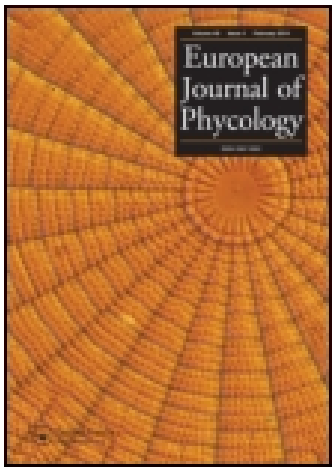

European Journal of Phycology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tejp20

Combined effect of temperature and bleaching herbicides on photosynthesis, pigment and fatty acid composition of Chlamydomonas reinhardtii

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Published online: 24 Nov 2014.

To cite this article: Annie Chalifour, Michael T. Arts, Martin J. Kainz & Philippe Juneau (2014) Combined effect of temperature and bleaching herbicides on photosynthesis, pigment and fatty acid composition of Chlamydomonas reinhardtii, European Journal of Phycology, 49:4, 508-515, DOI: 10.1080/09670262.2014.977962

To link to this article: http://dx.doi.org/10.1080/09670262.2014.977962

Eur. J. Phycol. (2014), **49(4)**: 508–515

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Combined effect of temperature and bleaching herbicides on photosynthesis, pigment and fatty acid composition of *Chlamydomonas reinhardtii*

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(Received 27 September 2013; revised 3 June 2014; accepted 23 June 2014)

Norflurazon (Nf) and fluridone (Fd) are phytoene desaturase inhibitor herbicides that are widely used for the control of grasses and invasive aquatic weeds, respectively. These herbicides enter aquatic environments where they can negatively affect non-target plant species (e.g. algae). Their toxicity towards algae may be modified by abiotic factors such as light intensity, temperature, pH and nutrients. Investigating the effect of low temperature on the toxicity of Nf and Fd is particularly important because both temperature and herbicides affect some of the same physiological process (e.g. carotenoid biosynthesis). Here we demonstrate that Nf reduced photosynthesis in the green alga *Chlamydomonas reinhardtii* more strongly at 15 than at 25ºC, while Fd showed stronger effects at 25 than at 15ºC. Neither herbicide significantly inhibited photosynthesis at 8ºC. Although the overall pigment content decreased with lower temperature, there was an increase in photo-protective carotenoids relative to chlorophylls at both 15 and 8ºC in the absence of herbicides. Moreover, most of the measured pigments decreased markedly in the presence of Nf and Fd at 15 and 25ºC, including β-carotene which fell to below detection limits. The fatty acid composition was modified by temperature and the level of unsaturation noticeably increased at 15 compared with 25ºC. At 8ºC, however, despite a 2.4 times decrease in fatty acid content, the unsaturation level was similar to 25ºC acclimated cells. Monounsaturated fatty acids increased concomitant with a decrease in polyunsaturated fatty acid in the 2.5 µM Nf treatment at 25ºC. Differences in the effect of Nf and Fd on photosynthesis at 15 and 25ºC can be attributed to the marked decrease in carotenoids, which play an important role in photoprotection. At 8ºC, the apparent lack of inhibitory effects compared with control cultures could be due to enhanced photoprotection and/or decreased uptake of herbicides by the alga.

Key words: algae, carotenoids, chlorophyll a fluorescence, fluridone, norflurazon

Introduction

Norflurazon (Nf) and fluridone (Fd) are phytoene desaturase inhibitors widely used as herbicides for the control of grasses and invasive aquatic weeds, respectively. Concentrations as high as 1.19 µM of Nf have been reported in surface run-off waters (Wilson *et al.*, 2007), while concentrations up to 0.46 µM of Fd per annual growth cycle of target plant are allowed to be applied directly to water bodies in the United States (HSDB, 2013). Their mode of action is the inhibition of phytoene desaturase, an enzyme essential for the conversion of the colourless phytoene in phytofluene and ζ-carotene, both precursors of important carotenoids such as β-carotene and lutein (Bartels & Watson, 1978; Arias *et al.*, 2005). The resulting depletion in photoprotective carotenoids leads to chlorophyll oxidation and thus foliage bleaching in the affected plants (Dalla Vecchia *et al.*, 2001).

When applied to the environment, the effect of Nf and Fd on target species will be influenced by abiotic factors such as temperature, light intensity, pH, nutrients and other contaminants (Di Baccio *et al.*, 2002; Puri *et al.*, 2009; Fischer *et al.*, 2010). Temperature, for example, affects many processes which are also targeted by herbicides including photosynthesis, pigment biosynthesis, enzyme activity and protein and lipid metabolism (Raven & Geider, 1988; Huner *et al.*,

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ISSN 0967-0262 (print)/ISSN 1469-4433 (online)/14/040508-515 © 2014 British Phycological Society
http://dx.doi.org/10.1080/09670262.2014.977962

1998). Some studies have found higher toxicity of xenobiotics, such as the photosynthesis-inhibiting herbicide atrazine, on microalgae or cyanobacteria at low temperature (Bérard *et al.*, 1999; Chalifour *et al.*, 2011). Alternatively, a higher toxicity of cadmium or polycyclic aromatic hydrocarbons at higher temperature has been observed in marine algae (Wang *et al.*, 2008; Vieira & Guilhermino, 2012). While Nf and Fd decrease the carotenoid content of cells, low temperature increases the proportion of those photoprotective pigments (Maxwell *et al.*, 1994). Therefore, we expect an antagonistic effect of the two factors on photosynthesis of *C. reinhardtii*. However, interactive effects of these herbicides at different temperatures on freshwater algae are as yet unknown. The aim of this study was thus to examine how acclimation to different temperatures affects the toxicity of Nf and Fd on *C. reinhardtii* and how these interactions may lead to differences in growth, photosynthesis and pigment and fatty acid composition.

Materials and methods

Algal culture and growth conditions

Chlamydomonas reinhardtii P.A.Dangeard wild type strain (CC125) was obtained from the *Chlamydomonas* Resource Center, University of Minnesota (USA) and was cultivated semi-continuously in Erlenmeyer flasks containing 300 ml of High Salt Medium (HSM; Sueoka *et al.*, 1967) in axenic conditions at a pH of 6.8. The cultures were acclimated to three different temperatures (8, 15 and 25ºC) for at least three weeks to achieve constant growth rates under a photosynthetic active radiation (400–700 nm) of 100 µmol photons m^{-2} s^{-1} provided by a combination of incandescent bulbs (Philips 60W) and white fluorescent lamps (Philips F72T8/TL841/HO, USA) with a 14 : 10 h light : dark cycle. To keep cells suspended, cultures were shaken for 30 seconds daily.

Algal cells were harvested during the exponential growth phase and transferred, at an initial density of 2×10^5 cells ml^{-1}, into 500 ml Erlenmeyer flasks containing 300 ml HSM. The cells were then exposed (in triplicate at each temperature) to two concentrations of Nf (1.25 and 2.5 µM; PS-1044, Sigma-Aldrich, Oakville, Canada) and one concentration of Fd (1.25 µM; Fluka 45511, Sigma-Aldrich), both dissolved in dimethyl sulfoxide (DMSO). The final amount of DMSO in control and herbicide-treated flasks was 0.25%.

Cell growth and photosynthesis

After dilution, growth rate (µ) was measured over a period of 96 h using a Coulter Counter particle analyser (Multisizer 3, Beckman Coulter Inc., Fullerton, USA), and all physiological measurements were taken 96 h after transfer to the new medium. Culture growth rates were estimated from the slopes of the linear regressions of the natural logarithm transformed cell biovolume (μm^3 ml^{-1}) versus time.

The maximum (Φ_M) and operational (Φ'_M) PSII quantum yields were evaluated from the fluorescence induction curves obtained with a Water-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) using these equations: $\Phi_M = (F_M\text{-}F_0)/F_M$ (Kitajima & Butler, 1975) and $\Phi'_M = (F'_M\text{-}F_0)/F'_M$ (Genty *et al.*, 1989). All measurements were taken at the growth temperature, using the temperature controller provided with the fluorometer. Prior to each fluorescence measurement, samples were kept in the dark for 15 min at their specific growth temperature to completely re-oxidize the PSII primary electron acceptors.

Pigments

Algal cultures (10 ml) were filtered on a 0.8 µm membrane filter (Whatman nucleopore, Piscataway, USA), frozen in liquid nitrogen and stored in the dark at −80ºC. Pigments were extracted from the filtered cells under green, dim light by adding 1.5 ml of acetone (90%) and sonicating for 30 s with an output power of 3W. The filters were kept in acetone at 4ºC overnight, after which the extracts were filtered through a 0.22 µm syringe filter (Millex-GV, Millipore, Billerica, USA). Extracts were stored at −20ºC until analysis. Chromatographic analyses were performed using a Waters reverse-phase HPLC system comprised of a Millennium 32 program, a model 510 pump, a Waters 2487 absorbance detector (Dual λ) set at 445 nm and a model 7725i Rheodyne injector. Chlorophylls and carotenoids were separated on an Inertsil ODS C18 column (4.6 × 150 mm, 5 µm particle size) protected by an Upchurch Scientific guard column (Oak Harbor, WA, USA) packed with C18 material and using 0.5 µm frits. All pigments were analysed according to the method of García-Plazaola & Becerril (1999). The injection volume was 30 µl for the 25 and 15ºC samples and 60 µl for the 8ºC samples. Pigments were identified and quantified by comparison of retention times and peak area with those of standards of chlorophylls (PPS-CHLA and PPS-CHLB, DHI, Horsholm, Denmark), xanthophylls (PPS-ANTH, PPS-LUTE, PPS-NEOX, PPS-VIOL and PPS-ZEAX, DHI, Horsholm, Denmark) and β-carotene (C4582, Sigma-Aldrich, Oakville, Canada).

Total lipids and fatty acids

For the total lipids and fatty acid (FA) analysis, 100 ml of algal culture were filtered on a pre-combusted, pre-weighed GF/F filter (Whatman, Piscataway, USA) and stored in the dark at −80ºC. Filters were then freeze-dried and re-weighed. Lipids were extracted and analysed as fatty acid methyl esters as in McMeans *et al.* (2012). Briefly, lipids were extracted by homogenizing samples, three times, in 2 ml of 2:1 (v/v) chloroform: methanol (Folch *et al.*, 1957). Fatty acid methyl esters were generated using the sulphuric-methanol (1:100) method, and were subsequently analysed on a Hewlett Packard 6890 GC using splitless injection on a Supelco SP-2560 column and identified using known FA standards. '∑SAFA' indicates the sum of all FA with no double bonds (i.e. saturated FA), '∑MUFA' indicates the sum of all FA with one double bond, and '∑PUFA' indicates the sum of all FA with ≥ 2 double bonds. The unsaturation index (UI) was calculated as the sum of the percentages of individual unsaturated fatty acids multiplied by the number of their double bonds.

Statistical analysis

For comparison of parameter values among the three test temperatures we used ANOVA and Tukey–Kramer *post-hoc* analysis using JMP ver. 10 software (SAS Institute, Cary, NC, USA). Differences were considered significant at $P < 0.05$.

Results

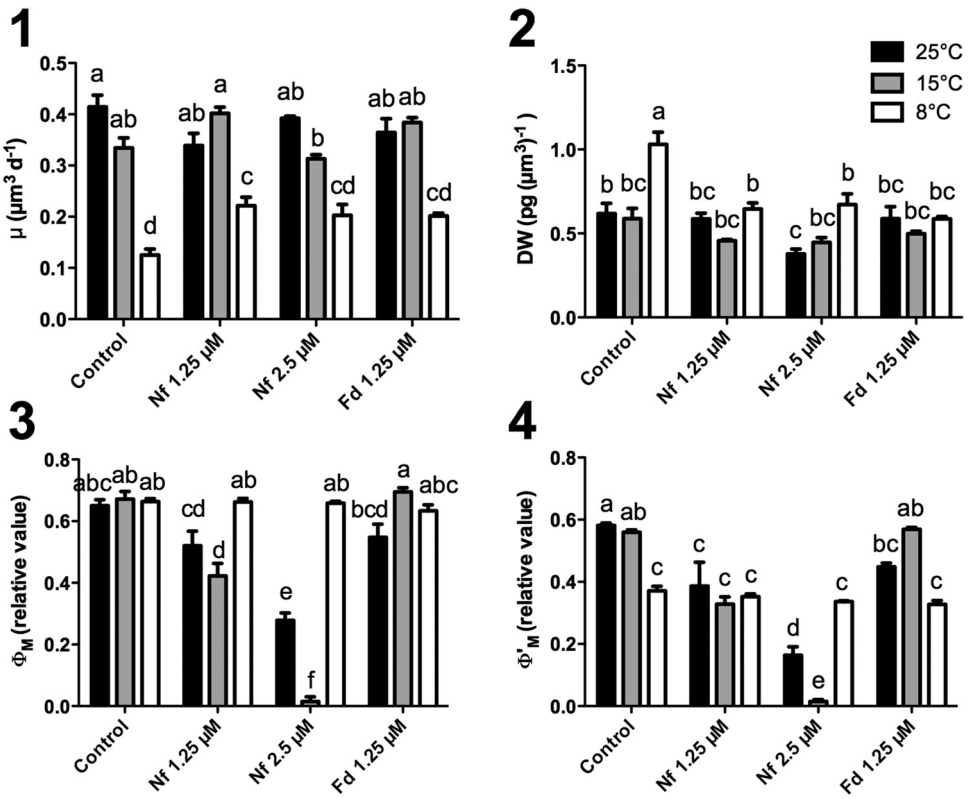
Growth rate (μ) of *C. reinhardtii* increased with temperature (Fig. 1). *Chlamydomonas reinhardtii* μ was not affected by herbicide treatment, except for cells exposed to 1.25 μM Nf at 8ºC, which had a 77% higher μ than the control. On the other hand, dry weight (DW) per cell biovolume (μm^3) was 67% higher at 8°C than at 25°C for the control condition (Fig. 2), and decreased by 39%, 24% and 35% with 2.5 μM of Nf at 25, 15 and 8°C respectively.

As for photosynthesis, the maximal PSII quantum yield (Φ_M) was significantly reduced by 57 and 98% in the 25ºC and 15ºC acclimated cells, respectively, after exposure to 2.5 μM of Nf (Fig. 3). The operational PSII quantum yield (Φ'_M) decreased even more in cells exposed to 2.5 μM Nf, with a 72% lower Φ'_M in the 25ºC cells and complete inhibition in the 15ºC cells (Fig. 4). Φ'_M was only affected by Fd at 25ºC. Neither herbicide significantly affected photosynthesis of *C. reinhardtii* acclimated at 8ºC.

Cold acclimation decreased the content of most pigments, except the photoprotective lutein and zeaxanthin (Table 1). Indeed, the content of these two carotenoids did not decrease at 8°C compared with 25°C and increased by 50% at 15°C compared with 25°C. There was 3.5 times more Chl *a*, 3 times more Chl *b* and 2.6 times more β-carotene at 25 compared with 8°C, resulting in lower Chl *a*:*b* and higher β-carotene:Chl *a* ratios at 8°C. Moreover, the lut+zea:Chl *a* ratio was 1.8 and 3.4 times higher at 15 and 8°C, respectively, than at 25°C.

Most pigments decreased markedly with Nf and Fd at 15ºC and 25ºC (Table 1). The β-carotene content was particularly affected, with a virtual disappearance of this carotenoid when algal cells were exposed to 2.5 μM Nf. Chlorophyll *a* decreased by 50 to 60%, while chlorophyll *b* decreased by 30 to 40% in the highest Nf treatment. The xanthophylls also decreased as a result of herbicide exposure, with ~50% less neoxanthin and lutein+zeaxanthin with Nf (2.5 μM) exposure. Most pigments were more affected by Fd at 25ºC than at 15ºC, but pigments were largely unaffected by herbicide exposure at 8ºC. There was, however, a small, non-significant decrease in β-carotene at 8ºC with both concentrations of Nf.

The total lipids per unit dry weight were about 10% lower at 15°C than at 25°C, but 2.4 times lower at 8°C (Supplementary table 1). The herbicide treatments, at



Figs 1–4. Growth rate of *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf) or fluridone (Fd) for 96 h. **Fig. 1**. Cell biovolume. **Fig. 2**. Dry weight. **Fig. 3**. Maximal quantum yield of photosynthesis. **Fig. 4**. Operational quantum yield of photosynthesis. Letters above bars represent significant differences between treatments at $P < 0.05$.

Table 1. Pigment content (μg mg^{-1} DW) and ratios of pigments of *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf) or fluridone (Fd) for 96 h.

Parameter	Treatment	25°C	15°C	8°C
Chlorophyll *a*	Control	393.12 ± 43.13 a	330.41 ± 36.31 ab	113.73 ± 3.87 f
	Nf 1.25 μM	253.09 ± 40.46 cd	223.75 ± 20.89 cde	116.00 ± 11.57 f
	Nf 2.5 μM	166.92 ± 15.47 ef	161.93 ± 19.22 ef	121.06 ± 15.66 f
	Fd 1.25 μM	215.55 ± 33.21 de	296.50 ± 15.40 bc	128.90 ± 6.39 f
Chlorophyll *b*	Control	142.14 ± 12.77 a	115.89 ± 5.96 bc	46.71 ± 1.12 f
	Nf 1.25 μM	111.03 ± 4.67 bc	98.97 ± 6.07 cd	47.99 ± 1.44 f
	Nf 2.5 μM	86.60 ± 5.21 de	79.24 ± 8.09 e	50.57 ± 1.17 f
	Fd 1.25 μM	99.21 ± 3.84 cd	119.66 ± 6.56 b	55.07 ± 2.82 f
β-carotene	Control	25.71 ± 3.14 a	20.81 ± 2.83 a	10.05 ± 2.27 bc
	Nf 1.25 μM	10.47 ± 2.53 b	4.20 ± 0.22 cd	8.82 ± 1.56 bc
	Nf 2.5 μM	0.00 ± 0.00 d	0.00 ± 0.00 d	7.73 ± 1.17 bc
	Fd 1.25 μM	7.07 ± 1.24 bc	12.80 ± 2.79 b	9.99 ± 2.02 bc
Violaxanthin	Control	1.84 ± 0.37 a	0.72 ± 0.20 b	0.60 ± 0.07 b
	Nf 1.25 μM	0.70 ± 0.43 b	0.32 ± 0.20 b	0.75 ± 0.32 b
	Nf 2.5 μM	0.30 ± 0.17 b	0.08 ± 0.09 b	0.53 ± 0.23 b
	Fd 1.25 μM	0.54 ± 0.33 b	0.77 ± 0.13 b	0.25 ± 0.02 b
Antheraxanthin	Control	0.49 ± 0.14 a	0.55 ± 0.01 a	0.22 ± 0.02 a
	Nf 1.25 μM	0.45 ± 0.12 a	0.40 ± 0.06 a	0.32 ± 0.07 a
	Nf 2.5 μM	0.31 ± 0.28 a	0.24 ± 0.21 a	0.35 ± 0.14 a
	Fd 1.25 μM	0.39 ± 0.09 a	0.43 ± 0.05 a	0.35 ± 0.01 a
Lutein + zeaxanthin	Control	2.25 ± 0.09 cd	3.36 ± 0.17 a	2.21 ± 0.11 cd
	Nf 1.25 μM	1.40 ± 0.09 fg	1.72 ± 0.11 ef	2.01 ± 0.16 de
	Nf 2.5 μM	1.21 ± 0.14 g	1.52 ± 0.14 fg	1.94 ± 0.14 de
	Fd 1.25 μM	1.19 ± 0.05 g	2.57 ± 0.14 bc	2.66 ± 0.15 b
Neoxanthin	Control	3.68 ± 0.24 a	1.95 ± 0.13 c	0.57 ± 0.01 c
	Nf 1.25 μM	1.94 ± 0.27 b	0.99 ± 0.09 c	0.60 ± 0.04 c
	Nf 2.5 μM	1.52 ± 0.23 b	0.92 ± 0.18 c	0.59 ± 0.12 c
	Fd 1.25 μM	1.71 ± 0.29 b	1.63 ± 0.11 b	0.67 ± 0.02 c
β-car: Chl *a*	Control	0.065 ± 0.006 ab	0.063 ± 0.004 ab	0.088 ± 0.017 a
	Nf 1.25 μM	0.042 ± 0.009 bc	0.019 ± 0.003 cd	0.076 ± 0.015 a
	Nf 2.5 μM	n.a.	n.a.	0.064 ± 0.009 ab
	Fd 1.25 μM	0.033 ± 0.005 c	0.043 ± 0.008 bc	0.078 ± 0.019 a
Lut + zea: Chl *a*	Control	0.006 ± 0.001 d	0.010 ± 0.001 c	0.019 ± 0.000 ab
	Nf 1.25 μM	0.006 ± 0.001 cd	0.008 ± 0.001 cd	0.017 ± 0.003 ab
	Nf 2.5 μM	0.007 ± 0.000 d	0.009 ± 0.001 c	0.016 ± 0.001 b
	Fd 1.25 μM	0.006 ± 0.001 d	0.009 ± 0.000 cd	0.021 ± 0.002 a

Data shown are means (n = 3) with standard deviation. The letters represent the significant difference between treatments for each pigment or ratio at $P < 0.05$.

Table 2. Relative amount (% of total measured fatty acids) of omega-6 (n-6), omega-3 (n-3), saturated fatty acid (SAFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and unsaturation index (UI) (as % of control) of *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf) or fluridone (Fd) for 96 h.

Parameter	Treatment	25°C	15°C	8°C
∑n-3	Nf 1.25 μM	96% ± 3% a	91% ± 15% a	117% ± 19% a
	Nf 2.5 μM	71% ± 3% *a	90% ± 31% a	108% ± 9% a
	Fd 1.25 μM	92% ± 6% b	93% ± 7% b	109% ± 3% a
∑n-6	Nf 1.25 μM	75% ± 7% *b	78% ± 15% b	111% ± 5% a
	Nf 2.5 μM	46% ± 5% *b	67% ± 28% b	112% ± 11% a
	Fd 1.25 μM	65% ± 5% *c	90% ± 2% b	106% ± 3% a
∑SAFA	Nf 1.25 μM	104% ± 2% a	88% ± 13% a	126% ± 41% a
	Nf 2.5 μM	91% ± 6% a	115% ± 23% a	107% ± 8% a
	Fd 1.25 μM	101% ± 1% b	100% ± 9% b	129% ± 6% a
∑MUFA	Nf 1.25 μM	95% ± 2% a	82% ± 13% a	126% ± 35% a
	Nf 2.5 μM	82% ± 5% *a	93% ± 24% a	108% ± 10% a
	Fd 1.25 μM	93% ± 4% a	96% ± 4% a	86% ± 45% a
∑PUFA	Nf 1.25 μM	93% ± 4% a	90% ± 15% a	116% ± 17% a
	Nf 2.5 μM	67% ± 3% *a	88% ± 31% a	109% ± 9% a
	Fd 1.25 μM	88% ± 6% b	93% ± 6% b	109% ± 3% a
Unsat index	Nf 1.25 μM	98% ± 2% b	102% ± 1% a	102% ± 2% a
	Nf 2.5 μM	92% ± 3% *a	92% ± 6% a	100% ± 0% a
	Fd 1.25 μM	97% ± 2% a	99% ± 4% a	96% ± 4% a

Data shown are means (n = 3) with standard deviation. The asterisk (*) represents a significant difference from the control condition (no herbicide) and the letters indicate a significant difference between the acclimation temperatures at $P < 0.05$.

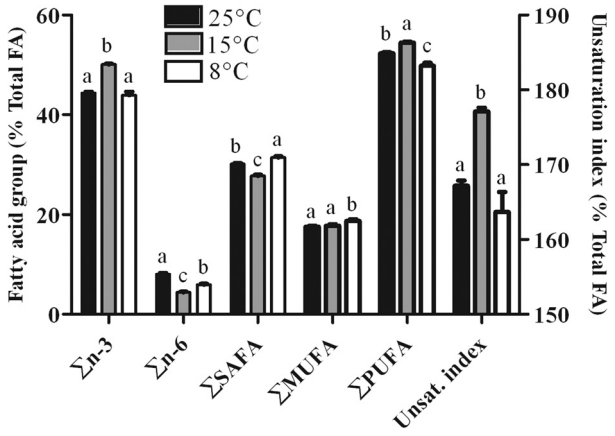


Fig. 5. Relative amounts (% of total measured fatty acids) of omega-6 (n-6), omega-3 (n-3), saturated fatty acid (SAFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and unsaturation index (UI) of *Chlamydomonas reinhardtii* acclimated to three different temperatures. Letters above bars represent the significant differences between treatments at $P < 0.05$.

both 25ºC and 15ºC, also caused a decrease in *C. reinhardtii* total lipid content. This was particularly evident in the 2.5 µM Nf treatment, where 45 and 37% less lipid (on a µg lipid mg DW^{-1} basis) was recorded at 25ºC and 15ºC, respectively (Supplementary table 1). There were significantly more n-3 FAs at 15°C and more n-6 at 25°C than at the two other corresponding temperatures, the 8°C-grown cells having only lower amounts of n-6 FAs when compared with 25°C (Fig. 5). The cells acclimated to 8°C also had higher contents of SAFA and MUFA per unit biomass, but less PUFA than cells grown at 15°C or 25°C. Cells acclimated to 15°C had significantly higher contents of PUFA and lower content of SAFA, resulting in a higher UI value than at the other two temperatures.

Cold acclimation decreased the mass fractions (µg mg^{-1} dry weight) of most individual FAs (Supplementary table 1). Some FAs (e.g. 16:1n-7, 20:0, 20:1n-9 and 20:2n-6) had similar mass fractions among the different temperatures. Traces of 20:3n-3, 22:1n-9 and 26:0 were also measured in *C. reinhardtii* grown at 8°C; these FAs were not present when algal cells were acclimated to higher temperatures in the control condition.

There was also a 7 times increase in 15:0i FA in cells exposed to 2.5 µM of Nf at 15ºC. A decrease in MUFA and PUFA was also observed (Table 2 and Supplementary table 1), particularly 18:2n-6 with the three herbicide treatments at 25ºC and Nf (2.5 µM) at 15ºC. We noticed a 2.7 times and 6.7 times increase in 26:0 at 25ºC and 15ºC respectively when algae were exposed to the highest concentration of Nf. At 8ºC, the FA composition of herbicide-treated *C. reinhardtii* was the same as in the control treatment. The decrease in some unsaturated FAs resulted in lower total omega-3 and omega-6 FA contents, particularly at 25ºC and when exposed to the higher Nf concentration (2.5 µM) (Table 2).

Discussion

Norflurazon (Nf) and fluridone (Fd) are phytoene desaturase inhibitors that interfere with carotenoid biosynthesis (Bartels & Watson, 1978). The depletion of carotenoids leads to chlorophyll oxidation and oxidative damage of photosynthetic components (Dankov *et al.*, 2009), which can explain the lower Φ_M and Φ'_M values we observed in algal cells exposed to these herbicides. *Chlamydomonas reinhardtii* acclimated to 25ºC was less sensitive to Fd (1.25 µM) than to Nf at the same concentration, which is probably due to differences in the location of the herbicide binding sites on the enzyme phytoene desaturase. Nf has been shown to displace the cofactor plastoquinone on its binding site (Breitenbach *et al.*, 2001), but the exact mode of action of Fd has not yet been elucidated.

Although Nf strongly or completely inhibited photosynthesis at the two highest temperatures, no significant decrease in growth rate was observed. This is in agreement with previous findings of only a slight effect on cell growth when *C. reinhardtii* was exposed to Nf 10 µM, a concentration 4 times higher than the highest concentration used in our study (2.5 µM) (Nestler *et al.*, 2012). Considering the strong decrease in photosynthetic efficiency with this herbicide, we suggest that, over the course of 4 d, *C. reinhardtii* was using its energy reserves to maintain a similar growth rate in cultures exposed to Nf or Fd, compared with the controls. This hypothesis is supported by the observation that dry weight per unit cell biovolume decreased by 25–40% in cells exposed to 2.5 µM of Nf at 8, 15 and 25ºC, compared with control (Fig. 2). Jahnke & Mahlmann (2010) also observed a decrease in dry weight per unit biovolume when *Phormidium autumnale* was grown under low light compared with high light, a condition that also decreased the growth rate and photosynthesis of various phytoplankton species (Deblois *et al.*, 2013).

We observed a large decrease in pigment concentration, particularly the chlorophylls, when algae were grown at lower temperatures. However, at 8°C, the 1.4 times higher ratio of β-car:Chl *a* and the 3.4 times higher ratio of Lut+zea:Chl *a* indicate a greater resistance to photoinhibition through heat dissipation by those carotenoids. The presence of a higher proportion of xanthophylls and β-carotene helps protect the photosynthetic apparatus against high excitation pressure (Maxwell *et al.*, 1994; Król *et al.*, 1999; Demmig-Adams & Adams III, 2006). The similar maximal PSII quantum yields (Φ_M) in control treatments for the different acclimation temperatures indicated that the integrity of PSII was conserved at all temperatures.

Carotenoids also act as natural antioxidants that quench triplet chlorophyll and singlet oxygen. There was only a slight decrease of Φ'_M, β-carotene and lutein+zeaxanthin in *C. reinhardtii* when exposed to these herbicides at 8ºC. The higher tolerance of 8ºC-grown *C. reinhardtii* photosynthesis to Nf was also observed with higher concentration of this herbicide (5 µM; data not shown). The absence of inhibitory effects of bleaching herbicides at 8ºC may be rationalized if there is an increased phytoene desaturase content or activity. This increase has been shown in response to high light (Huang *et al.*, 2002) and it is also known that low temperatures induce similar modifications in pigments (Maxwell *et al.*, 1994). For example, a mutant of *Synechococcus*, that had a deletion mutation in the promoter region of the *pds* gene, contained more carotenoids than the wild type and was resistant to Nf and Fd (Chamovitz *et al.*, 1993). It is also known that Nf interacts with the cofactors NADP or plastoquinone for the phytoene desaturase enzyme (Breitenbach *et al.*, 2001). Low temperature has been associated with a significant increase in plastoquinone (Griffith *et al.*, 1984; Gálvez-Valdivieso *et al.*, 2010). It is therefore possible that at low temperature the effect of Nf is less important since there is a competition with the larger amount of plastoquinone available.

At 25ºC and 15°C, carotenoid content of *C. reinhardtii* was greatly reduced in the presence of the two herbicides. It is noteworthy that, with a similar decrease in most pigments in the Nf treatment at 15ºC and 25ºC, Nf had much more effect on Φ_M and Φ'_M at 15ºC than at 25ºC. This observation is in line with the important role that carotenoid pigments play at low temperature in terms of protecting the photosynthetic apparatus (Huner *et al.*, 1998). The strong reduction in Φ_M at 15ºC also confirmed the importance of β-carotene in maintaining the stability of PSII as suggested by Trebst & Depka (1997). Wagner *et al.* (2002) also showed a drastic diminution in D1 protein content – another sign of photoinhibition – of tobacco leaves treated with Nf.

We found a decrease in total lipids and most individual fatty acids (FA) at 8ºC compared with 25°C. A similar decrease in total lipids was recently observed with *Chlamydomonas globosa* acclimated to 10ºC compared with 25°C (Piepho *et al.*, 2012) and these authors also found a similar FA composition at the two temperatures. Only the 15°C-acclimated cells had significantly higher and lower levels of 18:3n-3 and 18:2n-6, respectively. This difference was not measured for the algae acclimated to 8°C, resulting in a UI that was similar between the 25ºC and 8°C grown *Chlamydomonas*. Those results are in contradiction with many studies that related the increase in membrane fluidity at low temperature to an increase in unsaturated fatty acids (Murata & Siegenthaler, 1998; Los & Murata, 2004; Guschina & Harwood, 2006). On the other hand, Arts & Kohler (2009) pointed out that MUFA, in combination with certain PUFAs in phospholipids, are the most important FA with respect to membrane fluidity. We note that the relative MUFA content in 8°C acclimated cells was significantly higher than at 15 or 25ºC in our study.

Nf has also been shown to affect the FA composition of plants (Abrous *et al.*, 1998; Di Baccio *et al.*, 2002). These authors suggested that Nf could affect FA desaturases in addition to phytoene desaturase resulting in a decrease in PUFA (Abrous-Belbachir *et al.*, 2009). It is possible, as suggested by Abrous-Belbachir *et al.* (2009), that Nf inhibited some FA desaturases. In this case, as for the above explanation for pigment biosynthesis, Nf would have relatively less effect on 15°C-acclimated algae, in which a higher synthesis of PUFA is measured (shown by the higher UI at this temperature) than at 25°C. This hypothesis is corroborated by the non-significant decrease in MUFA and PUFA at 15°C in the presence of Nf, compared with the control. However, we also suggest that the effect of Nf exposure on FA desaturation was an indirect consequence of the inhibition of photosynthesis because, in addition to ATP and NADPH, FA desaturases also depend on ferredoxins as electron donors (Schmidt & Heinz, 1990). As measured in our study, the strong inhibition of Φ'_M indicates fewer electrons going through PSI to ferredoxin. Whether mono- or polyunsaturated fatty acids were decreased by FA desaturases or indirectly by decreased photosynthesis remains to be elucidated. Considering the importance of MUFA and PUFA in the stability of the required photosynthetic components (Sato *et al.*, 1996), their decrease in the presence of Nf and Fd might also have contributed to the observed inhibition of Φ_M and Φ'_M.

Kent & Caux (1995) found a significant relationship between the bioaccumulation of the lipophilic insecticide fenitrothion and algal lipid content; *C. reinhardtii* with > 6 times the total lipid content of *Chlamydomonas segnis* also accumulated 3 times the amount of fenitrothion. In our study, *C. reinhardtii* acclimated to 15 and 25ºC had ~ 2.4 times more lipid per dry mass than cells acclimated to 8ºC. We also observed that Fd had a greater effect on photosynthesis at 25ºC than at 15ºC, while Nf had a greater effect at 15ºC. This could also be linked to the octanol-water partition coefficient, known to influence the adhesion of herbicides to cell surface (adsorption) and uptake or penetration of herbicides into cells (absorption) (Dosnon-Olette *et al.*, 2011; Sun *et al.*, 2012), which is higher for Fd (3.16) than Nf (2.30) (Sabljic *et al.*, 1995). It was shown that the water solubility of chemicals, and therefore their octanol-water partition coefficient, varied differently with temperature (Shiu *et al.*, 1997), which might explain the discrepancy in the effect of Nf and Fd at 8, 15 and 25°C in our study. Moreover, the physical properties of the membrane can also influence the translocation and distribution of

toxicants having various lipophilicity (Baynes & Hodgson, 2004). Thus, the proportion of proteins, the presence of unsaturated fatty acids and other antioxidants (such as tocopherols and carotenoids), all of which are modified by temperature, will affect the movement of molecules such as herbicides, across membranes. The uptake of Nf and Fd by the cells might have been different at the different temperatures due to the modifications in FA composition. Indeed, this hypothesis would require more investigations, such as measurement of the amount of herbicide incorporated into the cells. Our findings that some herbicides can have a greater effect at a higher temperature is a major concern for water quality issues around the world, especially considering the increase in water temperature expected with global warming.

Acknowledgements

We would like to thank Martina Rudy and Jerry Chao (both at Environment Canada) and Francis Racine (UQAM) for their technical assistance in this project. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) (to P.J.), and by Environment Canada (to M.T.A.). M.J.K. was supported by the Austrian Science Fund grant P22006-B17. A.C. received an Alexander-Graham-Bell scholarship from NSERC and a travel scholarship from the International Relation Services of UQAM.

Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org/10.1080/09670262.2014.977962

Supplementary Table 1. Mass fractions of fatty acids (μg mg^{-1} DW) and total lipid (μg mg^{-1} DW) in *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf) or fluridone (Fd) for 96 h.

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