Journal of Phycology
October 2010, Volume 46, Issue 5, Pages 926–936
http://dx.doi.org/10.1111/j.1529-8817.2010.00878.x

Growth and phosphorus uptake by the toxic dinoflagellate *Alexandrium catenella* (dinophyceae) in response to phosphate limitation

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Abstract:

Alexandrium catenella (Whedon et Kof.) Balech has exhibited seasonal recurrent blooms in the Thau lagoon (South of France) since first reported in 1995. Its appearance followed a strong decrease (90%) in phosphate (PO_4^{3-}) concentrations in this environment over the 1970–1995 period. To determine if this dinoflagellate species has a competitive advantage in PO_4^{3-}-limited conditions in terms of nutrient acquisition, semicontinuous cultures were carried out to characterize phosphorus (P) uptake by *A. catenella* cells along a P-limitation gradient using different dilution rates (DRs). Use of both inorganic and organic P was investigated from measurements of $^{33}PO_4^{3-}$ uptake and alkaline phosphatase activity (APA), respectively. P status was estimated from cellular P and carbon contents (Q_P and Q_C). Shifts in trends of Q_P/Q_C and Q_P per cell ($Q_{P\cdot cell-1}$) along the DR gradient allowed the definition of successive P-stress thresholds for *A. catenella* cells. The maximal uptake rate of $^{33}PO_4^{3-}$ increased strongly with the decrease in DR and the decrease in Q_P/Q_C, displaying physiological acclimations to PO_4^{3-} limitation. Concerning maximal APA per cell, the observation of an all-or-nothing pattern along the dilution gradient suggests that synthesis of AP was induced and maximized at the cellular scale as soon as PO_4^{3-} limitation set in. APA variations revealed that the synthesis of AP was repressed over a PO_4^{3-} threshold between 0.4 and 1 µM. As lower PO_4^{3-} concentrations are regularly observed during *A. catenella* blooms in Thau lagoon, a significant portion of P uptake by *A. catenella* cells in the field may come from organic compounds.

Keywords: Alexandrium catenella; alkaline phosphatase; limitation; phosphorus; semicontinuous cultures

Abbreviations:

APA

alkaline phosphatase activity

DOP

dissolved organic phosphorus

MFP

methylfluorescein-phosphate

MUF-P

methyl-umbelliferyl phosphate

POC

particulate organic carbon

POP

particulate organic phosphorus

INTRODUCTION

Phosphorus (P) deficiency in marine systems has been reported for several open-ocean areas and coastal waters (Vidal et al. 2003, Labry et al. 2005, Thingstad et al. 2005) and may lead to growth limitation of both phytoplankton and heterotrophic bacteria communities (Thingstad et al. 1998). In such environments, a significant fraction of the total dissolved phosphorus pool (TDP) often corresponds to dissolved organic phosphorus (DOP) (Karl and Yanagi 1997, Karl and Björkman 2002, Suzumura and Ingall 2004), making the enzymatic remineralization of organic P compounds a key process in population competition.

In the Thau lagoon (South of France), the role of organic P compounds may have become more critical for organisms' nutrition as a strong decrease in phosphate (PO_4^{3-}) concentrations has been observed over the last four decades, with values in summer and winter dropping from 10 µM to 1 µM and from 3 µM to undetectable (<0.03 µM), respectively (Collos et al. 2009). This oligotrophication principally occurred during the 1970-1995 period, as a consequence of effective implementation of waste water collection and treatment facilities (La Jeunesse and Elliott 2004). The year 1995 coincided with the first report of the toxic dinoflagellate *Alexandrium catenella* (Whedon et Kofoid) Balech in Thau lagoon waters, followed by a first Paralytic Shellfish Poisoning (PSP) toxic event in 1998 (Lilly et al. 2002). Ever since 1998 seasonal recurrent blooms of *A. catenella* have been observed, suggesting the oligotrophication has created a niche where *A. catenella* may grow and become periodically dominant.

In the present study, P-uptake characteristics of *A. catenella* cells were analyzed to determine if this species manifests a competitive advantage for P-acquisition in PO_4^{3-} limited conditions. *Alexandrium* spp., and in particular *A. catenella*, are known to produce alkaline phosphatase (Oh et al. 2002, Ou et al. 2006) when PO_4^{3-} deficient. This enzyme hydrolyzes ester bonds between PO_4^{3-} and dissolved organic compounds, making PO_4^{3-} available for

cellular assimilation (Perry 1972). As the DOP resource might be critical for cellular growth in Thau lagoon waters, both the use of inorganic and organic P-sources were examined by carrying out PO_4^{3-}-uptake and alkaline phosphatase activity (APA) measurements along a P-limitation gradient.

MATERIALS AND METHODS

Experimental design. Two strains of *A. catenella* were tested: TL01 and ACT03, isolated respectively in 1998 and 2003 in Thau lagoon, France. Nonaxenic cultures were maintained on ESAW artificial seawater (Andersen et al. 2005) at 20±1°C and were illuminated with 100 µmol·photons·m^{-2}·s^{-1} under a 12h light : 12h dark cycle.

For each strain, one stock culture was used to inoculate 14 3L-flasks using ESAW medium with PO_4^{3-} concentration limited to ESAW/4 (N:P = 98). These cultures were run under batch conditions until PO_4^{3-} concentrations were reduced to less than 0.2 µM. Cultures were gently mixed prior to the regular monitoring of PO_4^{3-} concentrations. The cultures were then maintained semi-continuously renewing a part of the medium every 24 h. The fresh medium used for dilutions was based on ESAW composition with modified PO_4^{3-} and nitrate (NO_3^-) concentrations, corresponding to 9.1 µM and 882.5 µM respectively (N:P = 98). Seven dilution rates (DRs) were assayed (0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50 d^{-1}) with replicated cultures. After each renewal, the withdrawn water samples were used to measure PO_4^{3-}, NO_3^- + nitrite (NO_2^-), and *A. catenella* cell concentrations.

After nine days of semi-continuous conditions, equilibrium was presumed as changes in nutrient concentrations over 3d were <10% in all 3L-cultures and cell density varied by less than 20%, except for one DR under which density variations were <30%. Cultures were continuously subjected to daily dilutions until the end of the experiment. On days 1, 2 and 3 after equilibrium, additional measurements of maximal PO_4^{3-}-uptake rate, maximal alkaline

phosphatase activity (APA) and cellular composition parameters were performed for each DR. These measurements were done using withdrawn water samples from both replicated cultures mixed together in order to increase available sampling volume making it possible to perform duplicates. On following days 4, 5 and 6, the whole of a 3L-culture was used to do kinetics measurements of PO_4^{3-} uptake rate and APA. From the sacrifice of one culture per day, three different DR conditions were tested for each strain, 0.15 d^{-1}, 0.2 d^{-1}, 0.3 d^{-1} for TL01 and 0.1 d^{-1}, 0.15 d^{-1}, 0.2 d^{-1} for ACT03.

Chemical measurements and cell counts. Samples for PO_4^{3-} and $NO_3^- + NO_2^-$ determination were carefully filtered through glass fiber filters (Whatman GF/F, Maidstone, U.K.) with a syringe filtration system. Filtrates were used for nutrient concentration measurements on a Bran+Luebbe/Seal (Norderstedt, Germany) AutoAnalyzer 3 following Aminot and Kérouel (2007).

For measurements of particulate phosphorus and carbon, water samples were filtered through pre-combusted (12 h at 400°C) 25 mm Whatman GF/D filters which were then maintained at -20°C until analysis. Particulate organic phosphorus (POP) was measured according to Solórzano and Sharp (1980). Particulate organic carbon (POC) was determined based on the method of Aminot and Kérouel (2004) using a VarioEL III carbon-nitrogen elemental analyzer (Elementar, Hanau, Germany). POP and POC standardized by *A. catenella* cell densities were used to represent the P-status (Qp) and C content (Q_C), respectively. Cell concentrations were estimated from water samples fixed with formaldehyde (final concentration of 5%) using a haemocytometer. Cell counts were performed in duplicate, counting more than 400 cells per sample, and in triplicate otherwise.

PO_4^{3-}-uptake rate. Rates of PO_4^{3-} uptake were measured using the $^{33}PO_4^{3-}$ incorporation technique. On days 1, 2 and 3 after equilibrium, maximal uptake rates (Vp_{max}) were estimated for each DR on water samples varying from 150 mL (lowest DR) to 1000 mL

(highest DR) in polycarbonate bottles. After adjusting the PO_4^{3-} concentration of water samples to 6.4 µM, incubations started with the addition of 20 µCi $^{33}PO_4^{3-}$ and were ended by the addition of formaldehyde (4% final concentration). Different incubation times ranging from 5 min to 6 h were used and PO_4^{3-} uptake rates were calculated from the linear part of the ^{33}P incorporation time series. For kinetics experiments on days 4, 5 and 6, similar incubations were performed by adding graded PO_4^{3-} concentrations (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 µM KH_2PO_4) and 16 µCi $^{33}PO_4^{3-}$ to 250 mL-subsamples originating from the same 3L-flask. At the end of incubations, samples were filtered through 8 µm 25 mm cellulose ester (SCWP02500) Millipore (Billerica, Mass., U.S.A.) filters. Then, filters were rinsed twice with 5 mL of 0.2 µm filtered AW water and stored with 4 mL of scintillation cocktail until they were counted with a liquid scintillation counter (Wallac Model 1414, EG/G Istruments / Perkin-Elmer, Turku, Finland).

Alkaline phosphatase activity. APA measurements were performed in duplicate on subsamples obtained with and without pre-filtration of the water-samples through 0.2 or 10 µm polycarbonate filters. From the fractionated APA measurements, APA linked to *A. catenella* cells surface was determined by subtracting the APA in the <10 µm fraction from the total APA and APA obtained from the 0.2 µm filtrate was regarded as soluble APA. Fractionated APA was assayed by using the fluorogenic substrate methyl-umbelliferyl phosphate (MUF-P) (Hoppe 1983, Ammerman 1993). On days 1, 2 and 3 after equilibrium, a MUF-P final concentration of 250 µM was added to 2mL-water samples to determine maximal hydrolysis rates along the DR gradient. For kinetics experiments on days 4, 5 and 6, ten MUF-P final concentrations ranging from 0.5 to 250 µM were used. Incubations were carried out in the dark at 20°C and were ended by formaldehyde addition, with final concentration of 4 %, and by freezing the samples at -20°C. Incubations were stopped when APA variations with time were still linear, avoiding hydrolysis of the main portion of

152 available MUF-P. For estimations of maximal activities along the DR gradient, incubations
153 were 30 - 60 min, increasing with increasing DR; for kinetics experiments, incubations were
154 15 min when substrate concentration was lower than 2 µM, otherwise 30 min. The
155 concentrations of the dephosphorylated fluorescent product 4-methyl-umbelliferone (MUF) at
156 the end of incubations were estimated from fluorescence (365 nm excitation and 460 nm
157 emission) measured by flow injection analysis (Delmas et al. 1994, Labry et al. 2005) with a
158 buffered solution of borate (0.1 M, pH 10.5) as the carrier fluid. To convert units of MUF
159 fluorescence into concentration values, a calibration curve ($R^2 > 0.999$) was performed with
160 standard solutions of MUF in the range 0.02-20 µM.

161 *Data treatments – Analysis along the DR gradient.* Each strain had 2 replicate cultures
162 at each DR. For each replicate the PO_4^{3-} and cell concentrations obtained on days 1, 2 and 3
163 after equilibrium were averaged and then used to estimate the mean and standard deviation for
164 each strain TL01 (n=2) and ACT03 (n=2). Two tailed t-tests were used to test strain
165 differences in PO_4^{3-} concentrations and significance of cell concentration trends along the DR
166 gradient. Measurements of POP, POC, ^{33}P-uptake rate and APA were undertaken on water
167 samples of both replicated cultures mixed together, therefore these data are presented as an
168 average of days 1, 2 and 3 values along the DR gradient. They were used for linear regression
169 and curve fitting. All statistical analyses were undertaken using the Prism 4.0b software
170 (Graph Pad Software, San Diego, CA., U.S.A.).

171 *Data treatments – Kinetic and quota parameters.* From data collected on days 4, 5 and
172 6 after equilibrium, maximal uptake rates (V_{max}) and half-saturation constants (K_S) of PO_4^{3-}-
173 uptake rate and APA were determined using the Michaelis-Menten relation:

174 $$V = V_{max} \cdot [S] / (K_S + [S]) \quad (1)$$

175 where the uptake rate V (in h^{-1}) is function of the maximal uptake rate V_{max} (in h^{-1}), the half-
176 saturation constant K_S (in µM) and the substrate concentration [S] (in µM).

For APA, half of the data series did not show a clear intermediate saturation plateau, but a single Michaelis-Menten model did not correctly represent the data either, with a net underestimation of enzyme activity at high substrate concentrations. This was probably due to the high dependence of the kinetic parameters values upon the range of substrate concentration considered (McComb et al. 1979) rather than to a potential multi-enzymatic system such as the one described for the coccolithophorid *Emiliana huxleyi* (Riegman et al. 2000, Dyhrman and Palenik 2003). Taking into account the potential patchiness of nutrient concentrations at the cellular scale (Shanks and Trent 1979), we choose to consider biphasic patterns to avoid distorted estimations of kinetic parameters; one equation was used to obtain an estimation of the enzyme affinity (K_S) in the range of low substrate concentrations and the other to estimate the enzyme capacity (V_{max}) using data at high substrate concentrations. This approach is supported by the consistency between parameters values obtained from uniphasic and biphasic models (see results).

Quota measurements and theoretical growth rates estimated along the DR gradient were used to test the Droop relation (Droop 1968):

$$\mu = \mu_{max} \cdot (1 - q_o / Q) \qquad (2)$$

where μ (in d^{-1}) is the theoretical growth rate, Q is the quota (in $pg \cdot pgC^{-1}$ or $pg \cdot cell^{-1}$), q_o is the theoretical minimum quota (in $pg \cdot pgC^{-1}$ or $pg \cdot cell^{-1}$) and μ_{max} is the theoretical maximum growth rate at infinite Q (in d^{-1}).

Values of μ (in d^{-1}) were calculated from the dilution rate DR (d^{-1}) according to Tilman and Kilham (1976):

$$\mu = - \ln (1 - DR) \qquad (3)$$

RESULTS

Phosphate concentrations and cell enumerations. For both strains a net decrease in phosphate concentrations with decreasing DR was observed between 0.5 and 0.3 d^{-1} and reached values lower than 0.3 µM for cultures diluted at 0.2 d^{-1} and less (Fig. 1a). Along this decrease, phosphate concentrations were significantly lower (two tailed t test, $p < 0.002$) in the TL01 cultures compared to the ACT03 cultures but only at DR = 0.4 d^{-1}.

Along the dilution gradient cell concentrations were maximal at DR = 0.2 and 0.15 d^{-1} for TL01 and ACT03 cultures, respectively (Fig. 1b and 1c). The maximum was particularly evident for TL01 with a significant decrease (two tailed t test, $p < 0.01$) of 47 % between the maximal value and the density measured at the lowest DR (0.05 d^{-1}). For both strains, the proportion of single cells decreased with increasing DR. But the DR associated with the maximal cell concentration defined a threshold in chain formation: it corresponds to a shift where the increase in the percentage of two cell chains with DR was replaced by an increase in the percentage of four cell chains (Fig. 1b and 1c).

Cellular composition parameters. When expressed on per cell basis, overall Qp variation observed along the DR gradient followed the pattern of PO_4^{3-} concentrations (Fig. 2a). For DR $\leq$ 0.2 d^{-1}, no trend was visible in the relationship between cellular Qp ($Qp_{/cell}$) and DR, as $Qp_{/cell}$ values were constrained at low levels surrounding a mean value of 24.8 ± 2.6 pgP·$cell^{-1}$ compiled from both strains measurements, with a minimum value ($Qp_{/cell\text{-}min}$) of 20.3 pgP·$cell^{-1}$. At higher DRs, a net increase in $Qp_{/cell}$ with the DR was apparent only for the ACT03 strain with a Qp value ($Qp_{/cell\text{-}max}$) of 61.1 pgP·$cell^{-1}$ obtained at DR = 0.5 d^{-1}. For TL01, $Qp_{/cell}$ values appeared to be higher in the range of DR 0.3 – 0.5 d^{-1}, however they only reached 33.1 pgP·$cell^{-1}$ at the highest DR.

Trends in P quota values normalized to cellular carbon (Qp/Q_C) were different from the $Qp_{/cell}$ variations (Fig. 2b). From a minimal value ($Qp/Q_{C\text{-}min}$ = 0.010 pgP·pgC^{-1}) obtained at the minimal DR, Qp/Q_C increased regularly with increasing DR and reached a maximum

plateau at an intermediate DR: 0.4 d^{-1} for TL01 and 0.3 d^{-1} for ACT03. For both strains this plateau was characterized by a mean value of 0.021 ± 0.001 pgP·pgC^{-1}, slightly lower than the Redfield ratio (0.024 pgP·pgC^{-1}). This pattern of Qp/Q_C variation with DR was particularly visible for ACT03, for which a linear increase ($R^2 = 0.95$, $p < 0.005$) was observed between 0.05 d^{-1} and 0.3 d^{-1}.

As discussed below, only data obtained at DRs ranging from 0.2 d^{-1} to 0.5 d^{-1} for TL01 and 0.15 d^{-1} to 0.5 d^{-1} for ACT03 may be compatible with Droop's theory. Data from both strains were fitted to Droop's model (data not shown) to estimate the theoretical minimum quota (q_o) associated with a zero growth rate. From Qp values expressed on a per cell basis, a value close to the $Qp_{/cell\text{-}min}$ measurement was obtained, q_o = 16.4 pgP·$cell^{-1}$ ($r^2 = 0.54$). From Qp estimations per cellular C unit, a q_o value (0.010 pgP·pgC^{-1}) equal to the $Qp/Q_{C\text{-}min}$ measurement was calculated ($r^2 = 0.67$).

^{33}P-uptake rates. Data from DR = 0.5 d^{-1} for ACT03 strain were not included as they were clearly inconsistent with the rest of the data. Along the DR gradient, Vp_{max} showed a maximum at the lowest DR, corresponding to 1.9 fmolP·$cell^{-1}$·min^{-1} and 1.4 fmolP·$cell^{-1}$·min^{-1} (0.129 h^{-1} and 0.097 h^{-1} after division by $Qp_{/cell}$) for TL01 and ACT03 respectively (Fig. 3a). A regular decrease of Vp_{max} with the DR was observed as DR increased from 0.05 d^{-1} to 0.3 d^{-1} and a mean value of Vp_{max} of 0.2 ± 0.1 fmolP·$cell^{-1}$·min^{-1} (0.014 ± 0.009 h^{-1}) was obtained for DR ≥0.3 d^{-1}, compiling data of both strains.

Considering variation of Vp_{max} as a function of extracellular PO_4^{3-} concentrations (Fig. 3b): Vp_{max} higher than 0.42 fmolP·$cell^{-1}$·min^{-1} (0.039 h^{-1}) were associated with PO_4^{3-} concentrations lower than 0.34 µM. Above this threshold, Vp_{max} remained near 0.21 ± 0.11 fmolP·$cell^{-1}$·min^{-1}, corresponding to 0.011 ± 0.007 h^{-1}. No relationship was found between Vp_{max} and $Qp_{/cell}$, but an exponential decrease was observed representing Vp_{max} as a function of Qp/Q_C ($r^2 = 0.68$) (Fig. 3c).

From kinetics experiments testing three DR conditions for each strain, variations of PO_4^{3-} uptake rates as a function of concentrations followed the Michaelis-Menten model with $r^2 \geq 0.79$. The maximal P-uptake rate $V_{max(P)}$ showed a net decrease as the DR increased (Table 1), in accordance with previous trends observed in PO_4^{3-} uptake rate data (Fig. 3a). Associated $K_{S(P)}$ values ranged between 0.01 µM and 1.17 µM for TL01 and between 0.03 µM and 0.14 µM for ACT03 (Table 1).

APA measurements. The main part of the APA measured in the total fraction was cell-bound. The dissolved APA (< 0.2 µm) represented less than 13% of the total APA for all measurements, while for most of the data (77%) it reached less than 5% of the total fraction (data not shown). Considering the variation of maximal APA per cell as a function of DR, values showed an all-or-nothing pattern (Fig. 4a). Significant APA measurements were obtained for DR ≤ 0.3 d^{-1} for TL01 and 0.2 d^{-1} for ACT03, with no particular trend observed as the DR decreased. In this range, maximal normalized APA varied around respective means values of 13.4 ± 3.6 fmolP·cell^{-1}·min^{-1} and 10.0 ± 3.2 fmolP·cell^{-1}·min^{-1} for TL01 and ACT03, respectively.

Expressed as a function of PO_4^{3-} concentrations (Fig. 4b), values of maximal APA per cell showed a pattern clearly defined by a threshold value between 0.4 µM and 1.0 µM of PO_4^{3-}. A similar pattern was observed considering maximal APA per cell as a function of Qp/Q_C (Fig. 4c) and highlighted a strict threshold at $Qp/Q_C = 0.016$ pgP·pgC^{-1} (C:P ratio of 161) which separated large APA values from negligible ones. The relation observed between maximal APA and $Qp_{/cell}$ values was not so clear-cut, but large APA values were obtained for $Qp_{/cell} < 42.1$ pgP·cell^{-1} (Fig. 4d).

Along the MUF-P gradient of 0.5 – 250 µM, three series of specific APA measurements were done for each strain corresponding to three different DRs. The use of a single Michaelis-Menten equation did not allow a convergence of the hyperbolic regression

analysis with confidence intervals of 95% in all cases. For three of the six kinetic experiments, the use of a single equation did not lead to a random dispersion of normalized residuals along the substrate gradient, with a trend suggesting that the model tends to overestimate activities between 3.9 and 62.5 µM of MUF-P and underestimate them at higher substrate concentrations. To avoid distorted estimations of kinetic parameters, biphasic patterns described by two Michaelis-Menten equations were used to model these data, with a transition between both phases in the range 31.3 - 62.5 µM of MUF-P (Fig. 5). For biphasic models, K_S values of Phase I estimate AP affinity of ***A. catenella*** cells and V_{max} values of Phase II estimate enzyme efficiency under high substrate concentrations. Comparing these parameters values with the K_S and V_{max} values of uniphasic models (Table 1), no trend was clear in APA K_S and V_{max} values with DR.

DISCUSSION

P-limitation conditions. Phosphate pulses were almost entirely consumed after 24h in the cultures diluted at DR ≤ 0.2 d^{-1}, suggesting that the DR gradient from 0.05 – 0.5 d^{-1} imposed a severe P-limitation on ***A. catenella*** cells maintained at low DRs. The rate of chain formation reflected the graded phosphate conditions, probably due to a more general relationship between chain formation and growth rate. Considering total cell concentrations, a critical dilution rate can be defined for each strain from the maximum observed cell density (***DR_3*** on Figure 6). Maximal values at intermediate DRs were unexpected as cell concentrations often decrease linearly with DR, matching Droop's model (1968). Divergences from this theoretical trend may be observed under low DRs where cultures may reach particular steady states well described by Sciandra and Ramani (1994). According to these authors, decreasing cell density at low DRs (DR ≤ 0.3 d^{-1}) may be explained by additional controlling factors, such as competition with bacteria or negative effects due to accumulation

of excreted products. Shafik et al. (1997) and Le Floc'h et al. (2002) also reported cellular densities lower than the ones supported by Droop's model at low DRs ($DR \leq 0.11\ d^{-1}$).

At the cellular scale, the graded P-stress conditions led to a decrease in Qp per cell ($Qp_{/cell}$) followed by a decrease in Qp per C unit (Qp/Q_C) as the DR decreased (Fig. 6). These trends were particularly visible for ACT03. The uncoupling observed at highest DRs between $Qp_{/cell}$ and Qp/Q_C variations defines a first P-stress range where conditions are still P-sufficient for cell metabolism but the cell size decreases when the P-stress increases. The start of a decrease in Qp/Q_C when the DR decreases (DR_1 on Figure 6) indicates the threshold between P-sufficient conditions and P-limitation. P-starvation conditions are then encountered when $Qp_{/cell}$ reaches the minimum plateau (DR_2 on Figure 6). Below this P-starvation threshold (DR_2), Qp could not act as a buffer against external PO_4^{3-} depletion. Consequently, when PO_4^{3-} from the daily pulse was exhausted, cells could not use the internal P pool to support their growth. Under these P-starvation conditions, cell size probably increased with P-stress as $Qp_{/cell}$ was maintained when Qp/Q_C decreased with decreasing DR. This could explain the observed decrease in cell density at lowest DRs by a lag in cell division leading to growth rates that are lower than the ones predicted by Droop's theory.

Contrary to our results, Matsuda et al. (2006) observed a maximal cell density and a minimal $Qp_{/cell}$ at the lowest DR ($0.05\ d^{-1}$) in semi-continuous experiments performed with axenic cultures of *A. catenella* along a P-limitation DR gradient of $0.05 - 0.35\ d^{-1}$. Our non axenic experiments may have imposed additional controlling factors for *A. catenella* growth at low DRs such as: (i) competition with bacteria, resulting in more severe P-limitation conditions for algal cells, or (ii) cell density effects as the maximal cell concentration in the present study is seven fold higher than the one reported in Matsuda et al. (2006). The discrepancies noted along similar DR gradients also suggest that the French strains of *A.*

catenella may be more sensitive to P-stress than the Japanese strain TNY7 used by Matsuda et al. (2006).

P-storage capacities. The range of Qp values gives an indication of potential P-storage capacities of algal cells from the evaluation of the "luxury coefficient" defined by Droop (1974) as the ratio between the maximum and the minimum quotas, determined in non-limited and limited conditions respectively. Differences in patterns of $Qp_{/cell}$ and Qp/Q_C along the DR gradient, however, highlight the importance of the reference used (cell or Q_C) for quota estimations. Compiling data of ACT03 and TL01 compatible with Droop's theory (obtained at $DR \geq DR_3$), values of the theoretical minimum quota q_o (16.4 pgP·cell^{-1}; 0.010 pgP·pgC^{-1}) obtained from Droop's model were close to minimal Qp measurements ($Qp_{/cell\text{-}min}$ = 20.3 pgP·cell^{-1} ; $Qp/Q_{C\text{-}min}$ = 0.010 pgP·pgC^{-1}). Thus, neither ratios between the maximal and minimal Qp measurements (Qp_{max} / Qp_{min}) nor Qp_{max} / q_o revealed a particular P storage capacity of these strains because each ratio (not detailed) was low ($\leq$ 3.7).

A large range of minimal (1.3 – 83.5 pgP·cell^{-1}) and maximal (1.9 – 788.7 pgP·cell^{-1}) $Qp_{/cell}$ values have been reported for marine dinoflagellates (Sakshaug et al.1984, Ou et al. 2008), suggesting the existence of different P requirements and P-storage capacities between phytoplankton genera. For *Alexandrium* species, $Qp_{/cell\text{-}min}$ estimations were obtained for *Alexandrium tamarense* (23.6 pgP·cell^{-1}) and *A. catenella* (9.0 pgP·cell^{-1}) by Yamamoto and Tarutani (1999) and Matsuda et al. (2006) respectively, with a high P storage capacity highlighted for *A. tamarense* from Qp_{max} / q_o = 36 (cited in Yamamoto and Tarutani 1999). Cellular Qp in these studies, however, were not measured but calculated from the difference of PO_4^{3-} concentration between input and output media, which makes difficult a direct comparison with our $Qp_{/cell}$ data. More comparable results for *Alexandrium* species were obtained by Ou et al. (2008) and Béchemin et al. (1999). Ou et al. (2008) reported similar $Qp_{/cell\text{-}min}$ (24.7 pgP·cell^{-1}) but two times higher $Qp_{/cell\text{-}max}$ values for *A. catenella*, leading to

Qp_{max} / Qp_{min} = 7.68 which also suggests low P storage capacity of this species. According to Béchemin et al. (1999), *A. minutum* may present even lower P-storage capacities as they did not observe a trend in $Qp_{/cell}$ variations along a P-stress gradient, defined by N:P ranging from 16 to 160, with $Qp_{/cell}$ values oscillating between 8.7 and 14.3 pgP·cell^{-1}. Additional estimations under transient conditions, however, are required for a more ecologically relevant estimation of P-storage capacities of *A. catenella* cells (Spijkerman and Coesel 1998).

Alkaline phosphatase activity. Alkaline phosphatases activity is often used as another P-limitation indicator (Cembella et al. 1984, Dyhrman and Palenik 2003) as it is inducible by low extracellular PO_4^{3-} concentrations for many marine phytoplankton species. However, additional factors may regulate AP synthesis (Hoppe 2003) and, in particular for the *Alexandrium* genus, APA appeared to be a poor indicator of PO_4^{3-}-stress for *A. minutum*, *A. tamarense* and *A. affine* cells (Flynn et al. 1996). Among the potential controlling factors, a regulation by the intracellular P-pool has often been reported, for example by Xu et al. (2006) who noted a delay of several hours in the induction of genes associated with AP synthesis by *E. huxleyi* after cells transfer in P-depleted conditions.

In the present study, fractionated APA measurements were done to estimate cell bound and dissolved APA. Both AP synthesized by phytoplankton (Huang et al. 2005, Xu et al. 2006) and bacteria (Hoppe 1991) may be released into the water and contribute to the dissolved APA pool. The low levels of dissolved APA measured during our experiments indicate that APA was mainly cell bound in our cultures and that activity from the > 10 µm fraction described adequately APA originating from *A. catenella*.

Contrary to the observations of Flynn et al. (1996) on other species of *Alexandrium*, the regulation of AP synthesis by extracellular PO_4^{3-} concentrations appears to be straightforward in *A. catenella* cells, defined by a threshold value between 0.4 µM and 1.0 µM of PO_4^{3-}. Such threshold values have been reported for other marine phytoplankton

species (Table 2) revealing that this parameter is species specific, with a high inter-Class variability. The PO_4^{3-} concentration threshold observed for *A. catenella* is in accordance with the one characterizing *A. tamarense* (Oh et al. 2002), while values reported for other toxic dinoflagellates ranged from 0.2 µM for *Karenia mikimotoi* (Yamaguchi et al. 2004) to 3.3 µM for *Gymnodinium catenatum* (Oh et al. 2002). Regulation by the intracellular P-pool did not seem to be as direct as by extracellular PO_4^{3-} concentration. Indeed, the relation between APA and $Qp_{/cell}$ was not so clear-cut, even though a $Qp_{/cell}$ value lower than ~40 $pgP \cdot cell^{-1}$ appears necessary to allow AP synthesis. The control by the intracellular P-pool may take effect principally through the C:P stoichiometry because a Qp/Q_C value of 0.016 $pgP \cdot pgC^{-1}$ (atomic C:P = 161) appeared as a threshold for AP synthesis. Comparing variations of APA and Qp/Q_C along the DR gradient, normalized APA per cell increased drastically when Qp/Q_C started to decrease (*DR_1* on Figure 6) but did not vary further under more severe P-stress conditions. Thus, synthesis of AP by *A. catenella* cells appears to be induced and maximized as soon as P-limitation sets in. According to Ou et al. (2006), this maximization of APA synthesis may also be observed at the population scale. From batch cultures of *A. catenella*, these authors noted that a change in bulk APA of 0 to 1.42 $nmolP \cdot L^{-1} \cdot h^{-1}$ during two consecutive days was associated with a change of enzyme-labeled fluorescent cells from 1.4 % to 100 %, where cells were labeled on their active AP sites located on the cell surface by a fluorescent precipitate.

The characterization of APA in *A. catenella* cells may be complemented from the analysis of kinetic parameter values. Values obtained for TL01 and ACT03 appeared to be very close. Compiling data of both *A. catenella* strains, APA was characterized by an affinity constant (K_S) of 1.3 ± 0.7 µM and a potential maximal activity value (V_{max}) of 12.5 ± 2.8 $fmolP \cdot cell^{-1} \cdot min^{-1}$. Ou et al. (2008) determined lower kinetics parameters values for *A. catenella* cells (K_S = 0.55 µM, V_{max} = 2.88 $fmolP \cdot cell^{-1} \cdot min^{-1}$) using another fluorogenic

substrate, MFP. However, it is problematic to compare between studies when different AP substrates are being used (Dyhrman 2005). Comparing marine phytoplankton studies based on similar APA assays, values obtained for *A. catenella* reveal that this species shows a major competitive advantage for utilizing the DOP resource at low concentrations (Table 3).

Phosphate uptake characteristics. Metabolic adaptations of *A. catenella* cells to graded P-limitation in terms of PO_4^{3-} uptake include the control of Vp_{max} which was clearly linked with extracellular PO_4^{3-} concentrations and Qp/Q_C, but no characteristic influence of $Qp_{/cell}$ on Vp_{max} could be identified. Thus, as for AP regulation, PO_4^{3-} uptake rates appear to be regulated by the intracellular P quota only through an indirect pathway, the P:C stoichiometry of the cell. The exponential decrease in Vp_{max} as a function of Qp/Q_C reveals that *A. catenella* cells are able to optimize their PO_4^{3-} uptake capacity as the P-deficiency increases. Even if, under P-limitation, the specific APA per cell did not rise with the P-stress, this potential optimization of PO_4^{3-} uptake may also benefit to the DOP utilization as PO_4^{3-} released from APA may be more rapidly taken up. The maximal reached value of Vp_{max} was 1.9 fmolP·cell^{-1}·min^{-1} (for TL01) corresponding to 0.13 h^{-1}. This value is low compared to the one reported for *A. catenella* (2.5 h^{-1}) by Ou et al. (2008) and for the other dinoflagellates *A. tamarense* (1.8 h^{-1}, Yamamoto and Tarutani (1999)) and *G. catenatum* (0.31 h^{-1}, Yamamoto et al. (2004)) or the diatom *S. costatum* (2.6 h^{-1}, Ou et al. (2008)). Thus, Thau lagoon strains of *A. catenella* showed relatively poor competitive abilities under high PO_4^{3-} concentrations. Concerning abilities under low concentrations, K_S measured in this study (0.01 - 1.17 µM) are in accordance with the range (0.4 – 1.68 µM) reported for *Alexandrium* species by Cembella et al. (1984), Yamamoto and Tarutani (1999) and Frangópulos et al. (2004). Ou et al. (2008), however, characterized a Chinese strain of *A. catenella* by a higher K_S of 2.28 µM. Based on the summary of reported K_S values (0.01 – 2.8 µM) for marine phytoplankton species

proposed by Yamamoto and Tarutani (1999), K_S values obtained for Thau lagoon strains of *A. catenella* indicate a more efficient competitor for PO_4^{3-} at low concentrations.

Ecological considerations. The appearance of *A. catenella* in Thau lagoon (France) waters followed a long term decrease in PO_4^{3-} concentrations (Collos et al. 2009). This suggests that a modification of the bottom-up control of *A. catenella* bloom development may have occurred. The periodic dominance of this species may be partly explained by its competitive capacities to take up PO_4^{3-} at low concentrations and by its abilities to use DOP resources. Considering *A. catenella* blooms in spring 2002 and autumn 2003, AP synthesis has been likely to be induced during a large part of blooms durations as PO_4^{3-} concentrations lower than 0.4 µM were measured during 80 % and 33 % of the bloom duration in 2002 and 2003, respectively (unpublished data). Under such environmental conditions, *A. catenella* may have a competitive advantage from DOP use when APA is induced, as the range of *in situ* DOP concentrations (0.6-1.4 µM, Laugier, T.) was low but close to the K_S of APA (1.3 µM). Thus, *A. catenella* bloom developments in Thau lagoon in 2002 and 2003 may have been sustained by the DOP resource, with a significant part of P-uptake based on APA. A similar assumption was proposed by Ou et al. (2008) for *A. catenella* developments in Chinese coastal waters.

CONCLUSION

Shifts in trends of Qp/Q_C, $Qp_{/cell}$ and cell density variations along the DR gradient allow the definition of three successive P-stress thresholds for *A. catenella* cells. For each strain, a slight change in DR values, from 0.3 d^{-1} to 0.2 d^{-1} for ACT03 and from 0.4 d^{-1} to 0.2 d^{-1} for TL01, was sufficient to induce a shift from P-sufficient conditions to P-starvation. Thus, *A. catenella* cells show a very high sensitivity to P-stress, with the TL01 strain presenting slightly higher P-requirements than ACT03. APA was a robust indicator of PO_4^{3-}-

449 limitation for *A. catenella* cells. Additional *in situ* measurements have to be performed to
450 assess to what extent DOP utilization may be considered as a key competitive advantage for
451 *A. catenella* cells in Thau lagoon waters with regards to other controlling factors.
452
453 ACKNOWLEDGMENTS
454 This study was financed by CNRS (Centre National de la Recherche Scientifique) and Ifremer
455 (Institut Français de Recherche pour l'Exploitation de la Mer) ALTOX program. C.J. was
456 supported by a scholarship from the Région Languedoc-Roussillon and Ifremer.
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TABLE 1. Values of kinetic parameters (K_S in µM and V_{max} in fmolP·cell^{-1}·min^{-1}) obtained for PO_4^{3-} uptake rate and APA at different dilution rates (d^{-1}). APA results compiled data obtained using uniphasic and biphasic Michaelis-Menten models, with values detailed for each phase in case of biphasic patterns. Both phases of biphasic patterns are denoted as phases I and II and represent data modeling in the low and high substrate concentration ranges respectively. Dashes indicate which values of K_S (*) and V_{max} (**) for APA are comparable between uniphasic and biphasic patterns.

Kinetic parameters	Strain	Dilution rate	PO_4^{3-} uptake	APA		
				Uniphasic	Biphasic	
					Phase I	Phase II
K_S	TL01	0.15	1.17	1.22*	-	-
		0.2	0.44	-	0.82*	11.64
		0.3	0.01	-	0.29*	26.58
	ACT03	0.1	0.14	1.79*	-	-
		0.15	0.03	-	1.47*	27.55
		0.2	0.04	2.33*	-	-
V_{max}	TL01	0.15	1.97	11.8**	-	-
		0.2	0.77	-	6.61	9.64**
		0.3	0.49	-	5.24	8.89**
	ACT03	0.1	1.75	14.29**	-	-
		0.15	0.75	-	9.04	15.26**

	0.2	0.48	14.84**	-	-

8

TABLE 2. Values of PO_4^{3-} concentration threshold (in µM) reported to induce AP synthesis for marine phytoplankton species.

Classes	Species	PO_4^{3-} concentration threshold	Reference
Dinophyceae			
	Alexandrium catenella	0.4 - 1	Present study
	Alexandrium tamarense	0.43	Oh et al. (2002)
	Gymnodinium catenatum	3.3	Oh et al. (2002)
	Karenia mikimotoi	0.2	Yamaguchi et al. (2004)
	Ptychodiscus brevis	< 0.5	Vargo and Shanley (1985)
Bacillariophyceae			
	Phaeodactylum tricornutum	50	Garcia-Ruiz et al. (1997)
	Skeletonema costatum	0.25	Yamaguchi et al. (2004)

Prymnesiophyceae			
	Emiliana huxleyi	0.25	Dyhrman and Palenik (2003)
	Phaeocystis sp.	0.5	van Boekel and Veldhuis (1990)

TABLE 3. Comparison of kinetic parameter values (K_S in µM and V_{max} in fmolP·cell^{-1}·min^{-1}) reported for APA between *A. catenella* and other marine phytoplankton species. Compilation limited to previous works where the fluorogenic substrates MUF-P or MFP were used.

Classes	Species	Substrate	K_S	V_{max}	Reference
Dinophyceae					
	Heterocapsa circularisquama	MUF-P		3.44	Yamaguchi et al. (2005)
	Prorocentrum donghaiense	MFP	0.25	0.09	Ou et al. (2008)
	Alexandrium catenella	MFP	0.55	2.88	Ou et al. (2008)
	Alexandrium catenella	MUF-P	1.32	12.45	Present study
Bacillariophyceae					
	Phaeodactylum tricornutum	MUF-P	3.1	186	Garcia-Ruiz et al. (1997)
	Chaetoceros ceratosporum	MUF-P		1.04	Yamaguchi et al. (2005)
	Skeletonema costatum	MFP	1.38	0.03	Ou et al. (2008)

Prymnesiophyceae				
Emiliana huxleyi	MFP	1.9	3.17	Riegman et al. (2000)
Emiliana huxleyi	MUF-P	2.2	5100	Dyhrman and Palenik (2003)

Figure legends

Figure 1. Residual phosphate concentrations in semi-continuous cultures of *Alexandrium catenella* as a function of dilution rate (DR) for both TL01 and ACT03 strains (a), *A. catenella* cell concentrations (Cell nb) and cell distributions in chains of one (x1), two (x2) or four cells (x4) along the same DR gradient for TL01 strain (b) and ACT03 strain (c). Data correspond to means between replicate cultures at equilibrium and standard deviations are indicated for phosphate and cell concentrations.

Figure 2. Phosphorus content per cell of *Alexandrium catenella* (a) and phosphorus content per cell carbon (b) for both TL01 and ACT03 strains in semi-continuous cultures as a function of dilution rate (DR). Data correspond to averages of values obtained on days 1, 2 and 3 after equilibrium.

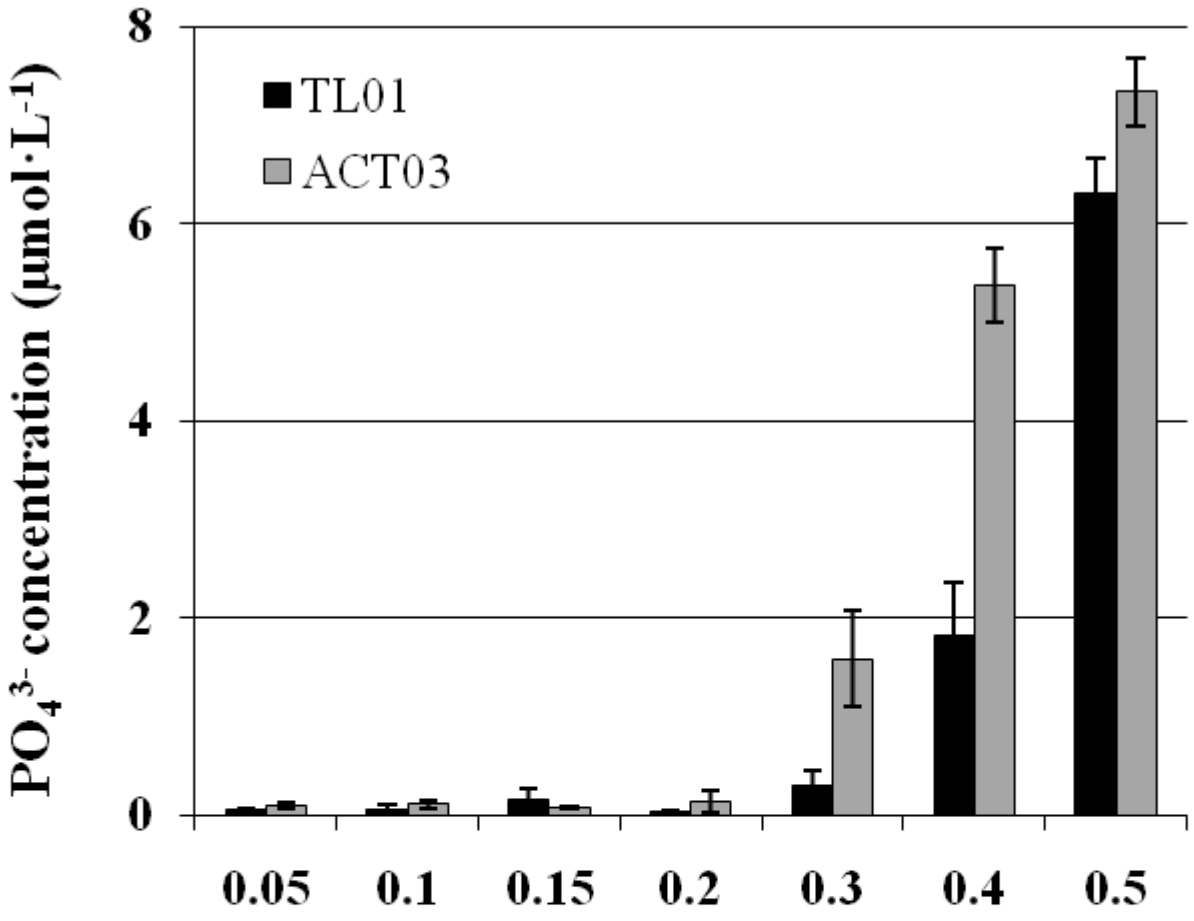
Figure 3. Maximal phosphate uptake rates per cell of *A. catenella* for both TL01 and ACT03 strains in semi-continuous cultures, expressed as a function of (a) the dilution rate (DR), (b) the residual phosphate concentration in culture medium and (c) the phosphorus content per cell carbon. Histogram data correspond to averages of values obtained on days 1, 2 and 3 after equilibrium.

Figure 4. Maximal alkaline phosphatase activity per cell of *A. catenella* for both TL01 and ACT03 strains in semi-continuous cultures, expressed as a function of (a) the dilution rate (DR), (b) the residual phosphate concentration in culture medium, (c) the phosphorus content per cell carbon and (d) the phosphorus content per cell of *A. catenella.* Histogram data correspond to averages of values obtained on days 1, 2 and 3 after equilibrium.

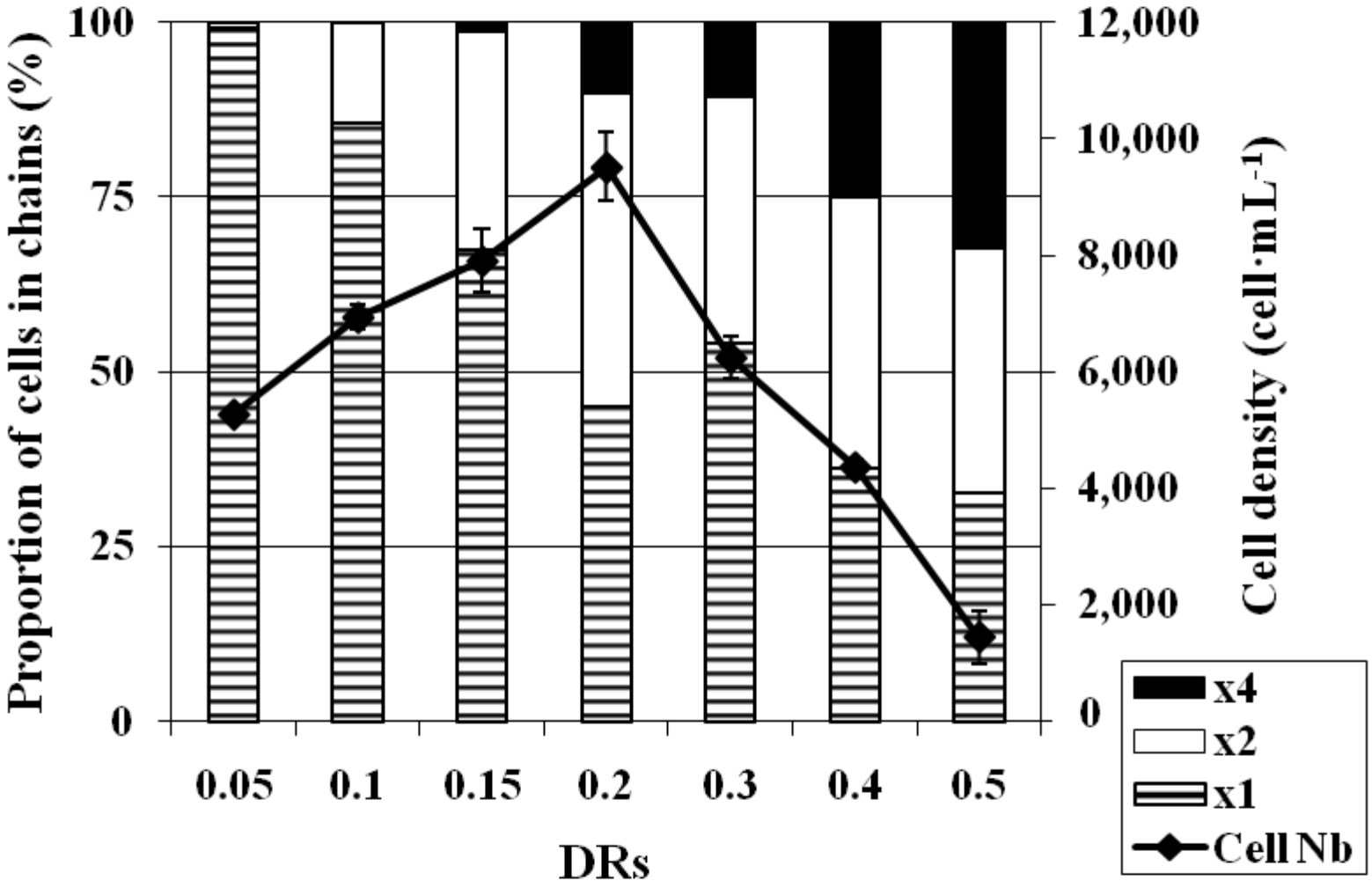
Figure 5. Alkaline phosphatase activity per cell of *A. catenella* as a function of methyl-umbelliferyl phosphate (MUF-P) concentration for TL01 strain at DR = 0.3 d^{-1} (a) and ACT03 strain at DR = 0.15 d^{-1} (b). Modeled curves correspond to biphasic kinetics based on the Michaelis-Menten model and are denoted as phases I and II for data modeling in the low and high substrate concentration ranges respectively.

Figure 6. Schematic representation of the different nutrient states of *A. catenella* assessed from trends in cell density (solid line), phosphorus content per cell (dashed line) and phosphorus content per cell carbon (dotted line) as a function of dilution rate (DR). Shifts in trends allowed the definition of three critical DR values (DR_1, DR_2 and DR_3) representing graded P-stress conditions.

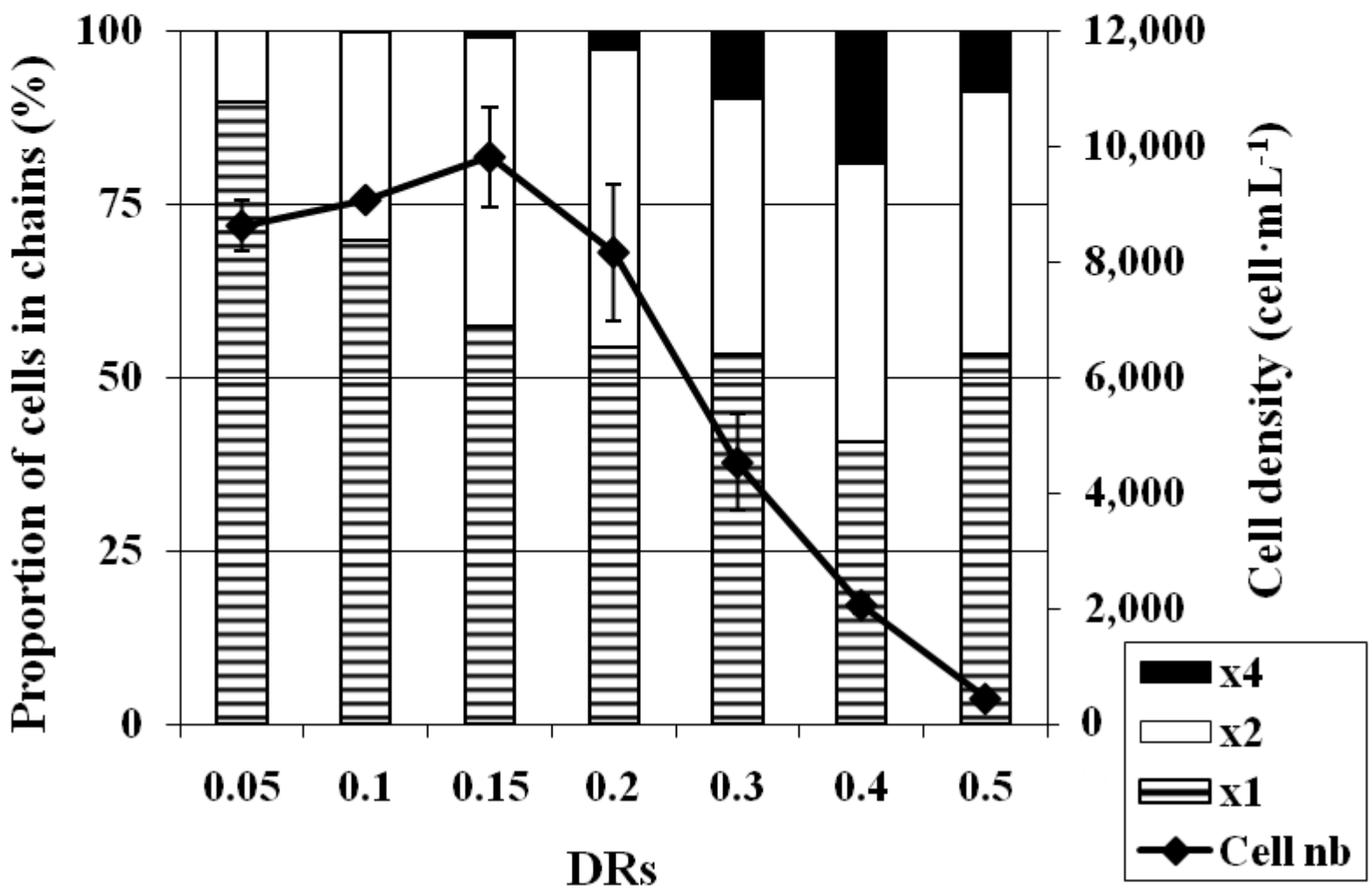
a)

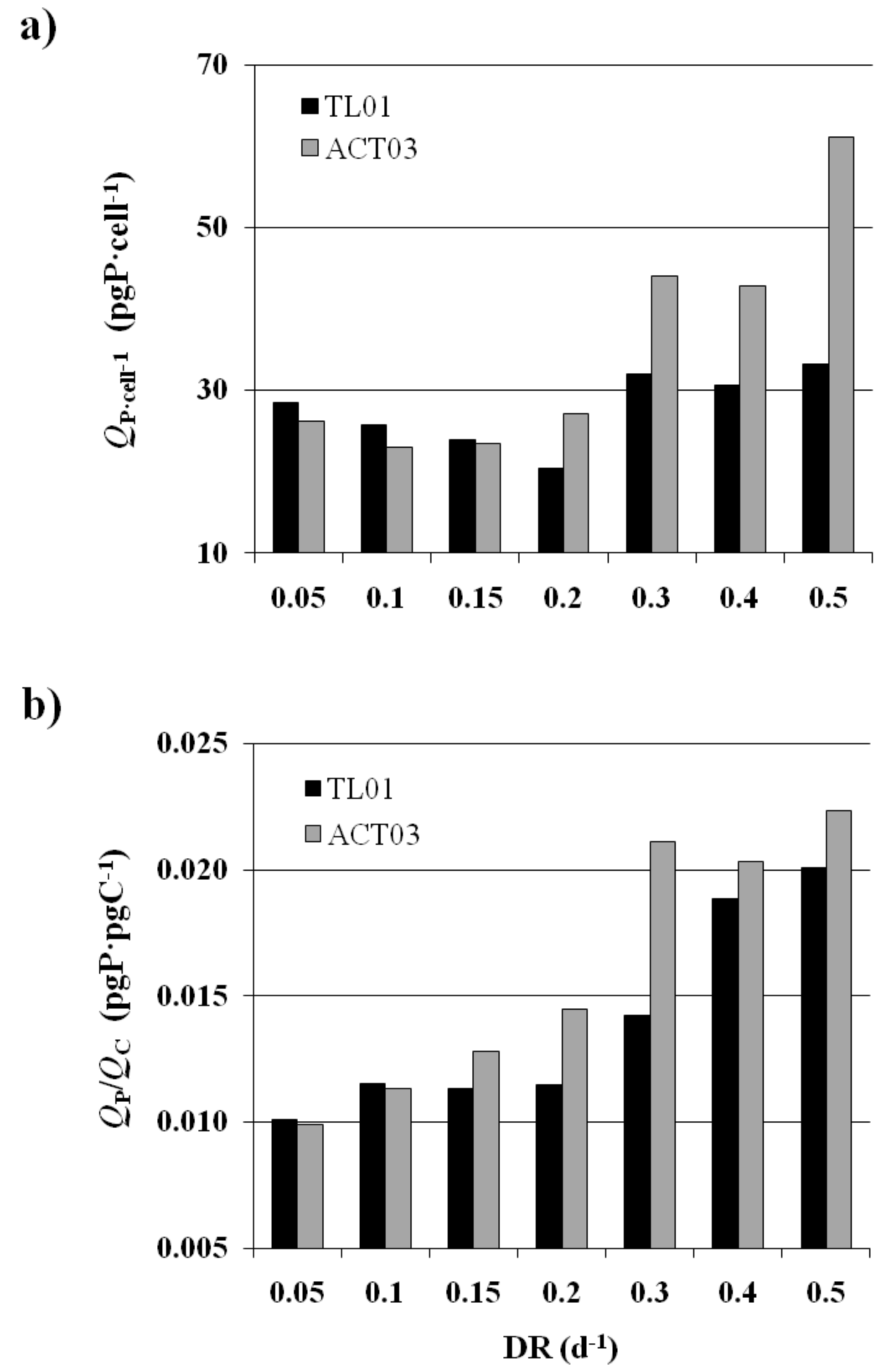

TL01
ACT03
PO_4^{3-} concentration (µmol·L^{-1})
8
6
4
2
0
0.05
0.1
0.15
0.2
0.3
0.4
0.5

b)

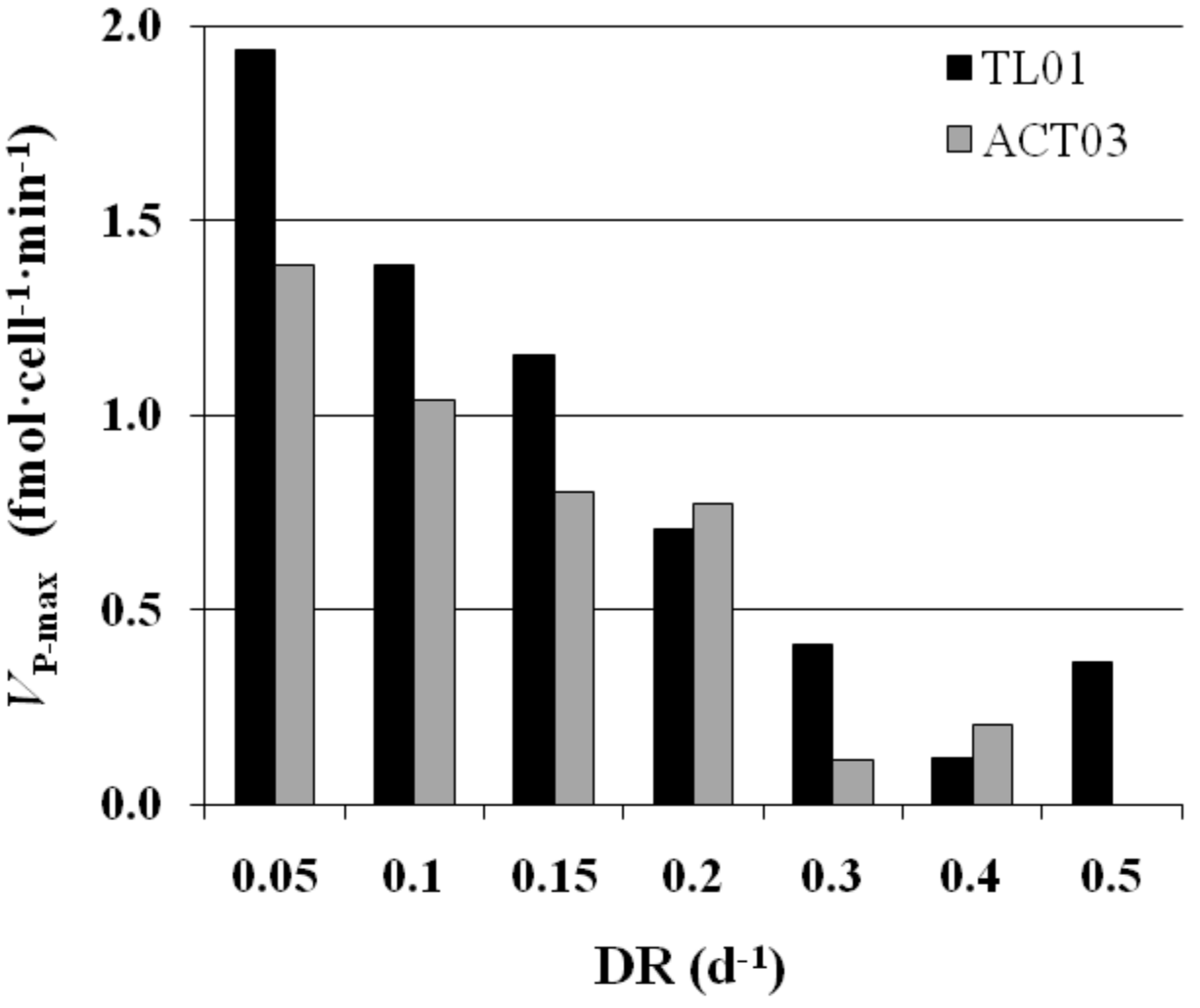

Proportion of cells in chains (%)
100
75
50
25
0
12,000
10,000
8,000
6,000
4,000
2,000
0
Cell density (cell·mL^{-1})
0.05
0.1
0.15
0.2
0.3
0.4
0.5
DRs
x4
x2
x1
Cell Nb

c)

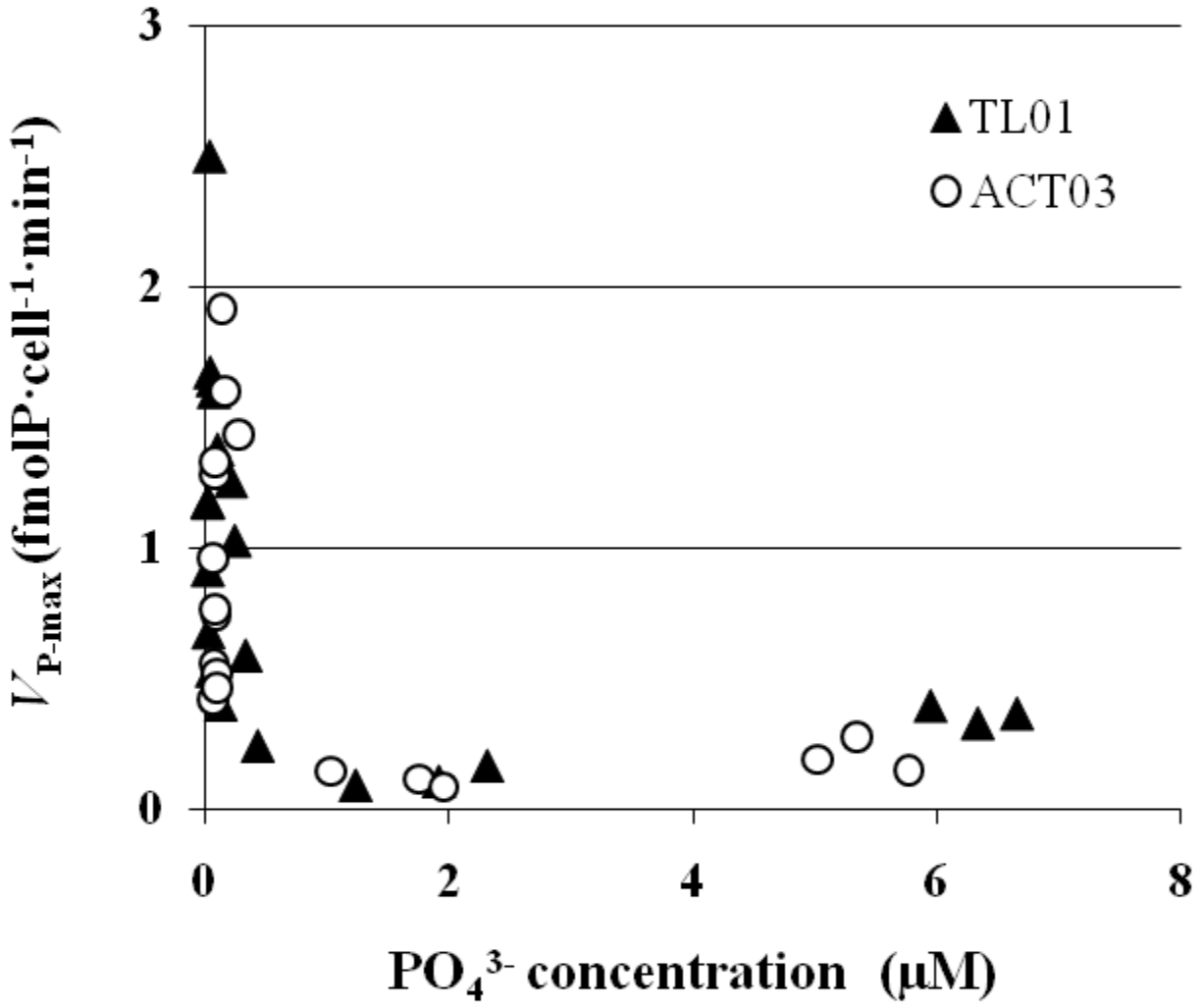

Proportion of cells in chains (%)
100
75
50
25
0
12,000
10,000
8,000
6,000
4,000
2,000
0
Cell density (cell·mL^{-1})
0.05
0.1
0.15
0.2
0.3
0.4
0.5
DRs
x4
x2
x1
Cell nb


a)
70
50
30
10
TL01
ACT03
$Q_{P\cdot cell^{-1}}$ (pgP·cell^{-1})
0.05
0.1
0.15
0.2
0.3
0.4
0.5
b)
0.025
0.020
0.015
0.010
0.005
TL01
ACT03
Q_P/Q_C (pgP·pgC^{-1})
0.05
0.1
0.15
0.2
0.3
0.4
0.5
DR (d^{-1})

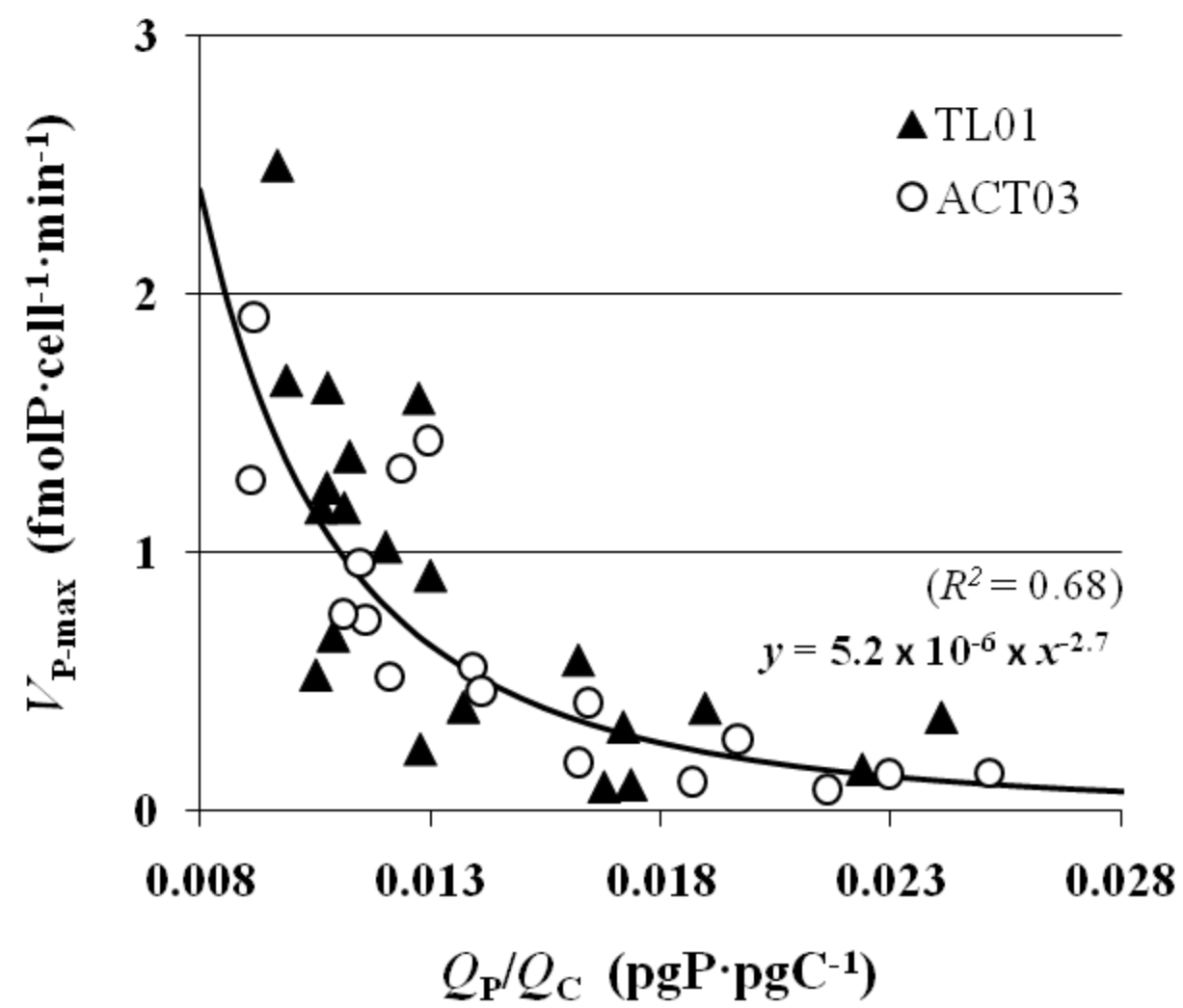
a)


2.0
1.5
1.0
0.5
0.0
TL01
ACT03
$V_{P\text{-}max}$ (fmol·cell^{-1}·min^{-1})
0.05
0.1
0.15
0.2
0.3
0.4
0.5
DR (d^{-1})

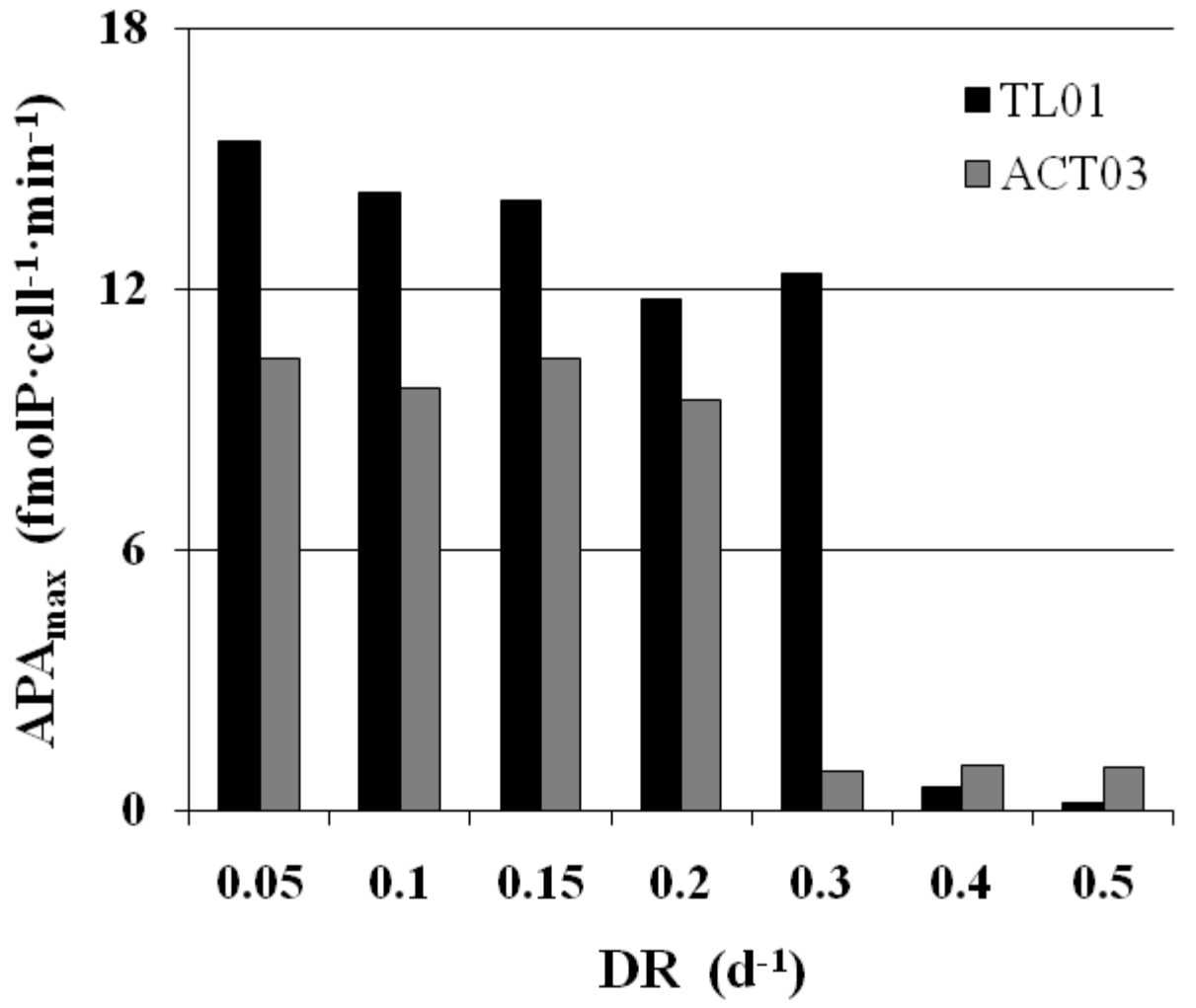
b)


3
2
1
0
TL01
ACT03
$V_{P\text{-}max}$ (fmolP·cell^{-1}·min^{-1})
0
2
4
6
8
PO_4^{3-} concentration (µM)

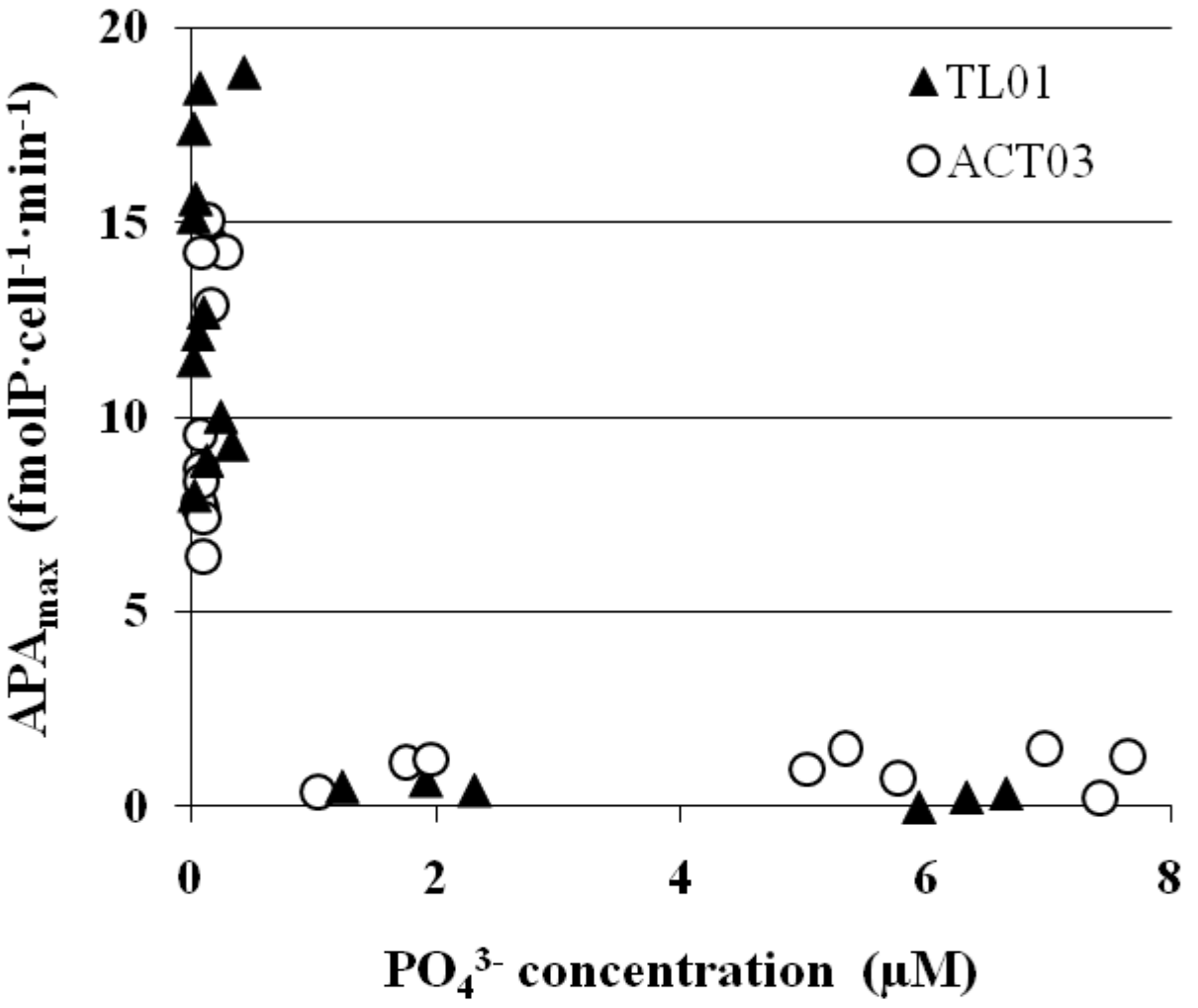
c)


3
2
1
0
TL01
ACT03
$(R^2 = 0.68)$
$y = 5.2 \times 10^{-6} \times x^{-2.7}$
$V_{P\text{-}max}$ (fmolP·cell^{-1}·min^{-1})
0.008
0.013
0.018
0.023
0.028
Q_P/Q_C (pgP·pgC^{-1})

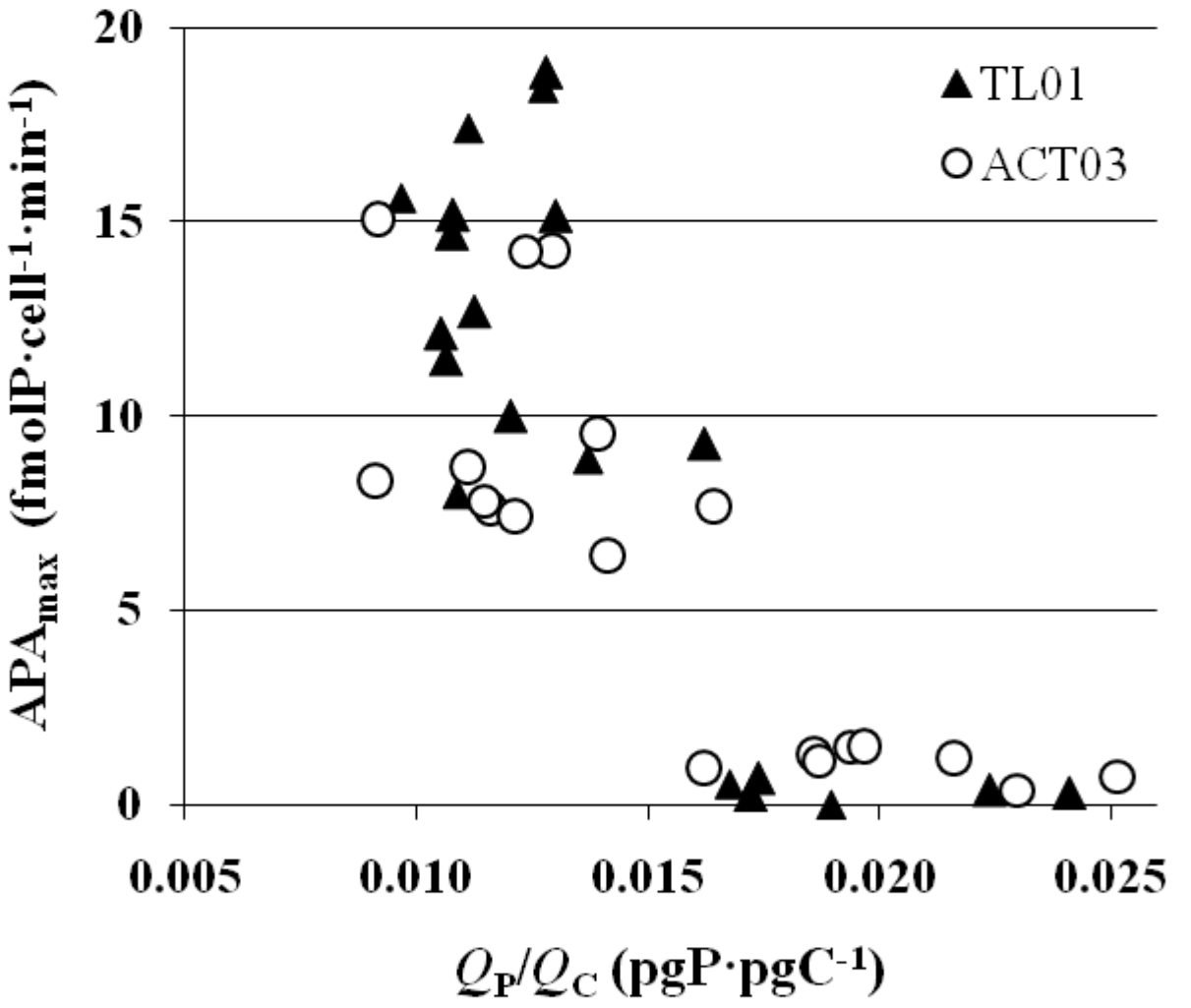
a)


18
12
6
0
TL01
ACT03
APA$_{max}$ (fmolP·cell^{-1}·min^{-1})
0.05
0.1
0.15
0.2
0.3
0.4
0.5
DR (d^{-1})

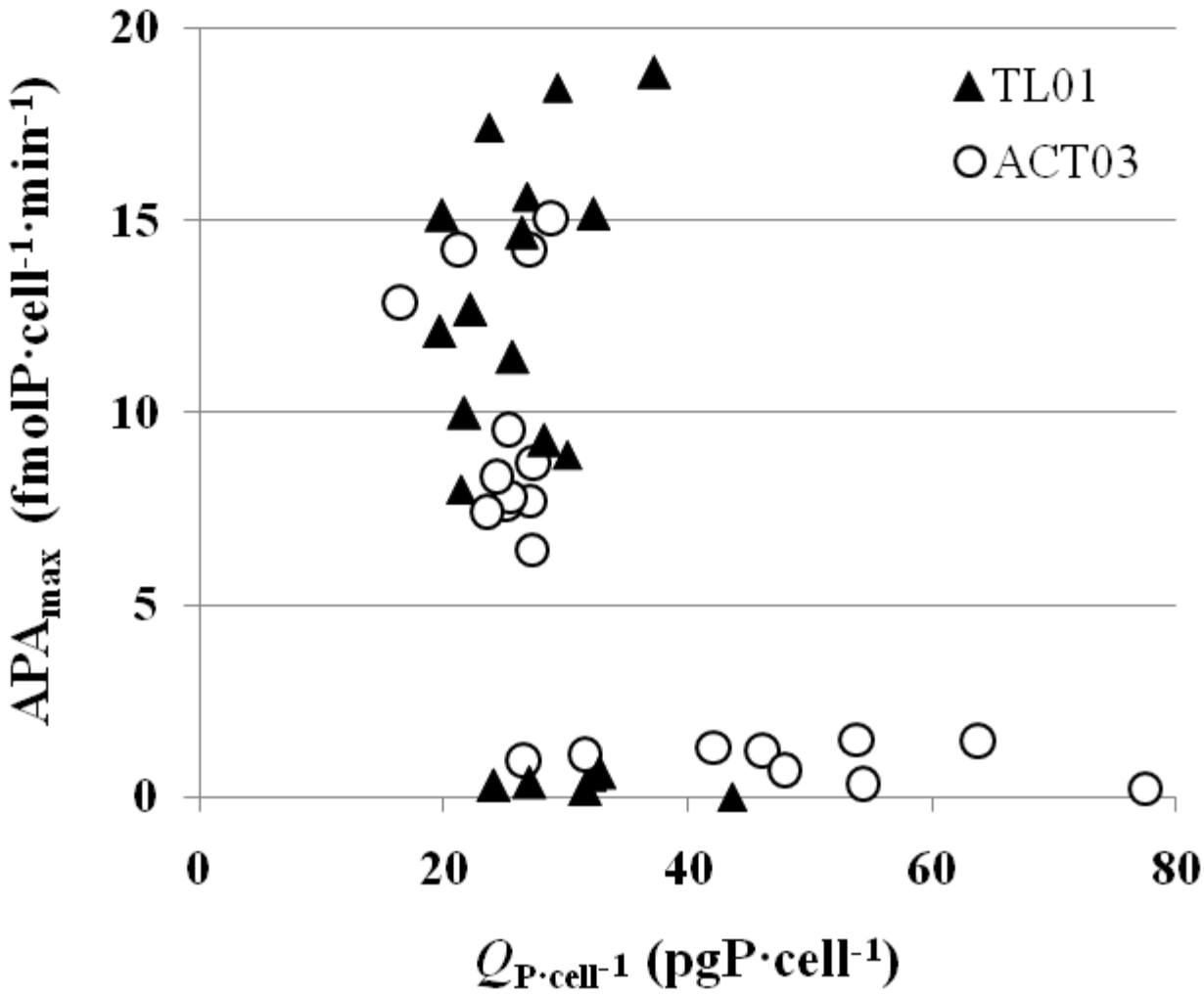
b)

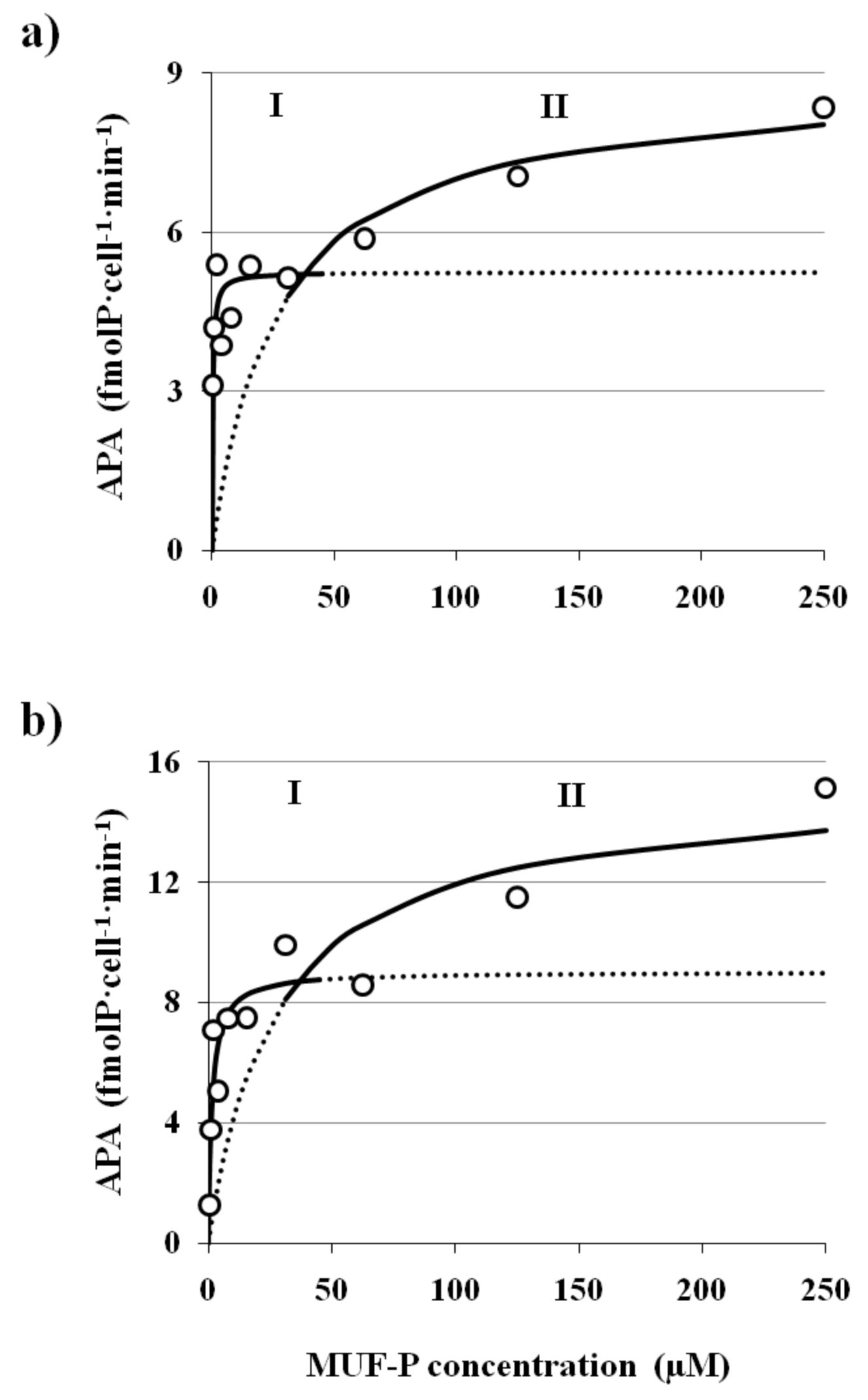

20
15
10
5
0
TL01
ACT03
APA$_{max}$ (fmolP·cell^{-1}·min^{-1})
0
2
4
6
8
PO$_4^{3-}$ concentration (µM)

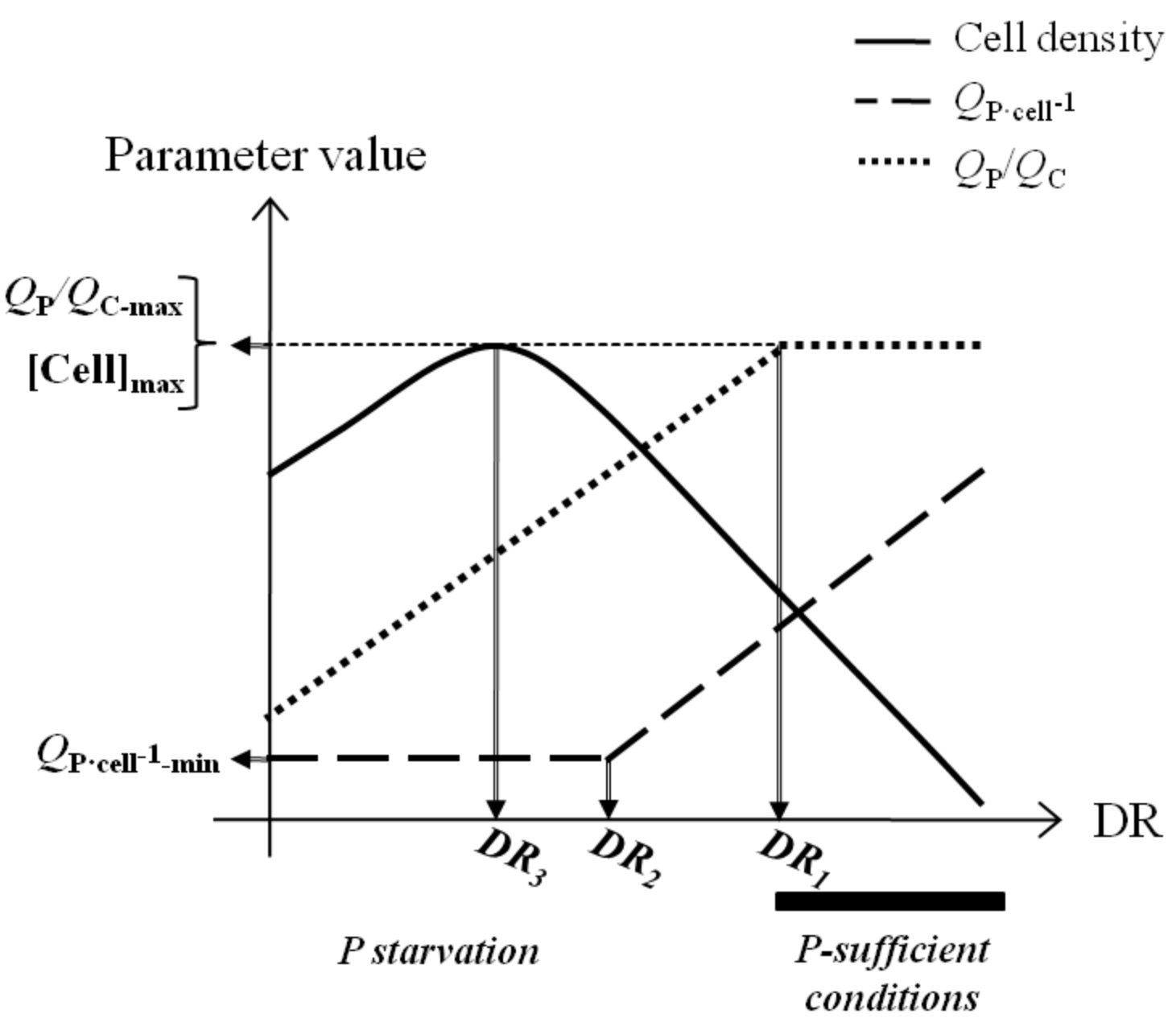
c)


20
15
10
5
0
TL01
ACT03
APA$_{max}$ (fmolP·cell^{-1}·min^{-1})
0.005
0.010
0.015
0.020
0.025
Q_P/Q_C (pgP·pgC^{-1})

d)


20
15
10
5
0
TL01
ACT03
APA$_{max}$ (fmolP·cell^{-1}·min^{-1})
0
20
40
60
80
$Q_{P\cdot cell^{-1}}$ (pgP·cell^{-1})


a)
9
6
3
0
I
II
APA (fmolP·cell-1·min-1)
0
50
100
150
200
250
b)
16
12
8
4
0
I
II
APA (fmolP·cell-1·min-1)
0
50
100
150
200
250
MUF-P concentration (µM)


Cell density
$Q_{P \cdot cell^{-1}}$
Q_P/Q_C
Parameter value
$Q_P/Q_{C\text{-}max}$
$[Cell]_{max}$
$Q_{P \cdot cell^{-1}\text{-}min}$
DR
DR_3
DR_2
DR_1
P starvation
P-sufficient conditions