

# Characterization of cell growth and starch production in the marine green microalga *Tetraselmis subcordiformis* under extracellular phosphorus-deprived and sequentially phosphorus-replete conditions

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**Abstract** Microalgal starch is a potential feedstock for bio-fuel production. Nutrient stress is widely used to stimulate starch accumulation in microalgae. Cell growth and starch accumulation in the marine green microalga *Tetraselmis subcordiformis* were evaluated under extracellular phosphorus deprivation with initial cell densities (ICD) of 1.5, 3.0, 6.0, and  $9.0 \times 10^6$  cells  $\text{mL}^{-1}$ . The intracellular stored phosphorus supported cell growth when extracellular phosphorus was absent. The maximum starch content of 44.1 % was achieved in the lowest ICD culture, while the maximum biomass productivity of  $0.71 \text{ g L}^{-1} \text{ day}^{-1}$ , starch concentration of  $1.6 \text{ g L}^{-1}$ , and starch productivity of  $0.30 \text{ g L}^{-1} \text{ day}^{-1}$  were all obtained in the culture with the ICD of  $3.0 \times 10^6$  cells  $\text{mL}^{-1}$ . Appropriate ICD could be used to regulate the intracellular phosphorus concentration and maintain adequate photosynthetic activity to achieve the highest starch productivity, along with biomass and starch concentration. The recovery of phosphorus-deprived *T. subcordiformis* in medium containing 0.5, 1.0, or 6.0 mM  $\text{KH}_2\text{PO}_4$  was also tested. Cell growth and starch accumulation ability could be recovered completely. A phosphorus pool in *T. subcordiformis* was shown to manipulate its metabolic activity under different environmental phosphorus availability. Though lower starch productivity and starch content were achieved under phosphorus deprivation compared with

nitrogen- or sulfur-deprived conditions, the higher biomass and starch concentration make *T. subcordiformis* a good candidate for biomass and starch production under extracellular phosphorus deprivation.

**Keywords** *Tetraselmis subcordiformis* · Starch · Phosphorus · Initial cell density · Productivity · Recovery

## Introduction

Microalgae, which possess faster growth rates, higher photosynthetic efficiencies, and better  $\text{CO}_2$  mitigation capacities than terrestrial plants and exert no competition for arable land and food crops, are currently considered to be the third-generation feedstock for biofuel production (Nigam and Singh 2011; Wang et al. 2008). Microalgal biomass is primarily composed of protein, carbohydrate (starch), and lipid. Microalgal carbohydrates can be converted to methane, bioethanol, biobutanol, and biohydrogen through anaerobic digestion, anaerobic fermentation, and biological biohydrogen production (Markou et al. 2012a). Bioethanol production from microalgal starch has recently been experiencing a remarkable surge (John et al. 2011). It has been estimated that approximately 46,760–140,290 L  $\text{ha}^{-1}$  ethanol can be produced from microalgae. This is several orders of magnitude larger than yields obtained from other feedstock such as corn or sugarcane (Mussatto et al. 2010). To make the process economically feasible, high starch content and improved biomass and starch productivity in the microalgae, especially marine microalgae that conserve fresh water, are desirable (Doan et al. 2012).

The accumulation of microalgal starch is species-specific and largely dependent on cultivation and environmental conditions. Macroelement (nitrogen, sulfur, or phosphorus) limitation is the most widely explored strategy for the enhancement of starch accumulation (Ball et al. 1990;

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Brányiková et al. 2011; Dragone et al. 2011). Phosphorus is an essential macroelement that is mainly incorporated into nucleic acid and phospholipids. It also serves as an important modifier of protein function and is indispensable for the basic processes of energy flow (Moseley and Grossman 2009). Phosphorus limitation can lead to restricted cell proliferation, downregulated photosynthesis (Wykoff et al. 1998), cessation of protein and chlorophyll synthesis, and accumulation of lipid and carbohydrate (Dean et al. 2008a, b; Markou 2012; Markou et al. 2012c; Sigee et al. 2007). Table 1 lists the biomass and starch production in some microalgae under nitrogen, phosphorus, or sulfur deprivation. Under phosphorus deprivation, the starch content, starch productivity, and starch concentration reached 53 %, 0.48 g L<sup>-1</sup> day<sup>-1</sup>, and 0.35 g L<sup>-1</sup>, respectively, in *Chlorella vulgaris*. This was a much better performance than under nitrogen starvation but somewhat weaker performance relative to that under sulfur deprivation (Brányiková et al. 2011). In contrast, nitrogen limitation seemed more efficient than phosphorus limitation in inducing carbohydrate accumulation in *Arthrospira platensis* (Aikawa et al. 2012; Markou et al. 2012b). It appears that which nutrient limitation induces the best performance in starch production differs among microalgal species. Particularly, owing to the storage of polyphosphate through “luxury uptake” and its function of regulating intracellular phosphate concentration (Powell et al. 2009), the manipulation of starch accumulation by extracellular phosphorus deprivation may be complex. Carbohydrate accumulation is reportedly triggered primarily by the intracellular rather than extracellular phosphorus concentration, and carbohydrate starts to accumulate when intracellular phosphorus drops below a critical level (Dean et al. 2008a, b; Sigee et al. 2007). Recently, Markou et al. (2012b) reported that the carbohydrate content in *A. platensis* increased to a maximum of 63.09±3.43 % when the alga was supplied with phosphorus at 1.82±0.16 mg g<sup>-1</sup> of dry biomass. This suggests that an optimal amount of intracellular phosphorus is needed to maximize carbohydrate content in combination with biomass production.

Microalgal cell density is an essential factor controlling light availability, which affects biomass productivity and the biochemical composition of microalgae (Richmond 2004). Generally, under a specific light intensity, lower cell density will allow more irradiance per unit cell, which facilitates starch accumulation (Brányiková et al. 2011). However, in terms of photosynthetic efficiency and biomass productivity, an optimal cell density is required (Hu and Richmond 1994; Hu and Richmond 1996). In batch cultivation mode, initial cell density (ICD), which denotes the inoculum size, can be used to regulate cell density and hence optimize biomass and starch production under stressed conditions, especially under extracellular deprivation of any specific nutrient of which there can be large intracellular stores.

*Tetraselmis subcordiformis*, a marine green microalga, has been proven to accumulate large amounts of starch in nitrogen- or sulfur-deprived environments (Yao et al. 2012). The highest starch content of 62.1 % and productivity of 0.62 g L<sup>-1</sup> day<sup>-1</sup> can be achieved under sulfur deprivation. It remains unknown whether and to what extent phosphorus deprivation can lead to starch accumulation in *T. subcordiformis*. In the present study, extracellular phosphorus deprivation was used to test this alga's cell growth and starch accumulation ability at various ICDs. As the recovery of microalgae that have accumulated large amounts of energy-rich compounds (starch or lipid) under stressed conditions is crucial for their cultivation in a semicontinuous mode in which the stressed microalgae serve as the inoculum for the next generation of cultivation, we also evaluated the recovery of cell growth and ability to accumulate starch in phosphorus-deprived cells by recultivating them in various phosphorus concentrations.

## Materials and methods

### Algal strain and culture conditions

*T. subcordiformis* FACHB-1751, a marine green microalga, was isolated from the Huanghai Sea near Dalian, Liaoning Province, China and maintained by the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB collection), Chinese Academy of Sciences.

The microalgae were previously cultivated in natural seawater by adding nutrients, as described by Yao et al. (2012). In the phosphorus deprivation experiment, algal cells were harvested during the late exponential phase and washed twice with phosphorus-free artificial seawater (ASW-P). The ASW-P contained (per liter): 27 g NaCl, 1.113 g KNO<sub>3</sub>, 6.6 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.6 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.5 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.04 g NaHCO<sub>3</sub>, 0.8 mg FeCl<sub>3</sub>, 0.4 mg MnCl<sub>2</sub>·4H<sub>2</sub>O, 33.6 mg H<sub>3</sub>BO<sub>3</sub>, 45.0 mg EDTA-2Na, 0.21 mg ZnCl<sub>2</sub>, 0.2 mg CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.09 mg (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, and 0.137 mg CuCl<sub>2</sub>·2H<sub>2</sub>O. The cells were resuspended in ASW-P and used to inoculate cultures to ICD of 1.5, 3.0, 6.0, and 9.0×10<sup>6</sup> cells mL<sup>-1</sup>. In the phosphorus repletion experiment, algal cells deprived of phosphorus for 4 days in the phosphorus deprivation experiment with the ICD of 1.5×10<sup>6</sup> cells mL<sup>-1</sup> were washed twice with ASW-P and inoculated with an ICD of 1.5×10<sup>6</sup> cells mL<sup>-1</sup> in ASW containing 0.5, 1.0, and 6.0 mM KH<sub>2</sub>PO<sub>4</sub>.

The cells were cultured in a 600-mL glass air bubble column photobioreactor (50 mm diameter, 400 mm height) with a working volume of 500 mL and an aeration of 0.4 vvm with air containing 3 % CO<sub>2</sub> at 25±2 °C as described by Yao et al. (2012). The cultures were continuously illuminated from two sides with cool white fluorescent lamps that provided an incident light intensity of 200 μmol m<sup>-2</sup> s<sup>-1</sup> measured by a

**Table 1** Biomass and starch production in different microalgae under nitrogen (–N), phosphorus (–P) or sulfur (–S) deprivation as reported in the literature

Microalgae	Stress conditions	Biomass (g L <sup>-1</sup> )	Biomass productivity (g L <sup>-1</sup> day <sup>-1</sup> )	Starch (g L <sup>-1</sup> )	Starch productivity (g L <sup>-1</sup> day <sup>-1</sup> )	Starch content (% of DW)	References
<i>T. subcordiformis</i>	–P (low ICD)	2.5	0.42	1.1	0.21	44.1	This study
	–P (medium ICD)	3.8	0.55	1.6	0.30	42.2	This study
	–N (low ICD)	1.2	0.65	0.6	0.49	54.0	Yao et al. (2012)
	–S (low ICD)	1.9	0.68	1.2	0.54	62.1	Yao et al. (2012)
<i>Chlorella vulgaris</i> Beijerinck CCALA924	–N	0.22	0.23	0.10	0.19	37	Brányiková et al. (2011)
	–P	0.65	0.75	0.35	0.48	53	Brányiková et al. (2011)
	–S	1.02	1.10	0.62	0.74	60	Brányiková et al. (2011)
<i>A. platensis</i> NIES-39 <sup>a</sup>	–N (3 mM nitrate)	1.6	NA <sup>c</sup>	1.03	0.29	65	Aikawa et al. (2012)
<i>A. platensis</i> <sup>b</sup>	–P (57.4 μM phosphate)	0.87	0.087	0.52	0.052	59.9	Markou et al. (2012b)

<sup>a</sup> The carbohydrate is assessed in the form of glycogen

<sup>b</sup> The starch production is evaluated from the carbohydrate reported by multiplying a conversion factor 0.9

<sup>c</sup> Not available

photosynthetically active radiation (PAR) detector (Optometer P9710 with PAR detector 3701, Gigahertz Optik Corporation, Germany). According to Brányiková et al. (2011), the initial mean light intensities for ICDs of 1.5, 3.0, 6.0, and 9.0 × 10<sup>6</sup> cells mL<sup>-1</sup> were measured and calculated to be approximately 73, 54, 48, and 44 μmol m<sup>-2</sup> s<sup>-1</sup>, respectively.

#### Growth measurement

Cells were counted in a hemocytometer under a light microscope (ECLIPSE TE2000-U, Nikon Corporation, Japan) after proper dilution. The cell dry weight (DW) was determined gravimetrically according to Yao et al. (2012). Maximum specific growth rate (μ<sub>max</sub>) was evaluated from DW at exponential phase, calculated as follows:

$$\mu_{\max} = \frac{\ln DW_2 - \ln DW_1}{t_2 - t_1} \quad (1)$$

where  $t$  represents cultivation time. Biomass productivity ( $P_b$ , in gram per liter per day) was calculated as follows:

$$P_b = \frac{DW_t - DW_0}{t} \quad (2)$$

where  $DW_t$  and  $DW_0$  are the cell dry weight at culture times  $t$  and 0, respectively. The cell density and DW fold increase were obtained by  $X_t/X_0$  where  $X_t$  and  $X_0$  are cell density or DW at culture times  $t$  and 0, respectively.

#### Biochemical composition analysis

The pigments were extracted from a 1–5 mg algal pellet by ethanol as described by Yao et al. (2012). The absorption of the extract at 470, 649, and 665 nm was measured with a spectrophotometer (Jasco V-530, JASCO Corporation, Japan). The chlorophyll (Chl, in milligram per liter) and carotenoid (Car, in milligram per liter) content were calculated using the following equation (Lichtenthaler and Wellburn 1983):

$$\text{Chl} = 6.63 A_{665} + 18.08 A_{649} \quad (3)$$

$$\text{Car} = 4.08 A_{470} + 3.31 A_{665} - 11.64 A_{649} \quad (4)$$

Starch was determined as described by Yao et al. (2012). Starch productivity ( $P_s$ , in gram per liter per day) was calculated as follows:

$$P_s = \frac{DW_t C_{st} - DW_0 C_{s0}}{t} \quad (5)$$

where  $C_{st}$  and  $C_{s0}$  are the starch content at culture times  $t$  and 0, respectively.

Some other variables denoting biomass or starch production in the present study are:  $X_{b\max}$ , maximum biomass concentration (in gram per liter);  $P_{b\max}$ , maximum biomass productivity (in gram per liter per day);  $X_{s\max}$ , maximum starch concentration (in gram per liter);  $P_{s\max}$ , maximum

starch productivity (in gram per liter per day); and  $C_{s,max}$ , maximum starch content (in percent of DW).

#### Chlorophyll fluorescence measurement

The chlorophyll fluorescence was measured using a chlorophyll fluorometer (Water-PAM WALZ, Germany). Algal cells were adapted in the dark for 10 min, and a measuring light (ML,  $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was applied to obtain the minimum initial fluorescence ( $F_0$ ). A saturating light pulse (SP, 0.6 s,  $330 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was then applied to evaluate the maximum fluorescence ( $F_m$ ). The maximum quantum efficiency of photosystem II (PS II), termed  $F_v/F_m$ , was calculated according to Schreiber (2004):

$$F_v/F_m = \frac{F_m - F_0}{F_m} \quad (6)$$

#### Phosphate and nitrate analysis

The intracellular total phosphorus (ITP) was measured as inorganic phosphate released from thermal acid digestion of cells. Microalgae were immersed in a mixture of nitric acid and perchloric acids (10:1, v/v) for 12 h and heated at  $140^\circ\text{C}$  until no precipitate could be observed. The solution was then appropriately diluted for phosphate determination.

A 2 mL aliquot of algal culture was centrifuged at  $8,000\times g$  for 3 min and the supernatant was used for extracellular phosphate (EP) and nitrate analysis. For intracellular free phosphate (IFP) measurement, an equal aliquot of algal culture was sonicated before centrifugation to obtain the total free phosphate (TFP). The IFP was evaluated by subtracting EP from TFP.

Nitrate was measured according to Yao et al. (2012). The colorimetric determination of phosphate was performed by reaction with molybdate and ascorbic acid catalyzed by antimony potassium tartrate and read at 660 nm. All the measurements were conducted using a SEAL AutoAnalyzer 3 (SEAL Analytical, Germany), according to the manufacturer's instructions.

## Results

### Extracellular phosphorus deprivation with different ICDs

#### Cell growth and starch accumulation

To determine the effect of extracellular phosphorus deprivation on cell growth and starch accumulation in *T. subcordiformis*, cells with ICDs of 1.5, 3.0, 6.0, and  $9.0 \times 10^6$  cells  $\text{mL}^{-1}$  (designated as low, medium, high, and

ultrahigh, respectively) were cultivated in ASW-P. Cell growth lasted for at least 4 days in all the cultures, with 3.8-, 2.6-, 1.7-, and 1.5-fold increase in cell density respectively for ICDs from 1.5 to  $9.0 \times 10^6$  cells  $\text{mL}^{-1}$  (Fig. 1a, Electronic supplementary material (ESM) 1). The volumetric cell DW also showed a continuous increase, and the maximum DW fold increase decreased with increasing ICD (Fig. 1b). The greatest increase in DW, 5.0-fold, was achieved in the lowest ICD culture on day 5 with a maximum DW of  $2.5 \text{ g L}^{-1}$  (Table 2, ESM 1). However, the best maximum biomass productivity,  $0.71 \text{ g L}^{-1} \text{ day}^{-1}$ , was achieved in the medium ICD culture (Table 2).

Starch concentration increased immediately upon extracellular phosphorus deprivation under low and medium ICD, while high and ultrahigh ICD caused a stagnation or even a decrease in starch concentration for 1 or 2 days before it started to rise (Fig. 1c). Notably, the medium ICD culture showed a stable starch accumulation rate, with the highest maximum starch productivity of  $0.30 \text{ g L}^{-1} \text{ day}^{-1}$  on day 5, whereas the low ICD culture maintained almost the same starch productivity ( $0.29 \text{ g L}^{-1} \text{ day}^{-1}$ ) only until day 2; thereafter, starch accumulation slowed down (Fig. 1c, Table 2). Thus, the maximum starch concentration of  $1.6 \text{ g L}^{-1}$  in the medium ICD culture was 1.5 times of that in the low ICD culture. The starch content based on DW increased sharply in the low ICD culture when exposed to extracellular phosphorus deprivation, from its initial level of 14.5–35.6 % on the first day, followed by a gentle rise until it reached its maximum of 44.1 % on day 5. Despite the much smaller increase of starch content obtained in the medium ICD culture, the maximum starch level resembled that in the low ICD culture on day 5. However, the starch content in the high and ultrahigh ICD cultures decreased on the first day, and the maximum starch level appeared to be nearly half of that in the low ICD culture (Fig. 1d, Table 2).

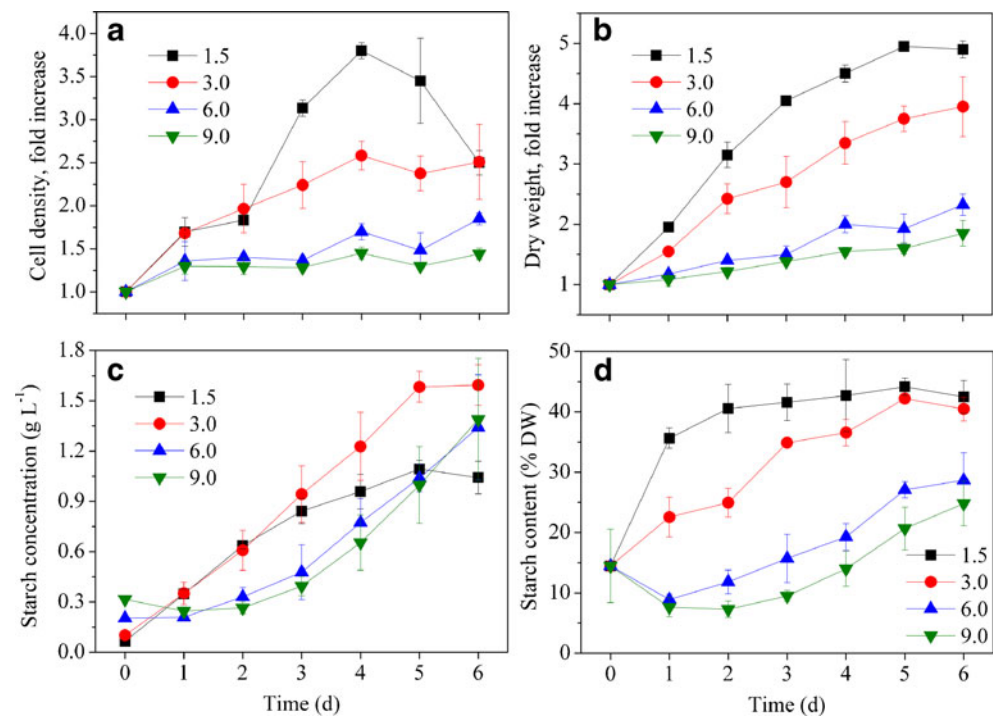
#### Nutrient availability

The extracellular nitrate concentration decreased in all four ICD conditions, indicating that *T. subcordiformis* can assimilate nitrogen for cell growth in the absence of extracellular phosphorus (Fig. 2a). The higher ICD resulted in a faster nitrate removal rate. The nitrate was completely removed in the high and ultrahigh ICD cultures on days 6 and 5, respectively, whereas 52 and 34 % of nitrate remained in the culture medium on day 6 in the low and medium ICD cultures, respectively.

The ITP also showed a decline in all four ICD conditions, with the most severe decline observed in the culture with the lowest ICD (Fig. 2b). Notably, from day 2, its ITP level diverged from that of the medium ICD culture, decreasing more sharply to reach its minimum of  $0.36 \text{ pg cell}^{-1}$  on day 4.



**Fig. 1** Increase of cell density (cells per milliliter) (a) and dry weight (gram per liter) (b), and variations in starch concentration (c) and starch content (d) in *T. subcordiformis* with initial cell densities (ICDs) of 1.5, 3.0, 6.0, and  $9.0 \times 10^6$  cells  $\text{mL}^{-1}$  under extracellular phosphorus deprivation. The initial dry weights for the four ICDs are: 0.4, 1.0, 1.9, and  $3.1 \text{ g L}^{-1}$ , respectively



#### Pigments and photosynthetic performance

The Chl content decreased in all four ICD conditions when cells were deprived of extracellular phosphorus, from the initial level of 4.3–1.3, 2.0, 3.1, and 3.2 %, respectively, on day 6 in the cultures with low to ultrahigh ICDs (Fig. 3a). The Car content also decreased during the first day (high and ultrahigh ICDs) or first 2 days (low and medium ICDs) but, unlike Chl, it increased somewhat thereafter (Fig. 3b). The Car/Chl ratio increased from the initial level of 0.07–0.17, and 0.13, respectively, by day 6 in the low and medium ICD cultures, while it decreased during the first 2 days in the high and ultrahigh ICD cultures, followed by an increase to 0.09 and 0.10, respectively, by day 6 (Fig. 3c).

$F_v/F_m$ , the maximum quantum efficiency of PS II photochemistry, reflects the potential photosynthetic activity and is an intrinsic and sensitive indicator of environmental stress (Baker 2008); nutrient stress is considered to be present when  $F_v/F_m$  declines (Shelly et al. 2011). The variation in  $F_v/F_m$  exhibited almost a reverse profile relative to the

Car/Chl ratio (Fig. 3d).  $F_v/F_m$  declined immediately in response to extracellular phosphorus deprivation in the low ICD culture, and a dramatic decrease occurred on day 2 from 0.73 to 0.68 on day 3; a more severe decline thereafter led to a value of only 0.41 on day 6. A similar trend was observed in the medium ICD culture, though  $F_v/F_m$  increased slightly on day 1 and then decreased slowly, and the final  $F_v/F_m$  was maintained at 0.72. Distinct from the low and medium ICDs,  $F_v/F_m$  increased in the first 2 days, peaking at 0.79 and 0.81 on day 2, respectively, for the high and ultrahigh ICDs, and thereafter both dropped slightly to 0.77 on day 6.

Recovery of phosphorus-deprived cells in phosphorus-replete conditions

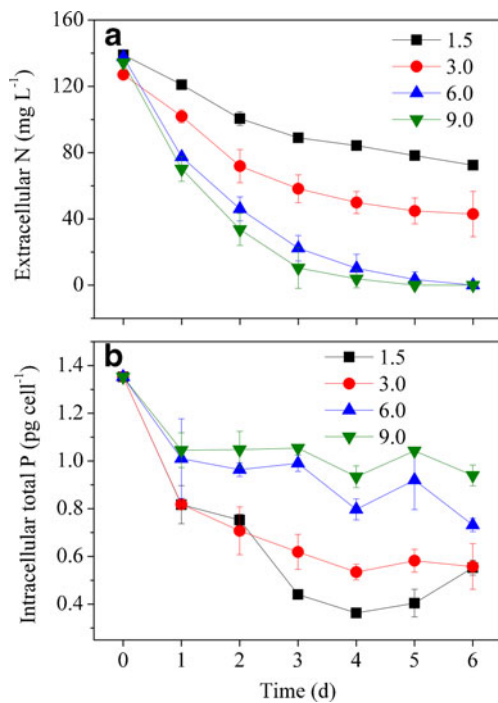
#### Cell growth and starch accumulation

*T. subcordiformis* cells that had been deprived of extracellular phosphorus for 4 days were used to inoculate fresh

**Table 2** Biomass and starch production in *T. subcordiformis* at different ICDs under extracellular phosphorus deprivation (means $\pm$ SD)

ICD ( $10^6$ cells $\text{mL}^{-1}$ )	$X_{bmax}$ ( $\text{g L}^{-1}$ )	$P_{bmax}$ ( $\text{g L}^{-1} \text{ day}^{-1}$ )	$X_{smax}$ ( $\text{g L}^{-1}$ )	$P_{smax}$ ( $\text{g L}^{-1} \text{ day}^{-1}$ )	$C_{smax}$ (% of DW)
1.5	$2.5 \pm 0.0$ (5) <sup>a</sup>	$0.59 \pm 0.02$ (2)	$1.1 \pm 0.1$ (5)	$0.29 \pm 0.01$ (2)	$44.1 \pm 1.4$ (5)
3.0	$4.0 \pm 0.5$ (6)	$0.71 \pm 0.12$ (2)	$1.6 \pm 0.1$ (5)	$0.30 \pm 0.02$ (5)	$42.2 \pm 0.1$ (5)
6.0	$4.7 \pm 0.4$ (6)	$0.53 \pm 0.11$ (4)	$1.3 \pm 0.3$ (6)	$0.19 \pm 0.05$ (6)	$28.7 \pm 4.6$ (6)
9.0	$5.6 \pm 0.6$ (6)	$0.42 \pm 0.09$ (6)	$1.4 \pm 0.4$ (6)	$0.18 \pm 0.06$ (6)	$24.8 \pm 3.7$ (6)

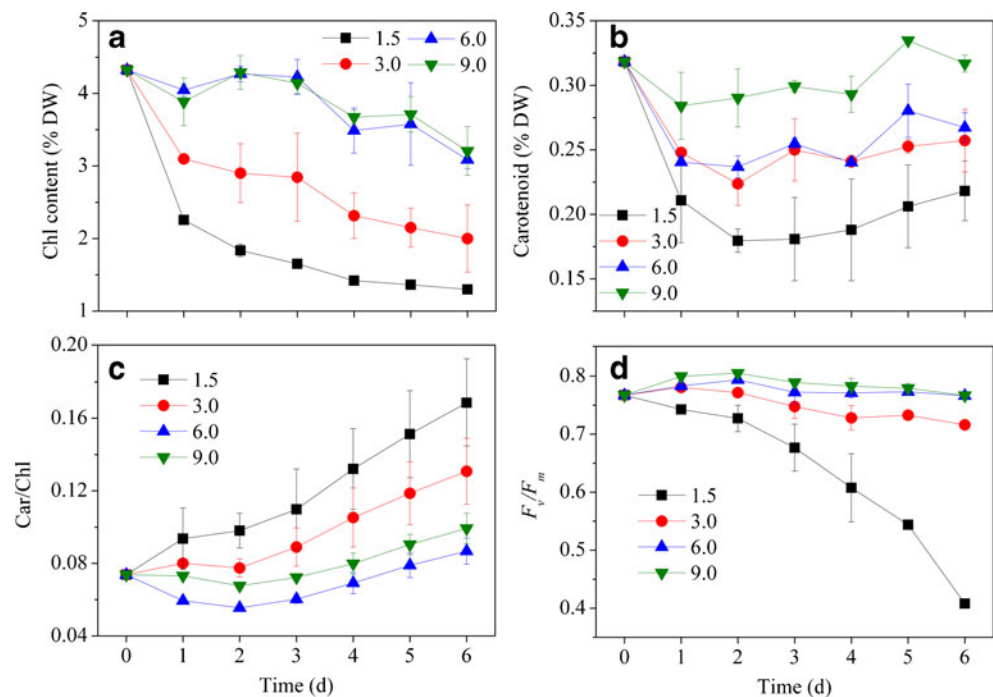
<sup>a</sup> The number in the parentheses denotes the corresponding cultivation day the parameter achieved



**Fig. 2** Extracellular nitrate consumption (a) and intracellular total phosphorus (ITP) variation (b) in *T. subcordiformis* cultures with ICDs of 1.5, 3.0, 6.0, and  $9.0 \times 10^6$  cells mL<sup>-1</sup> under extracellular phosphorus deprivation

cultures in ASW containing initial phosphate (KH<sub>2</sub>PO<sub>4</sub>) concentrations of 0.5, 1.0, and 6.0 mM to further test the alga's ability to recover. Cell density increased promptly in the media containing 0.5 and 1.0 mM KH<sub>2</sub>PO<sub>4</sub> (Fig. 4a). Stationary phase occurred on day 5, with both cultures

**Fig. 3** Changes in chlorophyll (Chl) content (a), carotenoid (Car) content (b), Car/Chl ratio (c) and photosynthetic activity ( $F_v/F_m$ ) (d) in *T. subcordiformis* cultures with ICDs of 1.5, 3.0, 6.0, and  $9.0 \times 10^6$  cells mL<sup>-1</sup> under extracellular phosphorus deprivation



attaining a maximum cell density of  $9.0 \times 10^6$  cells mL<sup>-1</sup>. In the recultivation with 6.0 mM KH<sub>2</sub>PO<sub>4</sub>, cells were stagnant during the first day, after which a drastic increase in cell density occurred, reaching  $11.0 \times 10^6$  cells mL<sup>-1</sup> on day 7. The changes in volumetric DW resembled those in cell density, with the maximal DW of 5.3 g L<sup>-1</sup> and biomass productivity of 0.68 g L<sup>-1</sup> day<sup>-1</sup> both achieved in 6.0 mM KH<sub>2</sub>PO<sub>4</sub> (Fig. 4b, Table 3).

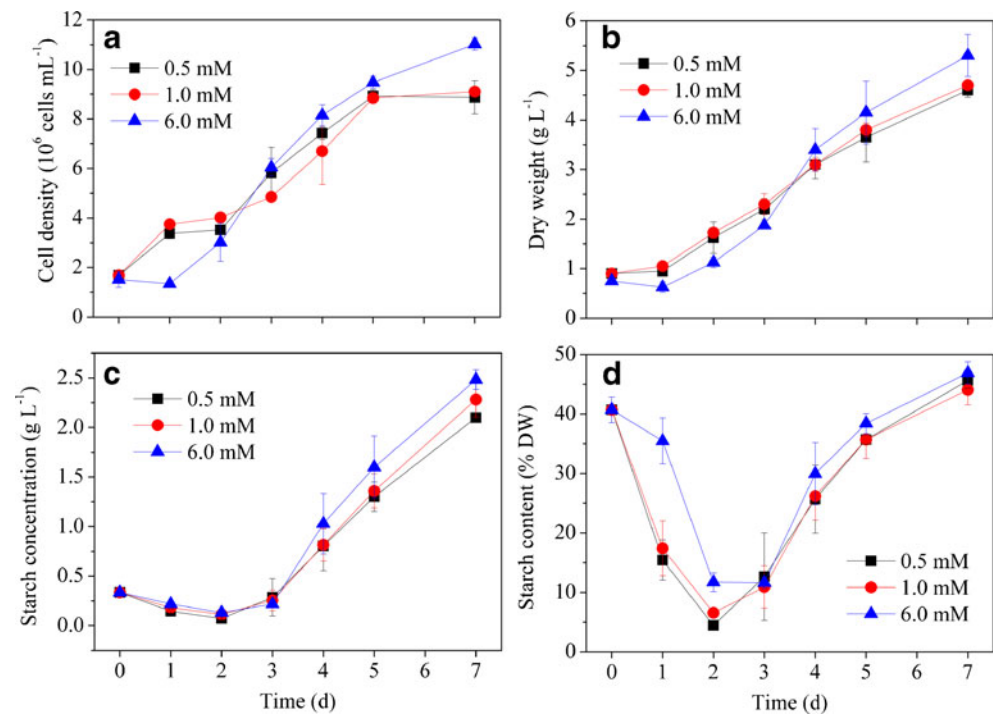
The starch concentration (Fig. 4c) and starch content (Fig. 4d) decreased during the first 2 days in all the recultivation conditions, indicating that starch was catabolized for cell growth in the nutrient-rich environment. The lowest starch content, 4.4 % DW, was obtained in the 0.5 mM KH<sub>2</sub>PO<sub>4</sub> culture on day 2. Then, starch accumulated throughout the following 5 days and the greatest starch concentration, starch productivity, and starch content of 2.5 g L<sup>-1</sup>, 0.31 g L<sup>-1</sup> day<sup>-1</sup>, and 46.9 %, respectively, were all achieved in the 6.0 mM KH<sub>2</sub>PO<sub>4</sub> culture on day 7 (Table 3).

#### Nutrient availability

The nitrate concentration in the culture medium declined in all four conditions in the first day (Fig. 5a), even though no cell proliferation occurred in 6.0 mM KH<sub>2</sub>PO<sub>4</sub>. Since no lag phase could be observed in the cultures with 0.5 and 1.0 mM KH<sub>2</sub>PO<sub>4</sub>, the nitrate removal rates in these cultures were faster than in 6.0 mM KH<sub>2</sub>PO<sub>4</sub>, but nitrate in all the treatments was completely consumed by day 3.

The EP was quickly removed and exhausted on days 1, 1, and 4, respectively, at initial concentrations of 0.5, 1.0, and 6.0 mM

**Fig. 4** Cell growth (a), biomass accumulation (b), starch accumulation (c) and starch content variation (d) in phosphorus-deprived *T. subcordiformis* recultivated in medium with 0.5, 1.0, or 6.0 mM  $\text{KH}_2\text{PO}_4$



$\text{KH}_2\text{PO}_4$  (Fig. 5b). The intracellular free P (IFP,  $\text{PO}_4^{3-}$ ) peaked on the day the EP was exhausted, with values at 2.5, 6.3, and 9.2  $\text{pg cell}^{-1}$ , respectively, for the three cultures, and thereafter declined to 0.6, 0.9, and 4.5  $\text{pg cell}^{-1}$  by day 7, respectively (Fig. 5c). Particularly in the culture with 6.0 mM  $\text{KH}_2\text{PO}_4$ , the IFP reached a plateau ranging from 8.0 to 9.2  $\text{pg cell}^{-1}$  during the first 4 days, despite continuous removal of EP.

#### Pigments and photosynthetic performance

Chl were synthesized immediately upon phosphorus repletion in the 0.5 and 1.0 mM  $\text{KH}_2\text{PO}_4$  cultures, which both attained a maximum Chl content of 4.2 % on day 3, after which a decline occurred, possibly due to the exhaustion of extracellular nitrate. A similar profile of Chl variation was observed in the 6.0 mM  $\text{KH}_2\text{PO}_4$  culture, except for the slower recovery rate and lower maximum Chl content, 3.2 %, on day 3 (Fig. 6a). The Car content in the 0.5 and 1.0 mM  $\text{KH}_2\text{PO}_4$  cultures increased during the first 3 days, remained stable for the subsequent 2 days, and thereafter dropped significantly. However, in the 6.0 mM  $\text{KH}_2\text{PO}_4$  culture, the Car content remained at the initial level until it increased on day 2; the maximum Car content was also

obtained on day 3, and was approximately 80 % of that of the 0.5 and 1.0 mM  $\text{KH}_2\text{PO}_4$  cultures (Fig. 6b). Correspondingly, in the cultures with 0.5 and 1.0 mM  $\text{KH}_2\text{PO}_4$ , the Car/Chl ratio decreased from the initial level of 0.15 to the minimum of 0.06 on day 2, followed by an increase to 0.13 on day 7. The Car/Chl ratio stayed at 0.15 in the culture with 6.0 mM  $\text{KH}_2\text{PO}_4$  on day 1, and then declined to 0.08 on day 3; afterwards, a sharp increase occurred and the ratio reached 0.18 on day 7 (Fig. 6c).

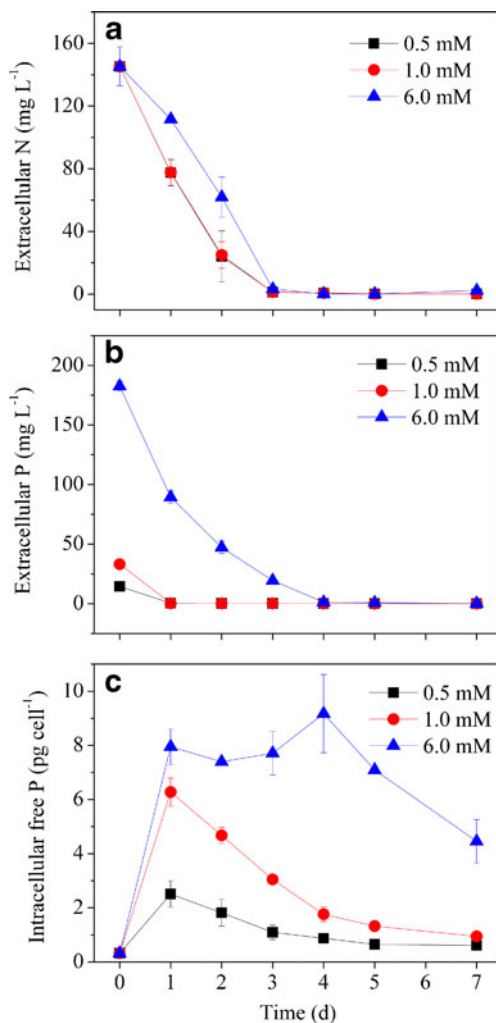
Generally, the trend in  $F_v/F_m$  showed an inverted profile relative to the Car/Chl ratio (Fig. 6d). In the cultures with 0.5 and 1 mM  $\text{KH}_2\text{PO}_4$ ,  $F_v/F_m$  recovered from 0.62 to its maximal level of 0.79 on day 2, and declined gently to approximately 0.70 on day 7. However, a marked decrease was observed in the culture with 6.0 mM  $\text{KH}_2\text{PO}_4$ , reaching 0.34 on day 1; thereafter  $F_v/F_m$  recovered promptly to 0.74 on day 3 and decreased to 0.66 by day 7.

#### Discussion

*T. subcordiformis* was able to grow for several days when deprived of extracellular phosphorus, though in the low ICD

**Table 3** Biomass and starch production in phosphorus-deprived *T. subcordiformis* recultivated in medium with different  $\text{KH}_2\text{PO}_4$  concentrations (means $\pm$ SD)

$\text{KH}_2\text{PO}_4$ (mM)	$X_{b\max}$ (g $\text{L}^{-1}$ )	$P_{b\max}$ (g $\text{L}^{-1}$ day $^{-1}$ )	$X_{s\max}$ (g $\text{L}^{-1}$ )	$P_{s\max}$ (g $\text{L}^{-1}$ day $^{-1}$ )	$C_{s\max}$ (% of DW)
0.5	4.6 $\pm$ 0.1 (7)	0.55 $\pm$ 0.10 (5)	2.1 $\pm$ 0.0 (7)	0.25 $\pm$ 0.00 (7)	45.6 $\pm$ 1.3 (7)
1.0	4.7 $\pm$ 0.0 (7)	0.58 $\pm$ 0.03 (5)	2.3 $\pm$ 0.2 (7)	0.27 $\pm$ 0.02 (7)	44.1 $\pm$ 2.5 (7)
6.0	5.3 $\pm$ 0.4 (7)	0.68 $\pm$ 0.13 (5)	2.5 $\pm$ 0.1 (7)	0.31 $\pm$ 0.01 (7)	46.9 $\pm$ 1.9 (7)



**Fig. 5** Nitrate consumption (a), extracellular phosphorus (EP) consumption (b), and intracellular free phosphate (IFP) variation (c) in phosphorus-deprived *T. subcordiformis* recultivated in medium with 0.5, 1.0, or 6.0 mM  $\text{KH}_2\text{PO}_4$

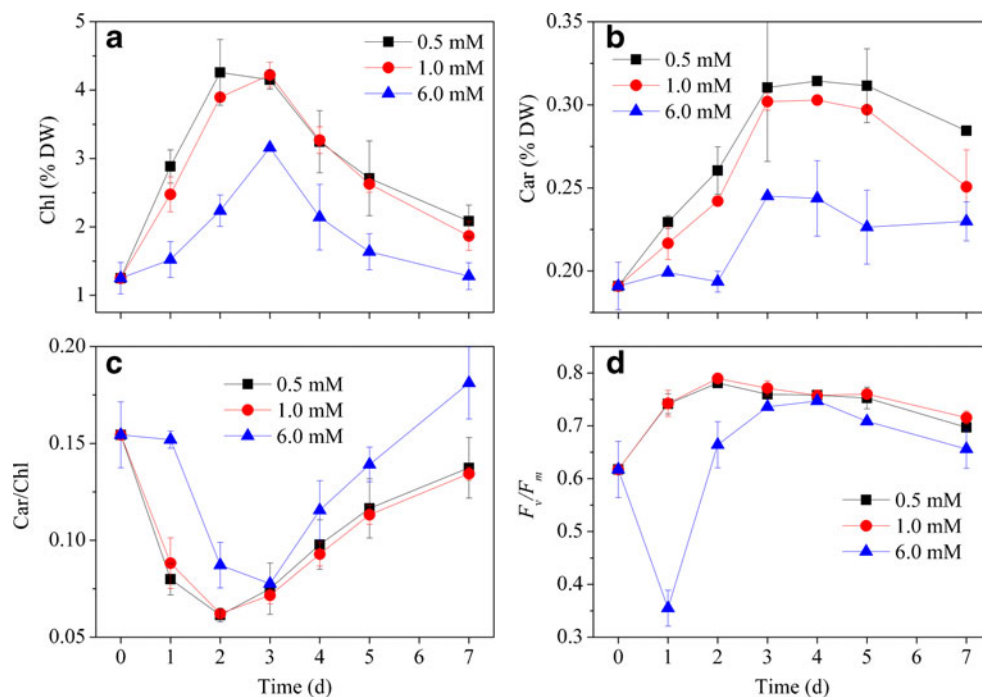
culture, only 55 % of the cell density and 53 % of the DW obtained under favorable conditions (Yao et al. 2012) with 0.5 mM  $\text{KH}_2\text{PO}_4$  could be achieved. The decline of ITP with the increase of cell density in *T. subcordiformis* suggests that intracellular phosphorus in the mother cells is sufficient to support cell proliferation until the minimum phosphorus content required for cell survival is attained (e.g., 0.36 pg P cell<sup>-1</sup> on day 4 in the low ICD culture, Fig. 2b). The allocation of intracellular phosphorus from mother cells into the offspring may involve the mobilization of stored polyphosphate, nucleic acid, and/or phospholipids. This is a common strategy used by many microalgae to acclimate to environmental phosphorus deficiency (Moseley and Grossman 2009). The lesser maximum cell densities and DW increments in the higher ICD cultures might be caused by other limitations, such as limited nitrogen and insufficient light.

The present work showed that in the low ICD culture, extracellular phosphorus deprivation caused starch accumulation and decreased Chl content in *T. subcordiformis* (Figs. 1d and 3a), the reverse of the result obtained under phosphorus-replete conditions as described by Yao et al. (2012) and the recovery experiment in this work. It is known that phosphorus deprivation can lead to the diversion of carbon allocation from growth-related protein and chlorophyll to energy-rich storage compounds such as carbohydrates and lipids (Dean et al. 2008a, b; Markou 2012; Markou et al. 2012c; Sigeo et al. 2007), as we have also demonstrated here. Particularly, our results showed that Car content increased slowly in the later period of phosphorus deprivation (Fig. 3b). This may be attributed to the synthesis of xanthophylls under stressful conditions that contribute to the activation of the xanthophyll cycle to dissipate surplus absorbed light energy as heat, rendering the microalgae less prone to photo-oxidative damage (Couso et al. 2012; Wykoff et al. 1998). Our results also show that the Car/Chl ratio is positively related to starch content and inversely related to  $F_v/F_m$  (Figs. 1d and 3c, d), suggesting that the increased Car/Chl ratio could be indicative of stress as the decreased  $F_v/F_m$  leading to starch accumulation in *T. subcordiformis*. An increased Car/Chl ratio has similarly been proposed to be an indicator of stress that induces lipid synthesis in *Nannochloropsis* sp. (Pal et al. 2011; Solovchenko et al. 2011).

Compared with nitrogen and sulfur deprivation ( $-N$  and  $-S$ , respectively; Yao et al. 2012), extracellular phosphorus deprivation ( $-P$ ) seems less effective in inducing starch accumulation in *T. subcordiformis* (Table 1). This may be ascribed to intracellular stored phosphorus supporting continuous cell growth, with a 3.8-fold increase in cell density under extracellular  $-P$ . Cell density increased by only 1.5- and 1.6-fold, respectively, in the  $-N$  and  $-S$  cultures (data not shown). In addition, the milder decline of  $F_v/F_m$  in  $-P$  relative to that in  $-N$  and  $-S$  also indicated that less stress was imposed on  $-P$  cells (Yao et al. 2012). The inverse relation between energy-rich compound (carbohydrate and lipid) accumulation and cell growth has been recorded in many other microalgae (Brányiková et al. 2011; Li et al. 2008; Wang et al. 2010) and is a challenge in the economic production of microalgal biofuel feedstock (Markou et al. 2012a). Several strategies, such as “two-stage” cultivation (Dragone et al. 2011; Rodolfi et al. 2009) or limited nutrient addition (Aikawa et al. 2012; Markou et al. 2012b), have been employed to solve this problem. Our results showed that unlike starch content and starch productivity, the starch and biomass concentration in the  $-P$  culture were comparable to or exceeded those in the  $-N$  and  $-S$  cultures, suggesting that extracellular phosphorus deprivation might serve as an alternative strategy if the cultivation is targeted to overall biomass and starch production. In this regard,



**Fig. 6** Changes in Chl content (a), Car content (b), Car/Chl ratio (c), and photosynthetic activity ( $F_v/F_m$ ) (d) in phosphorus-deprived *T. subcordiformis* recultivated in medium with 0.5, 1.0, or 6.0 mM  $\text{KH}_2\text{PO}_4$



phosphorus-deprived *T. subcordiformis* is competitive in biomass and starch production among other microalgae with various nutrient limitations (Table 1).

Higher ICD caused higher ITP and  $F_v/F_m$  but lower starch content under extracellular phosphorus deprivation. This may be because cells in the higher ICD cultures were exposed to less light and the consequent slower cell growth required less phosphorus, so there was less intracellular stored phosphorus mobilized and less stress on the cells, leading to less starch accumulation. The negative correlation between inoculum size and lipid or carbohydrate content as well as the optimization of ICD to achieve maximum biomass and lipid or carbohydrate productivity has been reported (Chen et al. 2012; Ho et al. 2012; Li et al. 2013). ICD can be used to regulate intracellular phosphorus concentrations in parallel with the degree of subsequent stress to induce starch accumulation under extracellular phosphorus deprivation. Our data show that the maximum starch content of 44.1 % was achieved in the low ICD culture, but the maximum biomass productivity of  $0.71 \text{ g L}^{-1} \text{ day}^{-1}$ , starch concentration of  $1.6 \text{ g L}^{-1}$ , and starch productivity of  $0.30 \text{ g L}^{-1} \text{ day}^{-1}$  were all obtained in the medium ICD culture (Table 2). This is due to the more severe stress on the low ICD culture, which is detrimental to photosynthesis. In the culture with low ICD,  $F_v/F_m$  declined immediately upon phosphorus deprivation but was maintained at 0.74, with a simultaneous sharp increase in starch content on day 1;  $F_v/F_m$  then dropped to 0.68 on day 3 when the ITP level decreased to  $0.44 \text{ pg cell}^{-1}$ , and thereafter starch accumulation slowed down. In contrast,  $F_v/F_m$  in the medium ICD culture declined less and stayed above 0.72 throughout

the cultivation period; the ITP level also stayed above  $0.53 \text{ pg cell}^{-1}$ , and thus continuous and stable starch production could be obtained (Figs. 1c–d, 2b, and 3d). Correspondingly, the Car/Chl ratio in the medium ICD culture was lower than that in the low ICD culture throughout the cultivation (Fig. 3c) indicating that the medium ICD culture was less stressed. This conclusion consolidated the results from  $F_v/F_m$ . The correlation of  $F_v/F_m$  and starch accumulation is similar to that seen in N and S stress (Yao et al. 2012). The improved photosynthetic activity enabled the medium ICD culture to produce more starch, which is in agreement with our opinion that the better starch accumulation under sulfur deprivation is attributable to photosynthetic efficiency being higher than it is under nitrogen deprivation (Yao et al. 2012). Similarly, Pan et al. (2011) concluded that high photosystem II efficiency during nitrogen starvation would lead to the accumulation of high levels of lipids in microalgae. Photosynthesis provides the fixed carbon and chemical energy that are essential for the synthesis of products such as starch and lipid in phototrophic microalgae (Fan et al. 2012). Hence, it is important to allocate more carbon to energy-rich molecules (starch or lipid) than to protein by providing appropriate stress conditions that do not damage the capacity of photosynthesis to fix carbon and supply ATP. In addition to the nutrient limitation strategy, our results have demonstrated the possibility of enhancing starch production by optimizing ICD without damaging photosynthesis.

Phosphorus-deprived *T. subcordiformis* recovered immediately in medium containing 0.5 mM  $\text{KH}_2\text{PO}_4$ , with increased cell growth, pigment synthesis, and photosynthetic

activity accompanying nitrate consumption, starch degradation, and a decreased Car/Chl ratio. The maximum specific growth rate of  $0.53 \text{ day}^{-1}$ , maximum Chl content of 4.2 %, and maximum  $F_v/F_m$  of 0.79 obtained in this recovery experiment are comparable to those obtained without phosphorus deprivation pretreatment and described previously (Table 4), suggesting that *T. subcordiformis* can recover almost completely from phosphorus deprivation. In addition, nitrate depletion from day 3 also caused starch accumulation in the recovery experiment, with the maximum obtained starch content of 45.6 % comparable to that without phosphorus deprivation pretreatment (Table 4), indicating that the recovered *T. subcordiformis* can be reused for starch production when re-exposed to stress conditions. The recovery of both cell growth and starch accumulation suggests that *T. subcordiformis* has the potential for semicontinuous production of microalgal biomass and starch.

In phosphorus-replete conditions, no significant difference in the parameters other than EP and IFP could be detected in the cultures with 0.5 and 1.0 mM  $\text{KH}_2\text{PO}_4$ , indicating that the supplied phosphorus in this concentration range had little effect on the physiological status of *T. subcordiformis* under our culture conditions. A similar phenomenon has been observed in other microalgae (Markou et al. 2012c; Wu et al. 2012). This may be attributed to the storage of excessive phosphorus as polyphosphate that can serve as an internal resource for cell survival when external phosphorus is limiting (Eixler et al. 2006; Nishikawa et al. 2006). Our results show that phosphorus was completely removed from the medium within 1 day (Fig. 5b), but cell density increased until day 5 (Fig. 4a). Correspondingly, the IFP peaked on day 1 and decreased thereafter (Fig. 5c), suggesting that aside from polyphosphate, cell growth may also be partially supported by free phosphate probably stored in the vacuole.

At the higher phosphorus concentration of 6.0 mM  $\text{KH}_2\text{PO}_4$ , phosphorus-deprived *T. subcordiformis* could not undergo prompt recovery; instead, no cell growth occurred in the first day though nitrate and phosphorus had been assimilated (Figs. 4a and 5a, b). The dramatic drop in  $F_v/F_m$  from 0.62 to 0.34 during the first day (Fig. 6d) suggested a high-phosphorus stress probably due to the rapid assimilation of phosphorus by the phosphorus-starved cells and the

consequent excess accumulation of free phosphate in the cytosol and chloroplast that is toxic to cellular metabolism, especially to the photosynthesis (Furbank and Lilley 1980; Nielsen and Rochon 1976). The subsequent recovery of  $F_v/F_m$  and other metabolic activity might be ascribed to the synthesis of polyphosphate from the free phosphate and the transportation of surplus-free phosphate to the vacuole. Polyphosphate and vacuole have been reported to act as phosphate reserve for the cytosol in microalgae and higher plants (Nishikawa et al. 2006; Rychter and Rao 2005). These regulations render cells to successfully acclimate to high-phosphorus environment. Interestingly, the 6.0 mM  $\text{KH}_2\text{PO}_4$  culture could not only recover from phosphorus deprivation and high phosphorus stress, but also seemed to have better starch accumulation ability compared with that of the 0.5 and 1.0 mM  $\text{KH}_2\text{PO}_4$  cultures (Table 3). This could be explained by the relatively tougher stress conditions (nitrogen depletion and high phosphorus stress) being manifested as less pigment, lower  $F_v/F_m$ , and a higher Car/Chl ratio (Fig. 6). Thus, more carbon fixed from photosynthesis was channeled to starch.

Starch synthesis in many microalgae and plants involves the conversion of glucose-1-phosphate and ATP to ADP-glucose and pyrophosphate, a rate-limiting step catalyzed by ADP-glucose pyrophosphorylase (AGPase; Ball and Morell 2003). AGPase can be activated by 3-phosphoglyceric acid (3-PGA) and inhibited by orthophosphate (Pi), and the activity is thus regulated by the 3-PGA/Pi ratio (Ball 1998). Li et al. (2011) reported a 39.2 and 45 % decrease of AGPase activity and starch synthesis, respectively, in the microalga *Pseudochlorococcum* sp. with the addition of 1 mM Pi under high irradiance with limited nitrogen availability. However, in the present study, extracellular phosphate with a concentration as high as 6.0 mM did not inhibit starch accumulation in *T. subcordiformis* (Fig. 4c, d). This might be ascribed to the limited IFP concentration throughout the cultivation. Although EP was continuously removed during the first 4 days, the IFP remained between 8.0 and 9.2 (Fig. 5b, c). It is estimated that only 41 % of the removed EP was detected as IFP on day 4; most of the rest of the removed EP might have been incorporated into stored polyphosphate. In addition, these IFP might largely locate in the vacuole instead of chloroplast, rendering chloroplastic AGPase less prone to be inhibited by excess phosphate. Moreover, owing to the fairly

**Table 4** Recovery of phosphorus-deprived (PD) *T. subcordiformis* in 0.5 mM  $\text{KH}_2\text{PO}_4$  compared with nonphosphorus-deprived (NPD) microalgae under the same cultivation conditions (means $\pm$ SD)

Treatment	$\mu_{\text{max}}$ ( $\text{day}^{-1}$ )	Maximum Chl content (% DW)	Maximum starch content (% DW)	Maximum $F_v/F_m$	References
PD	$0.53\pm 0.12$	$4.2\pm 0.1$	$45.6\pm 1.3^{\text{a}}$	$0.79\pm 0.00$	This study
NPD	$0.49\pm 0.05$	$4.9\pm 0.1$	$47.8\pm 1.3^{\text{b}}$	$0.80\pm 0.01$	Yao et al. (2012)

<sup>a</sup> The data were obtained on day 7 and might be higher with prolonged cultivation

<sup>b</sup> The data were obtained on day 8

sufficient nitrogen source, the photosynthetic activity in the 6.0 mM  $\text{KH}_2\text{PO}_4$  culture remained relatively high, with  $F_v/F_m$  being above 0.66 (Fig. 6d). The high photosynthetic activity might facilitate the production of 3-PGA and thus render the 3-PGA/Pi ratio high enough for the activation of AGPase as well as the consequent starch synthesis.

It seems that *T. subcordiformis* has a pool of stored phosphate that may support cell growth when extracellular phosphorus is limited and maintain metabolic activity (e.g., photosynthesis and starch synthesis) when phosphorus is in excess. The most probable forms of the pool are polyphosphate and/or free phosphate-enriched vacuole, but their size and regulatory functions need further demonstration. The existence of this “phosphorus pool” has to be taken into consideration when phosphorus stress is used to manipulate starch accumulation in *T. subcordiformis* and other phosphorus pool-containing microalgae.

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