

# Recovery kinetics of photochemical efficiency in winter stressed conifers: the effects of growth light environment, extent of the season and species

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Evergreens undergo reductions in maximal photochemical efficiency ( $F_v/F_m$ ) during winter due to increases in sustained thermal energy dissipation. Upon removing winter stressed leaves to room temperature and low light,  $F_v/F_m$  recovers and can include both a rapid and a slow phase. The goal of this study was to determine whether the rapid component to recovery exists in winter stressed conifers at any point during the season in a seasonally extreme environment. Additional goals were to compare the effects of species, growth light environment and the extent of the winter season on recovery kinetics in conifers. Four species (sun and shade needle) were monitored during the winter of 2007/2008: eastern white pine (*Pinus strobus*), balsam fir (*Abies balsamea*), *Taxus cuspidata* and white spruce (*Picea glauca*).  $F_v/F_m$  was measured in the field, and then monitored indoors at room temperature and low light for 6 days. The results showed that all species showed a rapid component to recovery in early winter that disappeared later in the season in sun needles but was present in shade needles on most days monitored during winter. There were differences among species in the recovery kinetics across the season, with pine recovering the most slowly and spruce the most quickly. The results suggest an important role for the rapidly reversible form of energy dissipation in early winter, as well as important differences between species in their rate of recovery in late winter/early spring which may have implications for spring onset of photosynthesis.

## Introduction

Evergreens living in seasonally extreme environments undergo a dramatic reduction in their maximal photochemical efficiency (measured as the chlorophyll fluorescence parameter,  $F_v/F_m$ ) during winter, which is associated with the maintenance of high levels of a sustained form of thermal energy dissipation (Ottander et al. 1995, Verhoeven et al. 1998, 1999, 2009, Adams et al. 2001, 2004, Öquist and Huner 2003). It is thought that during winter, when photosynthetic carbon

reduction is severely inhibited and growth ceases, the photosynthetic apparatus shifts from one that is highly efficient in harvesting light for photosynthesis to one that maximizes safe thermal dissipation of excessive light energy (for reviews see Adams et al. 2002, 2004, Öquist and Huner 2003, Demmig-Adams and Adams 2006, Ensminger et al. 2006). This transformation of the photosynthetic apparatus involves maintained high levels of the xanthophyll pigments zeaxanthin and antheraxanthin, and changes in the relative abundance

**Abbreviations** – A, antheraxanthin;  $F_v/F_m$ , the ratio of variable to maximal chlorophyll fluorescence; NPQ, non-photochemical quenching of chlorophyll fluorescence; qE, dependent quenching; qI, photoinhibitory; Z, zeaxanthin.

and/or organization of the photosynthetic proteins (Ottander et al. 1995, Savitch et al. 2002, Ensminger et al. 2004, Zarter et al. 2006, Busch et al. 2007, 2008, Verhoeven et al. 2009).

Upon removing winter stressed leaves to room temperature and low light,  $F_v/F_m$  will recover up to unstressed values over the course of several days (Ottander and Öquist 1991, Ottander et al. 1995, Verhoeven et al. 1996, 1998, 1999). The kinetics of recovery consists of two phases that differ in their relaxation kinetics. In some species and/or growth conditions, there is a rapid component to recovery that occurs on the time scale of minutes to hours after warming. This rapid component to recovery is only present on days when temperatures are below 0°C, and is more pronounced in leaves growing in shaded environments relative to sun-exposed leaves, which may have no rapid component to their recovery (Verhoeven et al. 1998, 2009). The rapid form of recovery has been induced in plants in growth chamber experiments (Hurry and Huner 1992, Gilmore and Björkman 1994, 1995, Verhoeven et al. 1998) but reported in only a limited number of field observations (Verhoeven et al. 1998, 1999, 2009), and thus has been relatively little studied. The slower phase to recovery occurs over several days after warming, is more pronounced in leaves acclimated to high light environments, and can be blocked by protein synthesis inhibitors (Ottander and Öquist 1991, Hurry and Huner 1992, Ottander et al. 1995, Verhoeven et al. 1998). This form of recovery has been observed in the field in a variety of species spanning temperate to boreal forests and has been studied somewhat more extensively (Ottander and Öquist 1991, Ottander et al. 1995, Adams et al. 2002, 2004, Öquist and Huner 2003, Demmig-Adams and Adams 2006, Ensminger et al. 2006).

Both the rapidly and slowly reversible components of reduced  $F_v/F_m$  correlate with the presence of zeaxanthin and antheraxanthin (Z + A), suggesting that xanthophyll cycle associated energy dissipation is involved in maintaining the observed low photochemical efficiencies (Verhoeven et al. 1996, 1998, 1999). Additionally, the difference in the kinetics of recovery (rapid vs slow) suggests two mechanisms are maintaining the sustained energy dissipation. One mechanism, which is maintained only at sub-zero temperatures, seems to be a flexible mechanism for retaining sustained energy dissipation only on nights when low temperatures will preclude photosynthesis the following morning. Because of its rapid reversal upon warming, this mechanism has been suggested to involve low temperature maintenance of a transmembrane  $\Delta pH$ , which relaxes quickly upon warming in darkness (Gilmore and Yamamoto 1992,

Gilmore and Björkman 1994, 1995, Gilmore 1997, Verhoeven et al. 1998). The second mechanism is sustained regardless of the external temperature and thus is less flexible in responding to changes in temperature. This mechanism for sustained energy dissipation has been suggested to involve protein reorganization such that the xanthophyll cycle is retained in a conformation for energy dissipation in the absence of a transthylakoid  $\Delta pH$  (Gilmore and Ball 2000, Öquist and Huner 2003, Ensminger et al. 2004, 2006, Demmig-Adams and Adams 2006, Verhoeven et al. 2009).

Preliminary examination of recovery of evergreens monitored in January in Minnesota showed an absence of the rapid component to recovery in several conifer species (unpublished observations). Most of the studies documenting the rapid phase to recovery have been done in a milder climate (the foothills of Colorado, mean temperature in January is 11°C warmer than in St. Paul, MN, Verhoeven et al. 1996, 1998). Therefore a major goal of the research presented here was to monitor the recovery kinetics of several conifer species over the course of the winter season, in order to determine whether the flexible rapidly reversible form of energy dissipation is observed at any point during winter in a seasonally more extreme environment. Multiple conifers species were compared in order to assess whether there are differences among species in their recovery kinetics, which might reflect species-specific differences in the mechanism, timing, or extent, of acclimation to winter stress. Selected species covered a variety of ranges, and included eastern white pine (*Pinus strobus*), balsam fir (*Abies balsamea*) and white spruce (*Picea glauca*), which have the following approximate latitudinal ranges; pine 36–51°N, fir 45–58°N and spruce 46–65°N. In three species, both sun and shade needles were monitored in order to probe the effect of the growth light environment on the recovery kinetics. Finally recovery kinetics was monitored on several dates throughout winter in order to determine whether the kinetics of recovery is altered as the season progresses.

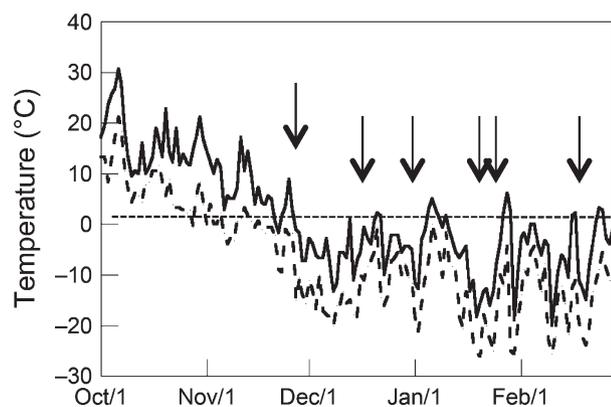
Recent studies examining desiccation-tolerant mosses and ferns have shown that upon desiccation, these species have dramatically reduced  $F_v/F_m$  values that also correlate with dark-retention of the xanthophylls (Z + A), suggesting nocturnal maintenance of a sustained form of xanthophyll-dependent energy dissipation in these species (Fernández-Marín et al. 2009, 2010, 2011, Proctor 2010). Upon rehydration, these species show a rapid recovery of  $F_v/F_m$  that correlates with reconversion of zeaxanthin to violaxanthin. In a recent study by Proctor (2010), recovery profiles were fitted to logistic curves in order to allow for better comparative data. A secondary goal of the present study was to parameterize

the recovery kinetics of winter stressed conifers by fitting logistic curves in a manner similar to Proctor (2010), both to determine whether there is a similar goodness of fit as occurs during rehydration of mosses, and to provide parameters for a statistical comparison of recovery kinetics.

## Materials and methods

### Plant material and growth environment

Four species of conifers were monitored from November of 2007 through February of 2008: eastern white pine (*P. strobus*), balsam fir (*A. balsamea*), *Taxus cuspidata* and white spruce (*P. glauca*). All plants were growing on the campus of the University of St. Thomas, in Saint Paul, Minnesota (44°59'40"N, 93°05'35"W). For each species, three individual trees were sampled on each date. For pine, only sun needles were sampled, while for the remaining three species both sun and shade needles were sampled. For balsam fir and *Taxus*, shade needles were collected from trees/shrubs on the north facing sides of buildings, while sun needles were collected from separate trees/shrubs growing in sun-exposed environments. For spruce, sun needles were collected on the south facing side of the trees, which were exposed to full sun throughout the day, while shade needles were collected from the shaded interior of the tree. Maximum and minimum temperatures during the period of sampling are shown in Fig. 1. Air temperature at the time of sampling was measured using a weather station (Texas Weather Instruments WRL-32, Texas Weather Instruments Inc., Dallas, TX, USA) located on top of a building on campus where all the sampling was performed. Dates of sampling, and the temperature at the time of sampling were



**Fig. 1.** Maximum (solid line) and minimum (dashed line) temperatures during the sampling period, with arrows indicating the approximate dates of sampling.

November 29, 2007 (−5°C), December 17, 2007 (−6°C), January 1, 2008 (−16°C), January 21, 2008 (−13°C), January 28, 2008 (4°C) and February 28, 2008 (−5°C).

### Monitoring recovery kinetics of winter stressed needles

The chlorophyll fluorescence parameter,  $F_v/F_m$ , was measured on dark-acclimated needles in the field using a field portable Fluorescence Monitoring System (FMS, Hansatech, King's Lynn, UK). Measurements were done at around 21:00 h, when all trees/shrubs had been in darkness for at least 3 h. For each species, two measurements were conducted on each of three trees/shrubs. After measuring  $F_v/F_m$ , the twig on which the measurement was made was cut and placed in a Petri dish and samples were taken indoors to room temperature and low light (20°C and light between 5 and 10  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). All needles were maintained in constant conditions for up to 6 days.  $F_v/F_m$  was measured after 0.5, 1.5, 10, 24, 48, 72, 96 and 144 h of recovery.

### Statistical analysis

In order to determine if there was a rapid component to recovery, paired *t*-tests were performed on the  $F_v/F_m$  values collected in the field compared with the  $F_v/F_m$  values measured after 30 min warming using Microsoft Excel.

Recovery curves were fitted to semi-log data (time was plotted on a log scale) using the software package GRAPHPAD PRISM. A four-parameter logistic curve was used to fit the data, following Proctor (2010). The built-in function for a dose–response curve was used which used the following equation where *L* is the lower asymptote or baseline  $F_v/F_m$  at time 0, *U* is the upper asymptote which represents the maximal range of  $F_v/F_m$  or final recovered value,  $t_{1/2}$  is the time of half recovery in hours and *S* is the slope of the curve.

$$Y = L + \frac{(U - L)}{1 + 10^{[\log_{10}(t_{1/2} - x) * S]}}$$

Both *U* and *L* were constrained such that *U* could not be greater than 0.85 and *L* could not be less than zero. An analysis of the residuals was performed on all curves, and showed that the residuals followed a Gaussian distribution. Values for  $t_{1/2}$  were determined from the fitted curves for each sample measured, and these data were analyzed with the software package SAS using a mixed model ANOVA with date and species as fixed effects. Additional comparisons were done using the Tukey–Kramer HSD comparison.

**Table 1.** Dark acclimated  $F_v/F_m$  measured in the field after at least 3 h of darkness. Data are means  $\pm$  SD,  $n = 3$ . Air temperature at the time of measurement is indicated.

Date		November 29	December 17	January 1	January 21	January 28	February 28
Temperature ( $^{\circ}$ C)		-5	-6	-16	-13	4	-5
Pine	Sun	0.38 $\pm$ 0.07	0.08 $\pm$ 0.03	0.05 $\pm$ 0.04	0.09 $\pm$ 0.04	0.13 $\pm$ 0.08	0.02 $\pm$ 0.01
Fir	Sun	0.34 $\pm$ 0.06	0.19 $\pm$ 0.12	0.13 $\pm$ 0.03	0.16 $\pm$ 0.11	0.29 $\pm$ 0.08	0.12 $\pm$ 0.04
	Shade	0.53 $\pm$ 0.09	0.50 $\pm$ 0.16	0.26 $\pm$ 0.07	0.14 $\pm$ 0.05	0.50 $\pm$ 0.08	0.22 $\pm$ 0.11
Yew	Sun	0.34 $\pm$ 0.03	0.15 $\pm$ 0.04	0.16 $\pm$ 0.04	0.12 $\pm$ 0.04	0.27 $\pm$ 0.15	0.14 $\pm$ 0.09
	Shade	0.67 $\pm$ 0.04	0.45 $\pm$ 0.14	0.31 $\pm$ 0.04	0.28 $\pm$ 0.03	0.53 $\pm$ 0.07	0.22 $\pm$ 0.12
Spruce	Sun	0.25 $\pm$ 0.03	0.12 $\pm$ 0.02	0.05 $\pm$ 0.02	0.15 $\pm$ 0.11	0.27 $\pm$ 0.09	0.11 $\pm$ 0.05
	Shade	0.52 $\pm$ 0.10	0.40 $\pm$ 0.06	0.22 $\pm$ 0.09	0.23 $\pm$ 0.06	0.47 $\pm$ 0.04	0.24 $\pm$ 0.03

## Results

Dark-acclimated values of  $F_v/F_m$  measured in the field on six dates during the winter of 2007 and 2008 are depicted in Table 1 for the four species monitored. Typical winter reductions in  $F_v/F_m$  were observed in all species and light conditions. For needles acclimated to sun conditions, the values of  $F_v/F_m$  in November (in the range of 0.25–0.38) were higher than they were for the remainder of the winter when measurements were conducted on sub-zero nights (values in the range of 0.02–0.2). For the shade needles, the magnitude of the reduction of  $F_v/F_m$  was less than that observed in the sun needles throughout the winter, as has been reported previously (Verhoeven et al. 1999, 2009, Porcar-Castell et al. 2008). The magnitude of the reduction of  $F_v/F_m$  in shade needles increased throughout the winter season on sub-zero nights. On the warm winter night (January 28) both sun and shade needles showed increases in  $F_v/F_m$  relative to the sub-zero nights, except for pine, which maintained very low  $F_v/F_m$ .

Recovery of  $F_v/F_m$ , upon artificially warming needles, was monitored for 6 days in the four species of conifers on five dates when the overnight temperatures were below  $0^{\circ}$ C (Fig. 2).  $F_v/F_m$  values increased throughout the recovery period in all species and light conditions. The shade needles, which had maintained higher  $F_v/F_m$  values, recovered more quickly and more fully than the sun needles in all cases. All species showed two phases (a rapid and a slow phase) to recovery early in the season. Following the November measurement, the recovery process proceeded much more slowly in the sun needles.

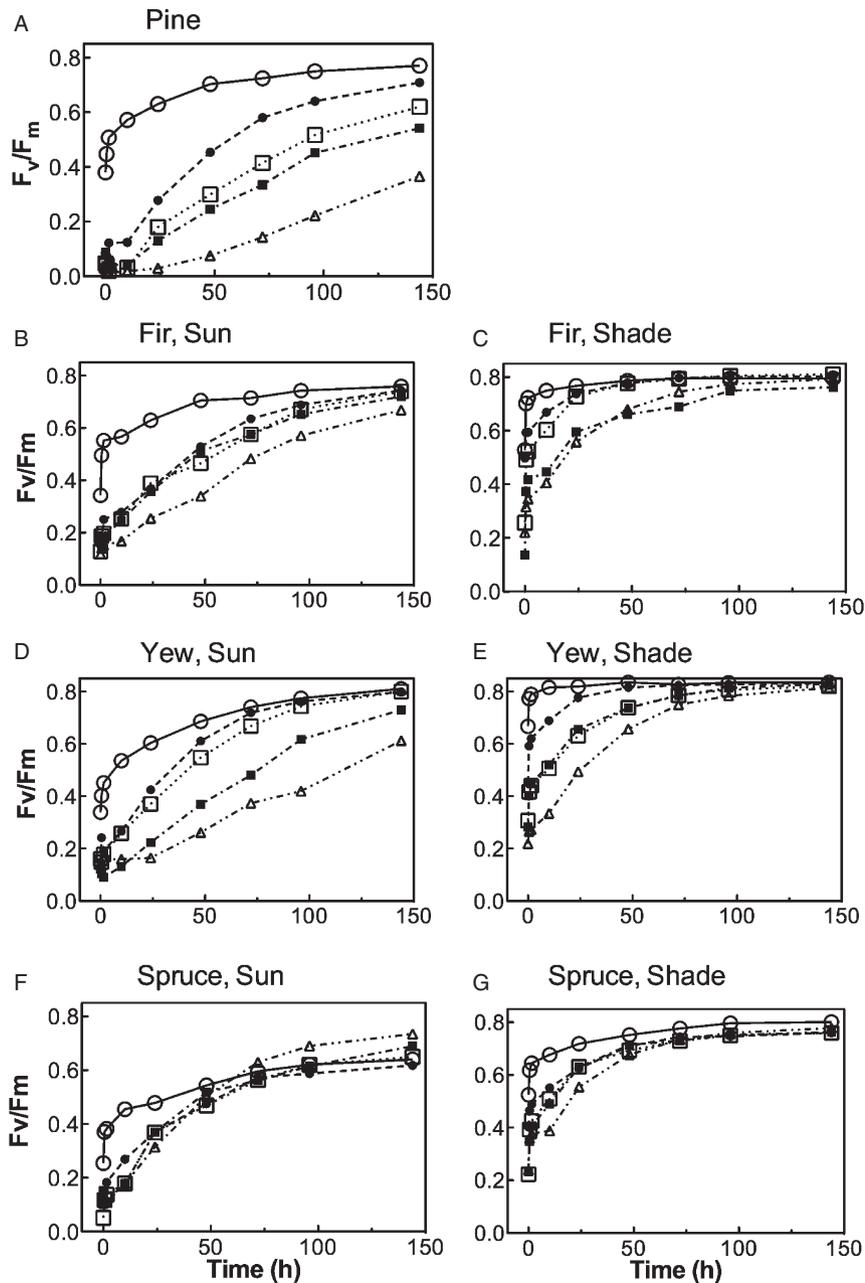
### The rapid component of recovery

A major goal of this study was to determine whether the rapid component of recovery occurs at any time during the season in a seasonally extreme winter environment. In order to answer this question, paired  $t$ -tests were performed on needles from all species and

light environments on all the sampling dates comparing  $F_v/F_m$  measured in the field with  $F_v/F_m$  of the same needles after 30 min at room temperature (Table 2). For all species from both high and low light environments there was a rapid component to recovery when measured early in the season (November), indicated by a significant increase in  $F_v/F_m$  upon warming for 30 min. During the rest of the season there were differences in the responses of needles collected from sun acclimated vs shade acclimated conditions. For sun-acclimated needles, there was typically no significant change in  $F_v/F_m$  upon warming the needles. The only exception to this was on January 1st when temperatures were the coldest of any measurement day ( $-16^{\circ}$ C), when both sun fir and sun spruce needles showed a small but significant increase in  $F_v/F_m$  upon warming the needles. In contrast, the needles collected from shade acclimated conditions showed significant increases in  $F_v/F_m$  upon 30 min warming on almost all the dates measured when temperatures were below  $0^{\circ}$ C. On January 28, when temperatures were above  $0^{\circ}$ C, there was no change in  $F_v/F_m$  upon warming in any of the species.

### Curve fitting of recovery kinetics

The recovery data were re-plotted on semi-log plots (Fig. 3) in order to better observe the rapid component to recovery, and in order to determine whether the recovery kinetics can be well described with a logistic curve, as reported by Proctor (2010) for fluorescence recovery of desiccated moss and ferns. These figures accentuate the difference in the recovery kinetics in the sun-exposed needles on November 29th compared with the remainder of the winter, suggesting a major transformation in the photosynthetic apparatus occurred between those dates as needles acclimated to winter conditions. Additionally, the data collected from shade needles shows a much more gradual change in the recovery kinetics during the first 10 h of warming, with both the magnitude of the reduction in  $F_v/F_m$  and the



**Fig. 2.** Recovery kinetics of  $F_v/F_m$  for all species and light acclimation conditions for pine (A) sun and shade fir (B and C) sun and shade yew (D and E) sun and shade spruce (F and G) on the following dates: November 29, 2007 (open circles), December 17, 2007 (closed circles), January 1, 2008 (open squares), January 21, 2008 (closed squares) and February 28, 2008 (open triangles). Time 0 was collected in the field after at least 3 h of dark acclimation. The remaining measurements were done on needles maintained at room temperature and low light. Each time point is an average of samples from three trees. The standard deviations ranged from 0.01 to 0.1.

rate of recovery decreasing steadily with the extent of the season in both fir and yew, while in spruce after January 1st there is very little change in the recovery kinetics.

Four-parameter logistic curves were fit to the average recovery data for each species (both sun and shade needles) for each date. The values for the calculated

parameters, in addition to the  $R^2$  for the best fit are reported in Table 3. Logistic curves describe the kinetics of recovery very well in all species where the rapid component to recovery was not observed. Fig. 4A depicts best fits for the recovery kinetics of the four species of sun needles sampled on January

**Table 2.** Results of paired *t*-tests comparing  $F_v/F_m$  measured at time 0 in the field with  $F_v/F_m$  measured after 30 min of warming at room temperature. The temperature in the field at the time of collection is indicated. The average magnitude of the increase in  $F_v/F_m$  upon warming the leaves is indicated in parenthesis in cases where there was a significant increase (i.e.  $F_v/F_m$  after 30 min warming  $-F_v/F_m$  measured in the field at the temperature indicated). A *P* value  $<0.01$  is indicated with \*\*,  $P < 0.05$  \* and NS is used to indicate no significant increase in  $F_v/F_m$  after 30 min of warming.

Date		November 29	December 17	January 1	January 21	January 28	February 28
Temperature (°C)		-5	-6	-16	-13	4	-5
Pine	Sun	*(0.07)	NS	NS	NS	NS	NS
Fir	Sun	*(0.15)	NS	*(0.06)	NS	NS	NS
	Shade	** (0.17)	*(0.10)	** (0.24)	*(0.24)	NS	*(0.10)
Yew	Sun	*(0.06)	NS	NS	NS	NS	NS
	shade	*(0.11)	*(0.14)	** (0.11)	*(0.12)	NS	NS
Spruce	Sun	** (0.12)	NS	*(0.07)	NS	NS	NS
	Shade	*(0.09)	NS	** (0.17)	*(0.11)	NS	*(0.12)

1st in addition to the actual data points. The logistic curves were constrained to have a top plateau of 0.85, and this constraint was reached on many dates, particularly for fir and yew. Despite this, the best fit was quite good, with  $R^2$  values consistently exceeding 0.95 (Table 3). The quality of the model was further analyzed by plotting the residuals. An average of the residuals for the data collected from each species on January 1st is depicted in the inset of Fig. 5A, showing a relatively even distribution above and below zero along the fit. Relatively even distributions of the residuals were observed for all species on all dates measured, suggesting that the model describes the relaxation of the slow component of sustained energy dissipation quite well.

On dates where there was a significant rapid component to recovery, the logistic curves were significantly improved if the first time point (time = 0, measured in the field) was excluded. Fig. 5B shows an example of this, depicting the recovery data from shade fir on January 1st with and without the initial time point included in the fit. Subsequently, for the shade species, curve fitting was performed excluding the first time point. A two phase model would be needed in order to model both phases of recovery; however, this would require more intensive sampling of recovery during the rapid phase, which needs to be done in future studies. However, even for samples where there was a significant rapid component to recovery, the slow component was well described by the logistic curve (Fig. 4B; Table 3).

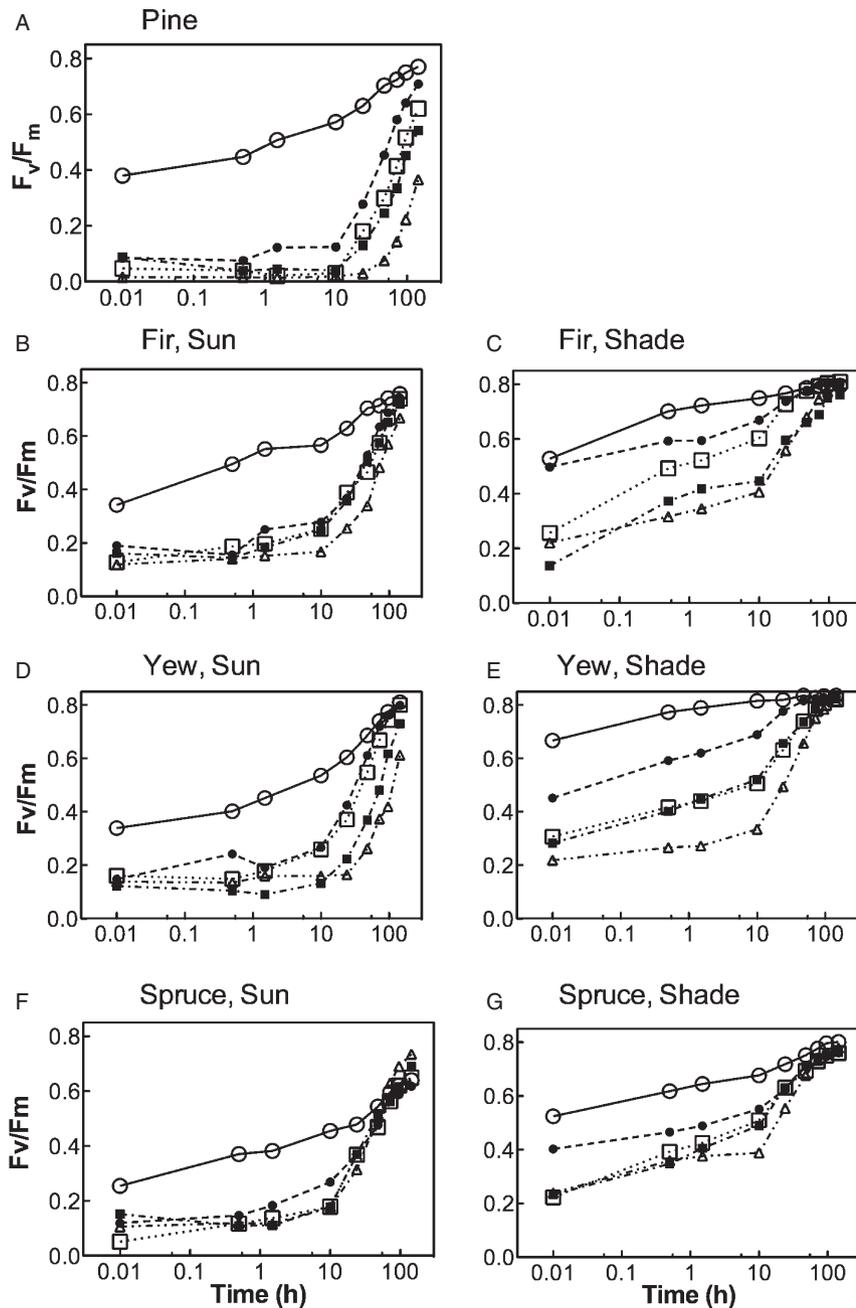
The model, which describes the relaxation of the slow component of energy dissipation, is useful in allowing the calculation of the half time of recovery for each species in order to perform a better quantitative comparison of recovery times between species, and over the course of the season. As such, curves were fit and  $t_{1/2}$  values were calculated for each individual recovery curve in order to better compare the slower component of recovery.

### The slower component of recovery

In addition to the rapid component of recovery, there were interesting differences among species, and dates of sampling in the slower component of recovery, which occurred for up to 6 days (Fig. 2). In order to explore these differences the  $t_{1/2}$  values of the sun needles were analyzed using a mixed model ANOVA with species and date as fixed effects (Fig. 5). There were significant differences between species in the half recovery time with pine recovering more slowly, followed by fir and yew (which were similar), followed by spruce which recovered more quickly than the other species at the later dates during the winter. In pine, fir and yew, there was a significant effect of sampling date on the half recovery time, with recovery occurring more slowly at the later dates, while in spruce the half recovery time was similar on all dates measured throughout the winter.

### Discussion

This study shows that conifers, growing in an environment with seasonally extreme winters, utilize the flexible temperature-dependent and rapidly reversible component to sustained energy dissipation early in the season (in November) when temperatures are more likely to fluctuate above and below zero (Table 2, Figs. 1–3). In needles acclimated to sunny conditions, this rapid component to recovery is not present for most of the winter, except perhaps in very cold conditions in some species. Instead, sun needles utilize the sustained, slowly reversible form of energy dissipation throughout the winter, from December to February. This is in contrast to recovery studies done in Colorado, where sun acclimated leaves and needles had a significant rapid component to their recovery when collected in January (Verhoeven et al. 1998, 1999). However, the rapid component to recovery was present throughout winter



**Fig. 3.** Semi-log plots of the recovery kinetics of  $F_v/F_m$  for all species and light conditions with the x-axis depicting log (time, h). All panels and symbols are identical to Fig. 2.

in shade needles in all three species examined. This is consistent with studies showing that shade leaves of *Euonymus kiautschovicus* had a much greater rapid component to their recovery relative to sun leaves on sub-zero nights in Colorado (Verhoeven et al. 1998). The fact that the rapid component to recovery occurs early in the season in all conifers monitored suggests that most evergreens are capable of utilization of this mechanism

of sustained energy dissipation, and that it may be an important photo-protective mechanism when temperatures fluctuate above and below zero in late fall when photosynthesis may still be occurring on warmer days. The disappearance of the rapid component to recovery in sun needles, and its persistence in shade needles, suggests that the rapidly reversible sustained energy dissipation is replaced by the more sustained form of energy

**Table 3.** Best fit values for non-linear curve fitting of semi-log plots of recovery kinetics. Best fits for the lower asymptote (L) which indicates the starting value of  $F_v/F_m$ , the upper asymptote (U) which indicates the fully recovered value of  $F_v/F_m$ , the time at half recovery ( $t_{1/2}$ ) in hours and the slope of the recovery curve (Slope) are reported. Additionally the  $R^2$  value for the goodness of fit is indicated. Note that the top asymptote was constrained not to exceed 0.85, and where the value is indicated as approximately 0.85 the value hit this constraint. Note for the shade samples, the time 0 was excluded from the analysis.

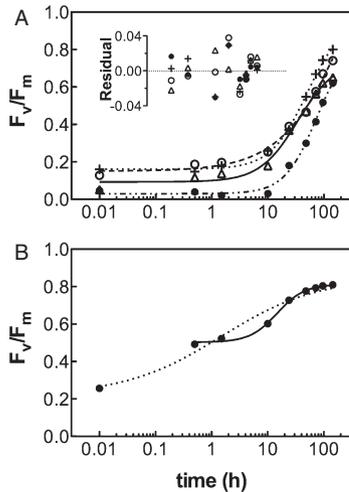
Parameter	Date	Pine	Fir		Spruce		Yew	
			Sun	Shade	Sun	Shade	Sun	Shade
L	November 29	0.39	0.30	0.68	0.26	0.62	0.37	0.72
	December 17	0.09	0.20	0.59	0.14	0.47	0.19	0.60
	January 1	0.03	0.17	0.50	0.09	0.40	0.17	0.43
	January 21	0.05	0.16	0.39	0.12	0.37	0.11	0.42
	February 28	0.02	0.14	0.33	0.11	0.36	0.15	0.27
U	November 29	~0.85	~0.85	0.82	~0.85	~0.85	~0.85	0.84
	December 17	0.79	~0.85	0.81	0.78	.79	~0.85	0.84
	January 1	0.83	~0.85	0.81	0.75	0.79	~0.85	0.85
	January 21	0.70	~0.85	0.80	0.72	0.78	~0.85	~0.85
	February 28	0.71	~0.85	0.83	0.81	0.78	~0.85	~0.85
$t_{1/2}$ (h)	November 29	14	3.8	11	44	33	20	1.0
	December 17	45	46	15	39	23	34	13
	January 1	73	49	15	35	18	39	25
	January 21	78	48	28	32	17	66	22
	February 28	144	76	27	40	28	109	31
Slope	November 29	0.6	0.4	0.6	0.4	0.9	0.9	0.5
	December 17	1.7	1.4	1.5	1.0	1.4	1.8	1.7
	January 1	1.5	1.3	1.8	1.3	1.3	1.7	1.6
	January 21	1.8	1.3	1.4	1.6	1.5	1.9	1.5
	February 28	2.2	1.7	1.6	1.7	2.2	2.1	1.8
$R^2$	November 29	0.986	0.955	0.987	0.969	0.992	0.979	0.978
	December 17	0.997	0.988	0.999	0.994	0.996	0.992	0.995
	January 1	0.994	0.985	0.997	0.989	0.997	0.996	0.999
	January 21	0.990	0.998	0.985	0.992	0.993	0.995	0.995
	February 28	0.999	0.995	0.999	0.999	0.997	0.990	0.999

dissipation as a function of the combined exposure to low temperatures and excessive light intensities.

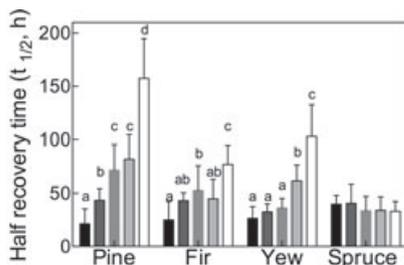
Although the mechanism for this flexible form of sustained energy dissipation is not known, it has been suggested to be a sustained form of the component of non-photochemical quenching (NPQ) referred to as energy-dependent quenching (qE), which requires acidification of the thylakoid lumen, formation of the xanthophyll cycle pigment zeaxanthin and protonation of the protein PsbS (Demmig-Adams and Adams 1996, Niyogi 1999, Müller et al. 2001, Holt et al. 2004, Ruban et al. 2012). This form of energy dissipation is normally characterized by relaxing rapidly upon darkening the leaf. In the case of plants exposed to sub-zero temperatures, it has been proposed that qE can be maintained in darkness via maintenance of a  $\Delta$ pH across the thylakoid lumen. Suggested mechanisms include an ATP-dependent reverse transport of protons through the thylakoid ATPase, or via low temperature-induced membrane permeability changes affecting proton leakage (Gilmore and Yamamoto 1992, Gilmore and Björkman 1994, 1995, Gilmore 1997, Verhoeven et al. 1998).

In contrast to the rapid component to recovery, all the conifers were characterized by maintaining high levels of the sustained, slowly reversible form of thermal energy dissipation in all species and light environments examined. This form of energy dissipation is more pronounced in sun needles relative to shade needles. The magnitude of the sustained energy dissipation increased dramatically between the November and December measurements (Fig. 2), indicating that the onset of this form of energy dissipation occurs during acclimation in late fall, corresponding with persistent sub-zero temperatures. The recovery kinetics of this form of energy dissipation was well described by four-parameter logistic curves (Fig. 4), allowing determination of half recovery times for each species and growth environment.

There were interesting differences between species in the slowly reversible recovery kinetics over the course of the season (Figs. 2 and 5). In spruce the recovery kinetics were very similar on all days measured throughout the winter, suggesting that this species engages a mechanism for sustained energy dissipation early in the winter, and maintains this such that there is no increase in the



**Fig. 4.** Examples of best fits of four-parameter logistic curves for sun and shade needles from the January 1, 2008 recovery experiment. (A) All four species with actual data depicted by symbols (pine, closed circles; fir, open circles; spruce, open triangles and yew, + symbol) and the lines showing the best fits. Curve fitting parameters are reported in Table 3. The residuals are plotted in the inset of (A), with the symbols indicating species as above. (B) The average recovery kinetics of shade fir on January 1, 2008 with two best fits plotted. The dashed line includes the time point taken at time '0' in the field, while the solid line excludes this time point.



**Fig. 5.** Average half recovery time ( $t_{1/2}$ ) in h, calculated from best fits using logistic curves for each sample for sun needles of all species. Different letters indicate there are significant differences between sampling dates within a species. Values are means  $\pm$  SD,  $n = 3$ . Different shading indicates dates of sampling on the following dates (from darkest to lightest, respectively) November 29, 2007; December 17, 2007; January 1, 2008; January 21, 2008 and February 28, 2008.

severity of the winter stress over the course of the season. The needles recover equally well in December as in February. However, in pine, fir and yew, the process of recovery was slowed throughout the winter season, such that needles in February recovered significantly more slowly than those in January, which were slower to recover than those collected in December. This suggests that for these species, changes in the photosynthetic apparatus continue to occur throughout the winter season, with pine showing more dramatic

changes compared with fir and yew. The resulting differences in the half recovery time late in the winter season were quite pronounced [the  $t_{1/2}$  for recovery in February was approximately 144 h for pine, 76 h for fir and 40 h for spruce (Fig. 5)]. These fairly dramatic differences have implications for spring recovery, suggesting species-specific differences in the phenology of onset of photosynthetic activity in the spring. Such differences in the timing of recovery of photosynthetic efficiency, and by extension carbon gain, will likely influence competitive interactions and productivity of forests in a changing climate. There is little data comparing species in their winter downregulation and recovery of photosynthesis and these results, illustrating pronounced differences between species sharing a similar environment, suggest that further comparative studies are needed.

The mechanism of the sustained energy dissipation that reverses very slowly upon warming is also not well understood. The very slowly reversible nature of this form of sustained energy dissipation is thought to be a form of NPQ, referred to as  $q_l$  or photoinhibitory quenching (Müller et al. 2001) which is characterized by reversing slowly upon darkening a leaf. Hypotheses that have been put forward to explain the slowly reversible nature of  $q_l$ , in the context of winter stress, have focused on either damage to the photosynthetic apparatus that is slowly repaired (e.g. Öquist et al. 1992) or the reconfiguration of light harvesting centers into dissipating centers (e.g. Ottander et al. 1995, Öquist and Huner 2003), or a combination of these phenomena. The differences among species in how the kinetics of the slow phase of recovery changed over the course of the winter season (Figs. 2 and 5) suggests species-specific differences in aspects of the mechanism of onset, reversal or maintenance of sustained energy dissipation.

Recently, severely reduced  $F_v/F_m$ , that correlates with the presence of Z + A in darkness, has been observed in plants that tolerate severe desiccation (Fernández-Marín et al. 2009, 2010, 2011). In these species the rate of recovery of  $F_v/F_m$  upon rehydration can occur extremely rapidly (on a time scale of minutes to hours, Proctor, 2001, 2010) in a similar manner to the rapid phase of recovery observed upon warming some winter stressed leaves (Verhoeven et al. 1998). In this study, a detailed analysis of recovery during the fast phase (first hour or so) was not performed, so the kinetics of that phase could not be analyzed for comparison to the desiccation-tolerant species. However, the slow phase of recovery of winter stressed conifers was shown to fit a similar logistic curve as the moss/fern (Proctor 2010), although occurring over much longer timescales. Thus the model describes the

relaxation of sustained forms of energy dissipation in both contexts.

Although the mechanism for the reduction in  $F_v/F_m$  upon desiccation, in desiccation-tolerant mosses and ferns, and its rapid reversal upon rehydration is not understood, it has been shown to involve the xanthophyll cycle pigments  $Z + A$ , which can be formed in darkness during desiccation (Fernández-Marín et al. 2009, 2011). Studies on lichens have suggested that the mechanism involves desiccation-induced conformational changes of an (as yet unknown) pigment–protein complex (Heber 2008). It is intriguing to speculate that the temperature-dependent nature of the very rapid phase to recovery observed in winter stressed conifers may involve a similar mechanism to that observed in the rehydration recovery observed in desiccation-tolerant plants. The very low temperatures that occur on sub-zero nights during winter have a similar physiological effect, as the low temperatures cause extracellular freezing of water resulting in dehydration within the protoplast (e.g. Levitt, 1980). Thawing of the leaves essentially results in rehydration, which is accompanied by rapid recovery of  $F_v/F_m$ . It therefore seems likely that a shared mechanism may be involved.

The method of monitoring the recovery kinetics of winter stressed evergreens upon artificial warming may be useful in assessing the potential of evergreens to respond to warming events, and become photosynthetically active, at different times throughout the winter season. It is known that both temperature and photoperiod lead to downregulation of photosynthesis in conifers during winter (Busch et al. 2007, 2008), and that temperature is the main factor constraining recovery of photosynthesis in spring (Lundmark et al. 1998, Ensminger et al. 2004, Thum et al. 2009, Gea-Izquierdo et al. 2010). Additionally, there is evidence that conifers, growing in mid latitude maritime forests can engage in photosynthetic activity at low levels during winter (Dolman et al. 2002). The data reported here suggest that species vary considerably in their ability to recover photosynthetic efficiency in response to warming, particularly later in the winter season (Figs. 2 and 5). Thus further studies examining recovery kinetics upon warming in a variety of species over the winter season can add important insights into the timing of downregulation and recovery of photosynthesis and the potential for different species to respond to warmer autumns, warming events during winter, and earlier springs.

## Conclusions

These results show that conifers growing in a seasonally extreme environment do utilize both a rapid and slowly

reversible component to sustained energy dissipation during winter. The flexible temperature-dependent rapid component to recovery occurs early in the winter season as acclimation to low temperatures is occurring, and to a lesser extent is utilized by needles growing in low light environments throughout the winter. This form of energy dissipation may be important in providing a flexible mechanism for responding to large temperature fluctuations in late autumn when temperatures may be warm enough during the day to allow photosynthesis. As winter progresses, in needles acclimated to both high and low light conditions, there is an increase in the slowly reversible sustained form of energy dissipation, such that in sun acclimated needles this is the only form of sustained energy dissipation present. Differences among species in the kinetics of recovery over the course of the winter season suggest species-specific differences in the mechanism or maintenance of sustained energy dissipation, with spruce maintaining consistent recovery kinetics throughout the winter season, while pine, fir and yew show increasingly slow recovery kinetics as the season progresses. The data suggest pronounced differences in the rate of spring recovery with pine recovering significantly more slowly than the other species and spruce recovering relatively quickly, likely impacting the spring onset of photosynthetic carbon gain. Further studies into the mechanism of both the flexible rapidly reversible and the slowly reversible forms of sustained energy dissipation are needed. Additionally, further studies of species-specific differences in the potential to recover photosynthetic efficiency upon warming will contribute to better predictions of forest responses to climate change.

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