^{14}C fixation in *Skeletonema costatum* (Beardall *et al.* 1976, Mortain-Bertrand 1987). On the other hand, incorporation of ^{14}C into photorespiratory metabolites was very low under all conditions (Table 2), a phenomenon commonly reported for other diatoms (Birmingham *et al.* 1982, Mortain-Bertrand 1987). Although the pathway of photorespiration was still not clear in diatoms, molecular evidence for a photorespiratory pathway in *T. weissflogii* was provided by Parker *et al.* (2004). In *T. pseudonana* high irradiance [300 µmol(photon) m^{-2} s^{-1}] among other factors, such as temperature or nitrogen source, seems to stimulate transcription of genes of phosphoglycolate phosphatase and glycine decarboxylase T-protein, both required for photorespiration pathway (Parker and Armbrust 2005). In our very different experimental approach with *H. ostrearia*, stimulation of photorespiratory pathway by high irradiance was not evidenced.

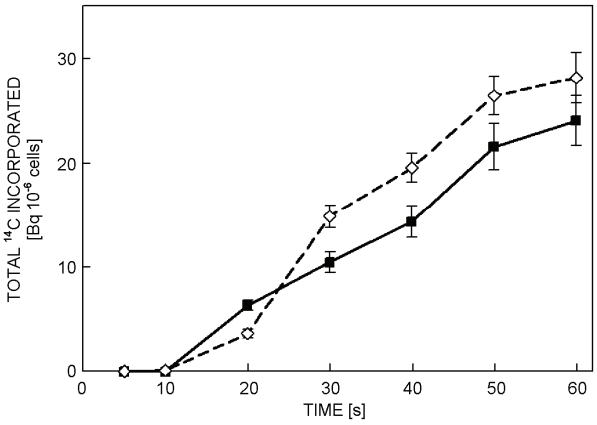

Fig. 1. $NaH^{14}CO_3$ incorporation in total alcohol soluble organic matter *versus* time (0–60 s) in *Haslea ostrearia* cells cultured under 25 (*dashes*) or 350 (*full line*) µmol(photon) m^{-2} s^{-1}. Means in Bq per million cells ± SE (n = 3).

The activity of CA, which plays an essential role in microalgal photosynthesis, was lower in BC than in GC. As suggested by Reinfelder *et al.* (2004) for another diatom, *T. weissflogii*, under low ambient CO_2 in *H. ostrearia* the C_4-like pathway might play an important role in CCM in BC, when CA_{ext} activity is reduced. Under both the irradiances tested here, CA_{int} remained stable, whereas CA_{ext} increased under low irradiance. This change in CA_{ext} activity was not related to the pH of the medium, which remained close to 8.1 under both irradiances at equivalent cell densities (data not shown). Dionisio *et al.* (1989a,b) showed in *Chlamydomonas reinhardtii* that radiant energy, and specifically blue radiation, is required for CA induction, and that CA_{tot}

Table 2. Distribution of ^{14}C [% of total incorporated ^{14}C] amongst Calvin-Benson (C_3), β-carboxylation (C_4), and photorespiration (R_P) metabolites during the first 60 s of incorporation in *Haslea ostrearia* cells cultured under two irradiances [25 or 350 µmol(photon) m^{-2} s^{-1}]. Means ± SE (n = 3).

	Irradiance	20 s	30 s	40 s	50 s	60 s
C_3	25	72.9 ± 3.6	47.8 ± 5.2	53.1 ± 5.8	64.2 ± 6.3	68.9 ± 6.5
	350	23.2 ± 5.8	31.2 ± 5.9	39.3 ± 6.2	43.8 ± 4.8	47.0 ± 4.2
C_4	25	13.8 ± 4.1	44.3 ± 5.8	37.8 ± 5.3	27.9 ± 6.1	23.8 ± 3.4
	350	66.5 ± 9.4	58.8 ± 8.6	42.7 ± 6.5	46.4 ± 4.8	41.1 ± 2.8
R_P	25	13.3 ± 3.1	7.9 ± 2.9	9.1 ± 1.8	7.9 ± 1.3	7.3 ± 1.2
	350	10.3 ± 3.4	10.0 ± 2.1	18.0 ± 1.9	9.8 ± 1.5	11.9 ± 1.6

activity increases with irradiance. In contrast, we found in *H. ostrearia* a negative correlation between the CA_{ext} activity and the irradiance. Under the lower irradiance, the C_3 pathway predominated; the high CA_{ext} activity promoted the supply of CO_2 favourable to RuBPCO activity. On the other hand, under the higher irradiance, the C_4-like pathway took a more active part in carbon assimilation; the CA_{ext} activity fell, even though CO_2 is a PEPCK substrate too. In a recent work (Morant-Manceau *et al.* 2007), we used inhibitors of CA and anion exchange proteins to show that in *H. ostrearia* grown under saturating irradiance about 27 % of fixed inorganic carbon could come from CO_2 uptake and about 13 % could come from CA_{ext} activity. This CO_2 source could be sufficient for PEPCK which has a high affinity for CO_2 (Granum *et al.* 2005). Further work is needed to identify the mechanism underlying the regulation of CA_{ext} in relation to irradiance in *H. ostrearia*.

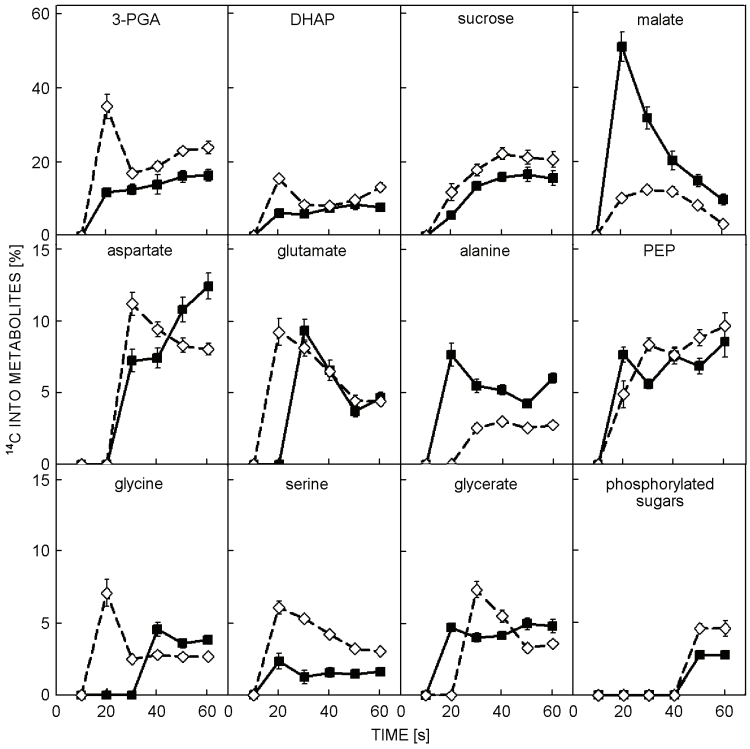

Fig. 2. Kinetics (0–60 s) of ^{14}C labelling into metabolites [3-phosphoglyceric acid (PGA), dihydroxyacetone phosphate (DHAP), phosphoenol pyruvate (PEP)] in *Haslea ostrearia* cells cultured under 25 (*dashes*) or 350 (*full line*) µmol(photon) m^{-2} s^{-1}. Means in % of total activity ± SE (n = 3).

Table 3. Total (CA_{tot}), extracellular (CA_{ext}), and intracellular (CA_{int}) carbonic anhydrase activity of *Haslea ostrearia* cultured under two irradiances [25 or 350 µmol(photon) m^{-2} s^{-1}]. Means ± SE (n = 3). For each column, values that are not significantly different ($p \leq 0.05$) carry the same superscripted letter.

Irradiance [µmol(photon) m^{-2} s^{-1}]	CA_{tot} [E.U. 10^{-6} cells]	CA_{ext}	CA_{int}
25	0.16 ± 0.04 ^{a}	0.09 ± 0.03 ^{a}	0.06 ± 0.01 ^{a}
350	0.10 ± 0.04 ^{b}	0.04 ± 0.01 ^{b}	0.06 ± 0.01 ^{a}

In terms of enzyme compartmentation, the C_4-like metabolism requires carboxylation in the cell cytosol, and decarboxylation in the chloroplast stroma containing RuBPCO. In *H. ostrearia*, only PEPCK activity has been detected (Tremblin and Robert 2001), and this could function as both a carboxylase and decarboxylase. Following Raven and Beardall (2003), we suggest the possibility that the carboxylase activity of PEPCK is promoted in the cytosol, whereas decarboxylase activity is promoted in the chloroplast depending on the relative contents of the substrates (*e.g.* CO_2 and ATP availability) in these cell compartments. As Granum *et al.* (2005) suggest, many aspects (such as the role and biochemical mechanism) of C_4-like carbon fixation in diatoms such as *H. ostrearia* need to be fully elucidated, and further studies are required. A study of the *Phaeodactylum tricornutum* genome has shown that a C_4-like pathway may exist, with the presence of NADP-malate dehydrogenase into the chloroplast that has to be confirmed (V. Martin-Jézéquel, personal communication). Results in *H. ostrearia*, where more than 50 % of the ^{14}C was incorporated into malate after 20 s of pulse (Fig. 2), confirm the importance of the C_4 pathway. Finally, the hypothesis of a possible shift of carbon fixation from a C_3 to a C_4-like pathway has previously been proposed (Tremblin and Robert 2001) when cells progress from the "green" to the "blue" form, and begin to accumulate marennine (Robert 1983). This hypothesis seems to be confirmed here with a lower CA_{ext} activity in BC. It will be interesting to look for a possible link between irradiance stress, carbon fixation pathway, and biosynthesis of the peculiar

secondary metabolite marennine. Pouvreau *et al.* (2006) have shown that marennine could be a polyphenolic compound but its biosynthesis pathway is still unknown.

To complete this biochemical approach, biomolecular investigation was currently in progress in order to quantify in these conditions gene expression of main enzymes of CCM (CA, PEPC, PEPCK) in diatoms.

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