

Enhanced biofuel production using optimality, pathway modification and waste minimization

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Abstract In response to their environment, algae in the wild may use an approximation to optimality of resource allocation in cellular structures, photosynthetic pigments, enzymes, transporters in membranes and RNAs and in their genetic material. However, under controlled conditions, when algae are grown for biofuel (lipid) production for example, some of these processes can be altered to increase the efficiency of photosynthesis and therefore, lipid yield. This suggests that there is scope for selecting mutations and for genetic engineering at various levels in the photosynthetic apparatus with the aim of increasing efficiency of photon use and the rate of transformation of resources per unit biomass to improve biofuel yields. More specifically, the wavelength range covered by photosynthetic pigments and photochemical reaction centres could be increased, the number of protons transported from the thylakoid lumen to the stroma per unit ATP synthesised by the ATP synthetase could be decreased, the fluctuating light effect could be utilised and photosynthetic pathways changed, e.g. replacing part or all of the current machinery for autotrophic fixation of inorganic carbon. There are also possibilities for decreasing carbon loss by decreasing ‘wasteful’ aspects of dark respiration and of dissolved organic carbon loss. Provided that the environmental fluctuations to which algal growth conditions are constrained, there are possibilities for decreasing the resource costs of protection from ROS, and by down-regulating photoprotective mechanisms, as well as limiting the capacity to repair processes related to

photoinhibition. Decreased protein turnover is also a potential energetic saving. These interventions apply to individual processes; however, this may not be immediately incorporated into the optimal allocation of resources by the alga, and further intervention using a system biology approach may be required.

Keywords ATP synthesis stoichiometry · Inorganic carbon assimilation pathways · Photochemistry · Photoinhibition · Protein turnover · Biofuel

Introduction

In an early paper reporting attempts to increase yield and light use efficiency in mass cultures of microalgae, Myers et al. (1951) wrote as follows. ‘If pushed to a state of practical development, the mass culture of algae will become an engineering problem. Before any such stage is reached, however, the problem is essentially biological’. Sixty-three years on, and after decades of algal mass cultures for high-value carotenoid products, there are still significant biological problems to overcome. The more recent attention to microalgal mass cultures to produce biofuels has focussed increased attention on how maximum production can be achieved at the level of algal physiology as well as, and in collaboration with, work at the level of engineering. Looking at what the evolution of pathways and the microalgae that contain them has given algal physiologists to work with, there is a balance that needs to be established between the rate at which vital processes occur, the efficiency with which resources are used and the safety with which the processes occur (Odum and Pinkerton 1955; Raven 1984a, b; Fabris et al. 2012; Flamholz et al. 2013; Hildebrand et al. 2013; Stettner and Segré 2013). Evolution, as described in the previous sentence, reaches local optima, both for individual

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processes and the integrated impact of these individual processes based on evolutionary fitness (Dawkins 2006). The means by which the individual processes interact in contributing to fitness is frequently considered to operate by means of optimal allocation of the available resources among the various critical functions (Rosen 1967). It is important to note that fitness does not invariably equate to maximising growth rate; there are many other factors which contribute to fitness, i.e. the ability to compete and leave offspring in a community involving other organisms at the same and other trophic levels. This has resulted in the evolution of algae taxa with a wide range of attributes related to maximum growth rate, defence against grazers and parasites and storage of materials against the probability (based on past evolutionary experience) of predictable or unpredictable variations in resource supply (Grime 1974; Shepherd 1981; Raven 1984a, b; Reynolds 1984; Flynn 2009). Resource allocation defines the ultimate value of a microalga as a biofuel feedstock.

Many studies have aimed at making algae as useful as possible for biotechnological applications, such as foodstuff or specific organic compounds, growing diazotrophs to increase soil fertility, and use in sewage treatment for the production of organic compounds (Myers et al. 1951; Kok 1952; Kok and van Oorschot 1954; van Oorschot 1955; Tamiya 1957; Myers and Graham 1959, 1961), with later work focusing on biofuels and high-value organic compounds (Borowitzka and Borowitzka 1988; Grobelaar 1991, 1994; Grobelaar et al. 1996; Grobelaar and Kurano 2003; Griffiths and Harrison 2009; Grobelaar 2009; Walker 2009; Kumar et al. 2010; Larkum 2010; Scott et al. 2010; Stephenson et al. 2010; Williams and Laurens 2010; Wilhelm and Jakob 2011; Day et al. 2011; Borowitzka 2013; Borowitzka and Moheimani 2013; Roleda et al. 2013). Such work can involve modifying the extent to which global optimality (defined by Rosen 1967; Levins 1968 in an evolutionary context) is approached, and, especially useful for biotechnology, increasing the maximum potential rate and efficiency of producing biomass or a specific product, such as neutral lipids with less optimal allocation (Fig. 1), see Williams and Laurens (2010).

Maximising the yield of oil as the fuel product from algal growth is currently mainly focussed on increasing the fraction of biomass allocated to oil. Two of the methods used to promote oil synthesis are selection of genotypes, which express high-oil traits, and growth of algal cultures under nutrient-limiting conditions (Greenwell et al. 2010; Williams and Laurens 2010; Schuhmann et al. 2012). Both of these methods decrease the growth rate of the algae. In an optimally allocating alga, an increased fraction of oil in the biomass means that a smaller fraction of the biomass is allocated to all the other processes (e.g. photosynthesis and protein synthesis) in the cell, with the exception of the enzymes specific to oil synthesis. However, Trentacoste et al. (2013) showed increased lipid accumulation in metabolically engineered

Thalassiosira pseudonana without compromising growth. Presumably, the unmodified diatom was not allocating resources optimally.

While at the culture level, the trade-off between algal growth rate and algal lipid content in optimally allocating algae can mean that the high-oil strains, growing under nutrient limitation, may not produce more oil per unit time than those cells which grow faster, but have a lower oil content, i.e. have a greater oil productivity (see excellent analysis by Williams and Laurens 2010, pp. 575–576; and also Griffiths and Harrison 2009). However, oil extraction may be easier if there is a larger fractional oil content per cell, with corresponding non-oil products containing algal nutrients, which should be recycled for subsequent algal cultures, as well as non-lipid organic matter. Metabolic engineering of algae can produce an increased fraction of oil in the biomass, as well as changing the ratio of oil components (e.g. saturated relative to unsaturated fatty acids, Schuhmann et al. 2012, Table 3) without changing the total pool size.

Rather than addressing ways of increasing the fraction of biomass allocated to oil (i.e. the yield of oil), the analysis in this paper addresses means by which the non-oil fraction of the biomass can be made to operate more effectively under modified culture conditions which could increase oil production. This means processing more photons and supporting a greater flux from inorganic to organic carbon, per unit time per unit non-oil biomass. Please note that we do not mention increasing the flux of inorganic carbon, nitrogen and phosphorus into the non-oil biomass, as our major objective is to examine the potential increase in the catalytic effectiveness of each unit of carbon, nitrogen and phosphorus in the non-oil biomass and/or to decrease the need for such catalysis (e.g. by waste minimization). The background to increasing photon and carbon flux per unit time per unit non-oil biomass has been examined, from a number of perspectives, by Raven (1984a, b, 1988), Bar-Even et al. (2010, 2011, 2012), Noor et al. (2010), Chen and Shachar-Hill (2012), Fast and Papoutsakis (2012) and Steuer et al. (2012).

An important point is that increasing energetic efficiency brings the reaction closer to thermodynamic equilibrium, which means a larger content of catalyst is required to bring about a given rate of substrate conversion (Odum and Pinkerton 1955; Raven 1984a, b; Bar-Even et al. 2010, 2011, 2012). The same principles used here to minimise non-oil biomass, can also serve to increase the rate of production of total biomass, though in this case, there would be an increase of the flux of all nutrient elements into biomass paralleling the increased cell growth rate and the increased rate of biomass production in the closed photobioreactor, open pond or re-circulating raceway. It should be clear that each of these three biofuel production formats has distinct optical (path length, package effect), physical (mixing) and chemical (nutrient, including CO₂ and availability) constraints that will

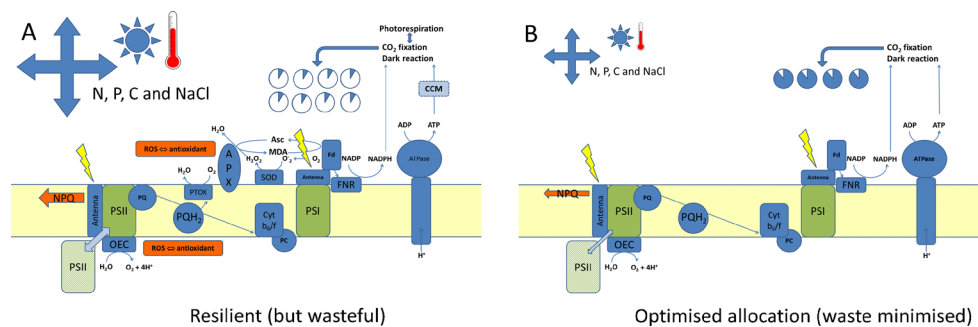


Fig. 1 Conceptual diagram of resilient cellular processes (able to withstand wide environmental fluctuation), with high biomass production, yet limited lipid production per cell (**a**); compared with cell with optimised

resource allocation that has a reduced biomass production with higher lipid yield per cell (**b**). Optimised cells have reduced flexibility to respond to fluctuating environmental conditions

be considered in this review. The algal biofuel industry does not overtly use a systems biology approach; while systems biology has been suggested as an important tool to improve the use of algae in producing biofuels, so far these are largely aspirations rather than achievements (Rupprecht 2009; Schmidt et al. 2010; Chang et al. 2011; Weckworth 2011; Wang et al. 2012).

An important point about the modifications discussed in this paper is that their full implementation may require significant control of environmental conditions, e.g. by light diffusers. These modifications are generally more readily attained in smaller-scale rather than very large-scale mass cultures (Grobelaar 2009; Borowitzka 2013).

Improving resource use efficiency of growth by altering the properties of the mechanisms of photon absorption and energy transduction to produce NADPH and ATP: optimality in a biotechnological context

Overall energetics of oxygenic photosynthesis

If the electron (or photon) efficiency of a candidate algal biofuel strain can be improved, then the overall cost efficiency of the production will increase; however, this is always constrained by the laws of thermodynamics. Energy transduction in oxygenic photosynthesis involves excitation energy from two photons moving one electron from water to NADP^+ coupled to the movement of three protons from the stroma (or cytosol in cyanobacteria) into the lumen of the thylakoid (Sacksteder et al. 2000; Falkowski and Raven 2007; Raven et al. 2014). The return of the three protons to the stroma through the CF_0CF_1 ATP synthetase is coupled to the phosphorylation of ADP to ATP according to the stoichiometry, from structural biology, of 3 ATP per 14 protons, so 3 protons should produce 0.643 ATP (Falkowski and Raven 2007; Raven et al. 2014). This would give an ATP/NADPH ratio of 1.286, or 2.57 ATP

per O_2 produced, although the measured protons moved per ATP produced is closer to 3.9 ± 0.3 (Petersen et al. 2012; Pogoryelov et al. 2012), corresponding to 3.1 ATP per 2 NADPH or 1 O_2 for 12 protons pumped per 4 electrons moving from water to NADP^+ . The requirement for two photons per electron moving from water to NADP^+ rather than three was argued by Hill and Bendall (1960) in their proposal of the “Z scheme” of two light reactions in series, and much subsequent experimentation (e.g. Redding et al. 1999) has shown alternative explanations to be in error.

The theoretical minimum photon cost (number of absorbed photons) for the transport of 4 electrons from water to NADP^+ in the ‘Z scheme’ is 8. However, it has been known for over 20 years that, while the photon yield of PSI is close to one electron per photon absorbed, that of PSII is significantly lower (Falkowski and Raven 2007; Johnsen and Sakshaug 2007; Hogewoning et al. 2012; Raven et al. 2014). This is reflected in the larger number of antenna pigment molecules per photochemical reactions centre (one of the definitions of photosynthetic unit size: Falkowski and Raven 2007) for PSII than PSI (see Johnsen and Sakshaug 2007). While this difference in the number of antenna pigments between the photosystems requires further analysis in terms of their different spectral absorption characteristics and the spectral characteristics of the radiation to which the organism is exposed, it is clear that more than 8 photons are needed to produce 1 O_2 , reduce 2 NADP^+ and phosphorylate 3.1 ATP.

This ATP/NADPH ratio of 1.55 is just adequate to account for the ATP/NADPH of 1.5 for the Rubisco–Benson–Calvin cycle in the absence of photorespiration and a carbon-concentrating mechanism (CCM) and also adequate to account for the ATP/NADPH of 1.48 for the Rubisco–Benson–Calvin cycle plus photorespiration for the conditions described in Table 3. However, the ATP/NADPH for an alga expressing a CCM (Hopkinson et al. 2011), assuming the residual photorespiratory carbon oxidation cycle flux makes a negligible contribution to the required ATP/NADPH ratio, is significantly

in excess of that needed by the carbon fixation cycle (Table 1) (Raven et al. 2014). Without a CCM or photorespiration the Rubisco–Benson–Calvin pathway needs three ATP and two NADPH per CO₂ converted to carbohydrate. An extra one ATP per C assimilated using a CCM gives four ATP, and two ATP per C where the cost is five ATP, in both cases per two NADPH. A non-cyclic electron flow gives 3.1 ATP per 2 NADPH, an extra 0.9–1.9 ADP per 2 NADPH is needed. Clearly, CCMs cost a significant amount of energy to run (Raven et al. 2014).

The energetic shortfall of a CCM could be made up by the Mehler-peroxidase reaction or water–water cycle involving PSII and PSI (Falkowski and Raven 2007; Raven et al. 2014). As this pathway has the same quantity of ATP produced per absorbed photon (3.1 ATP per 8 photons or 0.3875 ATP per photon), in which case the missing 0.9–1.9 ATP per O₂ evolved or CO₂ assimilated would need at least another 2.3–4.8 photons absorbed, giving a total of at least 10.3–13.8 photons per CO₂. The occurrence of the Mehler-peroxidase reaction can be estimated from measurements of the rate of gross photosynthetic oxygen production and of oxygen uptake in the light, using ¹⁸O-labelled oxygen and mass spectrometry (Badger et al. 2000; Suggett et al. 2009). For some microalgae (Badger et al. 2000; Suggett et al. 2009), there is insufficient flux through the Mehler-peroxidase reaction to power the CCM.

If the additional ATP comes from cyclic photophosphorylation involving only PSI, it has been known for almost two decades that the proton per photon ratio in cyclic electron flow is approximately 3, at least in flowering plants (Heber et al.

1995). The proton-pumping sites are in the NAD(P)H-PQ oxidoreductase and the cytochrome *b₆f* complex oxidising reduced PQ and reducing cytochrome *c₆* or plastocyanin (Falkowski and Raven 2007; Livingston et al. 2010). The crystal structure of the NADH-UQ oxidoreductase has four potential proton transporting sites which, with transfer of two electrons through the complex from NADH to UQ in each reaction cycle, is consistent with the 2 H⁺ per electron stoichiometry found by many experiments (Effemov and Sazanov 2011). However, the most recent estimate of the H⁺ per electron ratio give values of 1.5 (Wikström and Hummer 2012), i.e. a value lower than that predicted from structural biology, paralleling the situation with the H⁺ per ATP ratio in the ATP synthases. Less contentious is the ratio of 2 H⁺ per electron for the cytochrome *b₆f* complex oxidising reduced PQ and reducing cytochrome *c₆* or plastocyanin (Falkowski and Raven 2007). This gives a total of 3.5 protons moved into the thylakoid lumen per electron (or photon absorbed by PSI); however, Raven et al. (2014) assumed a value of 4 protons per photon absorbed by PSI. Assuming a value of 3.5 imposes an upper limit on the proton:electron ratio, as some oxygenic organisms lack the NAD(P)H-PQ oxidoreductase in their thylakoid membrane and rely on the PGR5 alternative pathway, which is not known to pump protons, from ferredoxin to PQ (Shikanai 2007; Alrie 2010; Iwai et al. 2010). Most oxygenic photosynthetic organisms have both the NAD(P)H-PQ oxidoreductase and the PGR5 pathways, while *Thermosynechococcus elongatus* has only the NAD(P)H-PQ oxidoreductase (Shikanai 2007). Clearly, maximising ATP generation per photon absorbed requires that the NAD(P)H-PQ oxidoreductase is functioning,

Table 1 Energy costs (NADPH, ATP) of fixation of 1 CO₂ into triose phosphate, half-saturation value for CO₂, occurrence of O₂ competitive inhibition of CO₂ fixation and occurrence of irreversible inhibition by O₂ for six pathways of autotrophic CO₂ fixation

Pathway	NAD(P)H per CO ₂	ATP per CO ₂	K _{1/2} CO ₂ (mmol m ⁻³)	O ₂ competitive inhibition	O ₂ damage
1. Rubisco–Benson–Calvin cycle; saturating CO ₂ and no O ₂ , diffusive CO ₂ entry	2	3	≥10	Not expressed in absence of O ₂	No
2. Rubisco–Benson–Calvin cycle; air-equilibrium medium with diffusive CO ₂ entry giving Rubisco oxygenase/carboxylase ratio of 0.25, using PCOC with glycolate dehydrogenase, glycine decarboxylase and serine hydroxymethyl transferase	2.5	3.7	>10	Yes	No
3. Rubisco–Benson–Calvin cycle in air-equilibrium solution with a CCM using 1–2 ATP to pump 1 CO ₂ (net) from medium to the site of Rubisco activity	2	4–5	<10	No	No
4. Reverse TCAC	2	1.67	>1,500	No	Yes
5. 3-HO-Propionate	2	2	≤10	No	No
6. 3-HO-Propionate–4-HO-Butyrate	2	3	>2,000	No	No
7. Dicarboxylate–4-HO-Butyrate	2	2.67	>2,000	No	Yes
8. Wood–Ljungdahl Pathway	2	1	40,000	No	Yes

For Rubisco, PCRC energy costs are given for diffusive CO₂ entry at saturating CO₂, diffusive entry of CO₂ for the present-day atmosphere with operation of a PCOC and supply of CO₂ to Rubisco from the present-day atmosphere using a CCM. Based on Raven et al. (2012), with O₂ damage from Bar-Even et al. (2012) and the energy costs of use of PCOC and of CCMs for Rubisco-CCM from the text

unless it has been shown that the PGR5 pathway has a proton:electron ratio at least as high as that of the NAD(P)H-PQ oxidoreductase. With 3.9 H^+ per ATP produced, cyclic electron flow with 3.5 H^+ per electron and 1 electron moved per photon absorbed generates 0.897 ATP per absorbed photon, so 0.9 additional ATP needs 1 photon absorbed by PSI and 1.9 additional ATP needs 2.1 photons absorbed by PSI; therefore, a total of at least 9–10.1 photons are needed (see also Raven et al. 2014). This has implications for the required ratio of antenna pigments to reaction centres, increasing that in PSI relative to PSII. The changes in relative antenna sizes could be achieved by a state transitions, moving antenna pigments away from PSII and associating them with PSI (Falkowski and Raven 2007), with a redox switch mechanism such as that shown by Terashima et al. (2012). Such balancing must accommodate the possibility of light-harvesting complexes dedicated to cyclic electron transport around PSI (Iwai et al. 2010). These photon requirements for ATP synthesis by cyclic electron flow around PSI are minimal estimates; in the green microalga *Chlamydomonas reinhardtii*, the pathway in a cyclic electron transport super-complex would only pump 2 protons per photon (Iwai et al. 2010) and so could only produce 0.51 ATP per photon. Producing an additional 0.9 or 1.9 ATP for a CCM needs 1.8–3.7 additional photons, i.e. a total of 9.8–11.7 photons. These calculations are for ATP within the stroma, while any component of the CCM involving ATP use at the plasmalemma requires ATP export from, and ADP and P_i import to, the stroma. We assume that this can be performed at no additional energy cost by use of the reductant and ATP (as triose) export on the phosphate exchanger with 3-phosphoglycerate import. Redox balance is maintained by reductant return to the stroma by malate import and oxaloacetate export using the C_4 dicarboxylate exchanger. The overall net stoichiometry is a net export of one ATP (Raven 1984b). This would suggest that the efficiency of carbon fixation can be altered by a wide diversity of biochemical processes.

An alternative way of powering CCMs is via photosynthesis to produce organic carbon followed by glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation as occurs in the eustimatoophyte alga *Nannochloropsis* (Huertas et al. 2002; Raven et al. 2014). Using the value of 2.9 H^+ /ATP for the mitochondrial F_0F_1 ATP synthetase (Petersen et al. 2012), the H^+ per electron moving through the mitochondrial electron transport chain of 4.5 for internal NADH oxidation and 3.0 for oxidation of internal reduced flavoprotein or external NADH (Wikström and Hummer 2012), 1 H^+ used for each ATP exported from (and ADP and P_i imported into) the mitochondrial matrix, and the metabolism of stored polysaccharide using hydrolytic production of hexose, each mole of organic C from hexose generates 4.7 ATP in the cytosol. Use of an additional 0.9 ATP per CO_2 in the CCM thus involves oxidation of 0.19 mol of C from carbohydrate, and 0.40 C if an extra 1.92 ATP are needed per CO_2 . As each (at

least) 8 photons only fixes 0.60–0.81 C net, the overall photon cost of fixation of 1 mol of CO_2 is 9.9–13.3 depending on the CCM stoichiometry. This less direct method of powering the CCM is, in terms of photon costs, similar to that using the Mehler-peroxidase reaction, but more efficient than that using cyclic electron flow round PSI to generate the extra ATP (Table 1, pathway 3).

From this discussion, it is clear that costs of the CCM in energy (photon) terms is significant, and that photon savings could be made, in principle, by supplying CO_2 at elevated concentrations in a biofuel context, by diffusive entry, at least as high a concentration of CO_2 at the Rubisco active site that is equivalent to using the CCM. This would also perform another function of the CCM, decreasing the metabolic flux through an energy-costly process by suppressing photorespiration (Table 1, pathways 2 and 3).

The considerations of the energetics of cyclic photophosphorylation and of oxidative phosphorylation have more wide-ranging implications for the energetic efficiency of the growth of algae than the functioning of CCMs or, especially in the absence of CCMs, the photorespiratory carbon oxidation cycle(s). These wider implications relate to maintenance and growth which involve energy inputs from oxidative phosphorylation and, sometimes, cyclic photophosphorylation (Falkowski and Raven 2007).

These predictions of the electromagnetic energy required to carry out partial reactions of photosynthesis must ultimately be summed and compared with the measured requirements for absorbed photons to bring about the reaction under study. Such measurements are difficult, and sometimes well-executed experiments yield photon costs (mole photons absorbed per gross mole O_2 produced yield values), which are lower than those predicted from the sum of the partial reactions on the basis of widely accepted mechanisms and stoichiometries (Welschmeyer and Lorenzen 1981; Geider et al. 1985, 1986). There are also well-executed experiments, using the same species (the diatom *Phaeodactylum tricornutum*) as was used by Geider et al. (1985, 1986), which yield significantly higher photon costs than predicted, admittedly for overall growth rather than photosynthesis (Frada et al. 2013). Of course, inefficiencies can ‘explain’ the high values, but the low values require more consideration.

The wavelength at which photochemistry occurs in relation to the sea surface or underwater spectrum

Limitations on the efficiency of the photochemical reactions of photosynthesis are imposed by a number of optical/biochemical constraints. Consequently, the greatest opportunities to advance algal biofuels as an economically viable alternative to fossil fuel is to understand the constraints and engineer solutions which allow small improvements in photosynthetic efficiency (Williams and Laurens 2010).

Photochemical reactions are restricted to the use of excitation energy from absorbed photons matching the difference in energy levels of the ground state and the appropriate excited state of the pigment molecule bringing about photochemistry (Falkowski and Raven 2007). These wavelengths are 680 nm for PSII and 700 nm for PSI. Regardless of the pigment bringing about the photochemistry, the wavelengths used can be related in natural selection to the wavelengths of radiation incident on the organism and the energy needed to produce usable products from the photochemistry with a limited capacity for back reaction. Black body radiation from the sun has a peak energy ($\text{W m}^{-2} \text{nm}^{-1}$) at about 550 nm, but the peak in photon flux ($\mu\text{mol photons m}^{-2} \text{s}^{-1} \text{nm}^{-1}$) is at about 630 nm (Wolstencroft and Raven 2002; Kiang et al. 2007a, b; Stomp et al. 2007). The photon-based irradiance curve decreases more rapidly with decreasing wavelength at wavelengths shorter than 630 nm, while the reverse is true for wavelengths longer than 630 nm: this is because the energy content of a photon decreases with increasing wavelength.

Biofuel production is also supported by artificial irradiance, provided mostly by LEDs (Matthijs et al. 1996). As technology drives LED technology, we will have a wider range of narrow bandwidth irradiances available. Matching the LED output to the optimal absorption region is critical in such PBRs (Gordon and Polle 2007). At least as critical is the costing of the energy input to the LEDs, which are highly efficient at converting electrical energy into electromagnetic radiation in a specific wavelength range, relative to the added value of biofuel production which is caused by the LEDs. Restricting photochemistry to 680 and 700 nm means that photons equivalent to wavelengths longer than 680 and 700 nm can only be used subject to certain restrictions, as the excitation energy transfer is energetically uphill; the approximate limits for such transfer are 730 nm for PSII and 750 nm for PSI (Pettai et al. 2005, 2006; Krausz et al. 2005; Wilhelm and Jakob 2006; Kiang et al. 2007a, b). Not all of the energy in the photons absorbed at shorter wavelengths can be used in photosynthesis, as the high-energy photons can only be used in photochemistry at energy contents corresponding to 680 and 700 nm (Falkowski and Raven 2007; Williams and Laurens 2010; Blankenship et al. 2011). This unavoidable loss is calculated by removing the fraction of the energy in photons at each wavelength shorter than 700 nm, which exceeds the energy content of a 700-nm photon. For the entire spectrum of shorter wavelengths down to 350 nm, assuming that there are pigments capable of absorbing the same fraction of photons at each wavelength, it can be calculated that at least 22 % of the energy contained within the photons of the range 350–700 nm is unavoidably lost in the use of photons at 680 and 700 nm in photochemistry (see Raven and Donnelly 2013). This agrees well with the 21 % calculated by Williams and Laurens (2010) for the wavelength range 400–700 nm. Some analyses of energy use (e.g. Weyer et al. 2010) do not explicitly

acknowledge this factor in the energy losses associated with use of absorbed photons in photochemistry and therefore overestimate the potential photon harvest.

Energy per photon is inversely proportional to wavelength. The energy content of three photons at 1,050 nm is the same as that of two photons at 700 nm; therefore, the same overall endergonic chemical reactions should in principle be energised by three 1,050 nm photons, as by two 700 nm photons. This is implicit in the ‘three photochemical reactions in series’ mechanism for the transfer of an electron from water to carbon dioxide mechanism. This was considered by Hill and Bendall (1960) when comparing the ‘two photochemical reasons in series’ mechanism which fitted the observations available in 1960 more readily, and which has now been vindicated. The three photon mechanism has been considered in an astrobiological context, predominantly for black body radiation from red dwarf (M) stars which are cooler than the sun and have a longer wavelength of maximum photon emission (Wolstencroft and Raven 2002; Raven and Wolstencroft 2004; Kiang et al. 2007a, b; Stomp et al. 2007; Raven and Donnelly 2013). However, in the context of life on Earth, it would have a number of drawbacks, especially for aquatic organisms because of infrared absorption bands of water in the 700–1,050 nm waveband. This absorption also has a significant impact, through atmospheric water, on the solar spectrum reaching the land and sea surface relative to that at the top of the Earth’s atmosphere. Furthermore, because 1,050 nm is 420 nm longer than the peak photon emission by the sun, the energy loss resulting from the use of photons absorbed at 350 nm as if they were photons absorbed at 1,050 nm, where the energy per photon is one third that at 350 nm, it can be calculated that at least 38 % of the energy in the shorter wavelength photons is unavailable for photochemistry. This, combined with the need to use three, rather than two photons, and absorption by water in the infrared, means that photochemistry at 1,050 nm is not likely to be favoured by natural selection for oxygenic photosynthetic organisms on Earth.

However, anoxygenic photosynthetic bacteria using bacteriochlorophylls as their photochemical catalysts do have photochemistry in the infrared extending out to about 1,000 nm but also with absorption down to about 350 nm (Kiang et al. 2007a, b; Stomp et al. 2007). Common rationalisations for photochemistry in the infrared is that these specialist autotrophic organism are confined to anaerobic habitats, usually under oxygenic photosynthetic organisms which absorb between 350 and 700 nm, and that the absorption peaks in the infrared, including those where photochemistry occurs, often occur at wavelengths where absorption by water is smaller than at nearby wavelengths (Stomp et al. 2007). However, this ‘explanation’ does not readily account for the aerobic anoxygenic photosynthetic bacteria in the upper mixed layer of the ocean. These organisms are not autotrophic, but seem to respire less than other heterotrophic bacteria because they can

spare respiratory production of proton gradients by using bacteriochlorophyll-based photochemistry to produce proton gradients (see Raven and Donnelly 2013). Raven and Donnelly (2013; see also Johnston et al. 2009; Raven 2009a) have attempted to quantify the decrease in CO₂ release from a cell resulting from replacing respiratory generation of proton gradients by proton gradients derived from photochemistry. The organisms involved are the otherwise heterotrophic aerobic anoxygenic photosynthetic bacteria using bacteriochlorophyll and the otherwise heterotrophic bacteria with photochemistry catalysed by proteorhodopsins. The upper limit on the decrease in CO₂ production resulting from this use of photochemistry may be as large a contribution to the global C cycle as CO₂ fixation by anaerobic autotrophic anoxygenic photosynthetic bacteria plus chemolithotrophs, i.e. about 0.4 Pg C year⁻¹ (Raven and Donnelly 2013).

Blankenship et al. (2011) have suggested a way of using wavelengths longer than 700 nm by genetically engineering a bacteriochlorophyll *b*-based photosystem from an anoxygenic photosynthetic bacterium into an oxygenic photosynthetic organism, replacing the present PSI with what they term RC1. This would extend the photosynthetically usable range of absorbed photons to about 1,050 nm. Starting with an organism with chlorophyll *d* as the photochemical catalysts in both photosystems, as well as a major antenna pigment for, PSII, it would cover the wavelength range 400–730 nm. It would be necessary to limit the excitation of the engineered RC1 to the 730–1,100 nm range to avoid competition between the two systems for the radiation at wavelengths shorter than 730 nm (Blankenship et al. 2011). Among the problems with such a scheme is that the bacteriochlorophyll *b*-based prototype for RC1 does not function in vivo in directly reducing NAD(P)⁺ using the photochemically produced reductant, but has a cyclic electron flow generating a proton gradient which brings about ‘reversed electron transport’ from the external reductant to reduce NAD(P)⁺ (Falkowski and Raven 2007). This would require additional input of photons to power the carbon fixation reaction, as much, or all, of the proton gradient generated in electron transport from water to the oxidising end of RC1 would be used in reversed electron transport rather than in phosphorylating ADP to produce ATP that can be used in CO₂ assimilation. Alternatively, engineering the redox range covered by RC1 to a more reducing (negative) value and allowing direct photochemical reduction of NAD(P)⁺ would eliminate the possibility of generating as much ATP in the water to the oxidising end of the RC1 segment of the pathway, again requiring additional photon input to provide sufficient ATP for CO₂ fixation. Finally, modifications involving the use of solar radiation at wavelengths beyond 750–800 nm for photochemistry are more applicable to terrestrial plant leaves where, having passed through the atmosphere, the aqueous path length for radiation in each individual leaf is only some 0.2–0.4 mm. This contrasts with algal bioreactors

or raceways, where the aqueous radiation path length is 100–1,000 times greater than the thickness of a leaf, and consequently, greatly increases absorption of those infrared wavelengths by water relative to bacteriochlorophylls.

A role for long-wavelength forms of chlorophyll *a* and chlorophyll *d*?

Having considered the spectral distribution of black body radiation from the sun and its impact on the wavelength at which photochemistry occurs, we need to consider the relationship of the energy available from photons at 680 and 700 nm to the energy stored in the primary and subsequent products of the photochemical reactions. While it is possible to rationalise the use of chlorophyll *a* as the photochemical catalyst in oxygenic photosynthesis with a long-wavelength cut-off (‘red edge’) at about 700 nm (Milo 2009; Raven 2009a; Björn et al. 2010), it is also important to realise that there are aquatic oxygenic organisms with an extension into the near infrared of their absorption and action spectra (Schliep et al. 2013). For organisms whose main photosynthetic pigment is chlorophyll *a* there are a number of algae with a shoulder or small peak in their photosynthetic action spectrum (Fork 1960); this has also been reported for flowering plants (Pettai et al. 2005, 2006). Chen and Blankenship (2011) point out that extending the photosynthetically active radiation (PAR) range from 400–700 to 400–750 nm would increase the number of photons available from the solar radiation at the sea or land surface by 19 %. The question that remains is how widespread is this extension of the PAR range, and is it significant for productivity in the organisms in which it is found?

To optimise the use of incident irradiance, it has been suggested that mixed cultures of algae should be used that preferentially absorb different wavelengths such as *Acaryochloris marina* (Scott et al. 2010). In this way, a chlorophyll *a*-containing algae would absorb the majority of irradiance but would allow the secondary strain to capture unused wavelengths such as those in the far-red. The most studied of such far-red organisms is the ulvophycean marine green alga *Ostreobium* which typically lives inside massive corals where shading by the *Symbiodinium* photobionts gives irradiances at the outer surface of the *Ostreobium* zone which may be as low as 0.19–0.15 % of those incident on the coral in the wavelength range 340–680 nm; this increases to 0.6 % at 700 nm and 2.0 % at 720 nm (Halldal 1968; Shibata and Haxo 1969; Magnusson et al. 2007). The action spectrum of photosynthesis in *Ostreobium* shows a major shoulder at 720 nm (Halldal 1968; Wilhelm and Jakob 2006), as does the fluorescence excitation action spectrum (Fork and Larkum 1989), and growth can occur under monochromatic 700 nm irradiation (Wilhelm and Jakob 2006).

A long-wavelength-absorbing chlorophyll *a*-protein complex has been identified in extracts of *Ostreobium* (Koehne et al. 1999), and Wilhelm and Jakob (2006) suggest uphill energy transfer from this complex to PSII. Such transfer has a relatively low probability which decreases as the difference in wavelength between the far-red absorbing chlorophyll *a*-protein complex and the reaction centre increases, and depends on thermal energy to provide the activation energy and so is temperature dependent (Pettai et al. 2005, 2006). Schlichter et al. (1997) and Ralph et al. (2007) showed that the light compensation point for oxygen exchange is less than 2 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, the lowest reported value for growth of an oxygenic photosynthetic organism is about 0.01 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Raven et al. 2000). Cherbady and Propp (2008) reported *Ostreobium* at 220–240 m depth in the South China Sea where the flux densities of near-infrared radiation is extremely low, so any capacity to absorb radiation at 700–730 nm would not be significant: this is also the case for deep-growing anoxygenic photosynthetic bacteria whose absorption, and photochemistry, occurs further into the infrared.

Other oxygenic organisms, which have significant photosynthetic activity at wavelengths in excess of 700 nm are those cyanobacteria which use chlorophyll *d* in both light harvesting and in the photochemistry of PSI and PSII. Chlorophyll *d* was originally described from some red algae collected from their natural environment (Manning and Strain 1943), and so were not axenic; it is now known that the chlorophyll *d* of these red algae is in endophytic cyanobacteria such as *Acaryochloris marina* (Larkum and Kühl 2005). Primary photochemistry catalysed by chlorophyll *d* at longer wavelengths than that found for the common chlorophyll *a* wavelengths of 680 nm (PSII) and 700 nm (PSI) incurs no obvious change to the energetics and kinetics of photochemistry (Larkum and Kühl 2005; Duxbury et al. 2009; Chen and Blankenship 2011; Mielke et al. 2011, 2012; Larkum et al. 2012; Tsuchiya et al. 2012). Interestingly, although there are cyanobacteria in corals, no chlorophyll *d* has been found in the coral endophytes (Magnusson et al. 2007), so absorption of the infrared-enriched radiation field is left to chlorophyll *a* in *Ostreobium* and to the anoxygenic photosynthetic bacteria whose bacteriochlorophyll has been detected deep within corals (Magnusson et al. 2007; Ralph et al. 2007). Chlorophyll *d*-containing cyanobacteria occur endolithically under crustose coralline red algae (Behrendt et al. 2011) and in association with gelidiaceous red algae under mangrove canopies (Larkum et al. 2012); both of these habitats are enriched in radiation beyond 700 nm. *Acaryochloris* grows rapidly in biofilms, similar to its natural habitats, as well as planktonically in suspension cultures (Behrendt et al. 2011).

How much further can photochemistry be moved into the infrared to improve algal production without compromising the performance of oxygenic photosynthetic organisms using two photochemical reactions (Schliep et al. 2013)? In this

regard, further characterisation of the role of chlorophyll *f*, which was recently discovered in cyanobacteria (Chen et al. 2010; Akutsu et al. 2011; Chen and Blankenship 2011; Li et al. 2012), is eagerly awaited. The information available so far suggests that chlorophyll *f* is a relatively minor component of the pigments, dominated by chlorophyll *a*, in the cyanobacterium and has a light-harvesting role which presumably involves uphill energy transfer to the chlorophyll *a*-containing photosynthetic reaction centres, as is the case for the organisms with chlorophyll *a* complexes absorbing in the infrared (Pettai et al. 2005, 2006; Wilhelm and Jakob 2006). In these respects, the organisms containing chlorophyll *f* differ from those with chlorophyll *d*. It would be of interest to determine if the predicted decrease in photon yield in the infrared, for chlorophyll *f* organisms where uphill excitation energy transfer occurs, which contrasts that of the chlorophyll *d* organism *Acaryochloris* which maintains high photon yield in the infrared (Mielke et al. 2011, 2012). It is likely that coculturing a chlorophyll *a* and a chlorophyll *f* organism within a single culture that could enhance the overall biomass production of the raceway or PBR in comparison to coculturing a chlorophyll *a* and a chlorophyll *d* organism.

Significance of UVA for algal photosynthesis

The wavelength range of UVA is defined here as 320–400 nm, with the invariably damaging UVB occupying the 280 to 320 nm range. The short wavelength tails of absorption by chlorophylls and light-harvesting carotenoids can be used to power photosynthesis. However, at the cell level, absorption of UVA by photosynthetic pigments faces increasing competition at decreasing wavelengths of UVA from absorption by the long-wavelength tail of absorption by proteins and of pigments produced by the organism. These pigments absorb and thus limit damage by UVB, while there are also inhibitory effects of UVA (e.g. Lesser and Lewis 1996). The natural radiation environment also has decreasing photon flux densities of radiation with decreasing wavelengths of UVA in the solar spectrum reaching the land and sea surface. Coloured dissolved organic matter and planktonic organisms has variable effects on UVA fluxes incident on organisms relative to PAR (400–700 nm), with less attenuation of UVA relative to PAR in the open ocean, than in some coastal and inland waters (Kirk 2011).

The sum of these cell level and environmental influences has different results with different alga–environment combinations. In some cases there is dominance of the photoinhibitory effects of UVA, added to the universal inhibitory effects of UVB, with (general) increasing photon yields of photodamage with decreasing wavelengths (Holm-Hansen et al. 1993; Häder et al. 1996; Franklin and Forster 1997; Dring et al. 2001; see Takahashi et al. 2010 for UV and PAR photoinhibitory effects of the solar spectrum on a terrestrial flowering plant). However,

for other alga–environment combinations there can be significant photosynthesis using wavelengths below 400 nm (Halldal 1964; Mengelt and Prézélin 2005; Gao et al. 2007; Xu and Gao 2010). The work considered here shows that there is a variation in the action of UVA on algae, from inhibition of photosynthesis and growth to powering photosynthesis by extending absorption to wavelengths below 400 nm.

The impact of solar UVA on outdoor algal biofuel cultures clearly needs testing on a case by case basis, and preventative (UV screening) measures taken as appropriate. Tentatively, the photosynthetically usable spectrum can be extended to include radiation from 400 to 350 nm, which has half the wavelength, and twice the energy per photon, of the classic oxygenic photosynthetic cut-off of 700 nm. However, it must be borne in mind that the damaging effects of UVA on an absorbed photon basis can be much greater than those in the 400- to 700-nm range. The impact and response to UV needs to be carefully examined for all strains that are to be used in outdoors ponds.

Photon absorption as a function of wavelength 350–730 nm and of cell size

Returning to the use in photosynthesis of wavelengths below those at which photochemistry usually occurs, it is clear that the chlorophylls involved in photochemistry, predominantly chlorophyll *a* and, in a few organisms, chlorophyll *d*, do not absorb a constant fraction of incident solar radiation or sub-surface radiation in water. This conclusion is based on the absorption spectra of these two chlorophylls in very dilute solutions in organic solutes (Chen and Blankenship 2011). The wavelengths of maximum absorption are altered in vivo by association with apoproteins, with minor changes in the specific absorption coefficients. However, the same conclusions can be drawn about very different fractional absorption of incident photons as a function of wavelength despite variations in the incident spectrum at different depths in the aquatic habitats, provided the package effect is small. The package effect is the decrease in the effective specific absorption coefficient of chromophore molecules as the content of the chromophores per unit cell volume for cells of a given volume, or the cell size is increased at a given content per unit volume of cells, or both an increase in content per unit volume and an increase in volume (Raven 1984a, b). The same argument applies to multicellular organisms as to cells.

A small package effect at the single cell level requires a small pigment content per unit cell volume and/or small cells. This means a small optical path length for each cell allowing each cell to express the spectral absorption characteristics of its individual pigments, and this also means that the time needed to recoup the energy cost of synthesising a given quantity of pigment–protein complex in a given light field is less than in a larger cell or multicellular organism with a large

optical path length and a greater package effect (Raven 2011; Raven and Hurd 2012). This process corresponds to a smaller effective specific absorption coefficient for the pigments, so that each pigment molecule has a lower rate of photon absorption in the given light field than would the same pigment in a smaller cell or organism (Raven 1984a, b, 1996, 2009a, 2011; Raven and Hurd 2012). This has implications for flat plate photobioreactors, such that the light field within a PBR changes with cell density and size and consequently influences overall productivity.

Raven (1996) points out that the chemical and spectral diversity of photosynthetic pigments in small algal cells is, across all taxa, greater than that in larger algal cells and multicellular algae. The extreme state for a very optically thick organism is to have almost complete (more than 90 %) absorption averaged across the 400 to 700 nm wavelength range (Lüning and Dring 1985; Frost-Christensen and Sand-Jensen 1992). Given the asymptotic approach to a fractional absorption of 1 (i.e. totally absorbent), the energy (and other resource) costs of making the chromophores that provide each subsequent increment in absorption as the fractional absorption gets nearer and nearer to 1, the cost becomes increasingly large. It would take an infinite amount of energy and other resources to reach a fractional absorption of 1, and even for a slightly smaller fractional absorption, the energy cost could never be recouped in terms of the lifetime absorption of photons by the pigment–protein complex. Thus, a delicate balance must be established between absorption and optimum resource allocation. Nevertheless, some large and long-lived algal structures (perennial parts of thalli of very long-lived seaweeds) do have a very high (0.90 or more) fractional absorption averaged over the 400 to 700 nm range. These highly absorptive structures can presumably recoup the energy (and other resource) costs of synthesis of the pigment–protein complexes over the lifetime of the complexes by photon trapping and photochemistry, and subsequent investment of the organic products into producing and operating the machinery which acquires inorganic C and N (and P and other elements). This, in turn, allows the production of more pigment–protein complexes and other components of the organism (Raven 2011; Raven and Hurd 2012). These cellular responses also impact on the cost effectiveness of a particular biofuel strain.

The argument of Raven (1996) that small cells benefit more than larger cells and organisms from a greater diversity of pigments in making use of all wavelengths from 400 to 700 nm in the natural radiation field, warrants further investigation especially for candidate biofuel strains. Even macroalgae, some of which are very large, with a substantial optical thickness and large package effect at maturity, have small germling diploid stages and, for laminarians, small haploid gametophyte phases in their life cycle; these small stages have small package effects (Raven 2011; Raven and Hurd 2012). If competition for

light energy among taxa at the early stages of their life history is evolutionarily more important than photon absorption properties in later stages of the life cycle, it would be expected that natural selection would favour a greater diversity of pigments, at least in younger cells. This does not seem to be the case, because either selection is not more intense for photon absorption attributes in younger than older stages, or that the spectral nature and diversity of photosynthetic pigments is less important than Raven (1996) suggested. Equally, the hypothesis of complementary chromatic adaptation of algae to the spectral qualities of their radiation environment does not have as broad an application as was originally believed (Stomp et al. 2004; Raven 2011; Raven and Hurd 2012).

A further point for consideration is that there are many more algal classes containing small, mainly unicellular organisms than those with large multicellular organisms. Thus, the observed class fidelity of pigmentation means that there is a much greater possibility of diversity of pigmentation among the taxa with only small representatives. The diversity of pigmentation does not necessarily apply to individual classes, for example, the very restricted range of photosynthetic pigments within the Eustigmatophyceae, the class to which the biotechnologically important microalga *Nannochloropsis* belongs (Raven 1996). However, some very small cells do have a great diversity of pigments, for example some members of the Prasinophyceae and the 'green' cyanobacterium *Prochlorococcus* have a chlorophyll *c*-like light-harvesting pigment, as well as chlorophyll *b* (or allo-chlorophyll *b* in *Prochlorococcus*), as well as the various phycobilins found with chlorophyll *a* and carotenoids in *Synechococcus* (Raven 1996). This suggests that algal strains can be selected on the basis of their optical absorption and this may provide a competitive advantage in some irradiance regimes

Fluctuating light

Natural PAR can oscillate by two orders of magnitude over periods of less than a second. One example is sun-flecks reaching understorey photosynthetic organisms in terrestrial and, to a lesser extent underwater environments, because direct sunlight is more diffuse in these environments than in air. Another example is wave focussing for benthic photosynthetic organisms in a shallow lagoon (Nakamura and Yamasaki 2008). More relevant to the pelagic phytoplankton, the source of many potential biofuel microalgae, are the light fluctuations due to periodic vertical migration within a stratified photic zone (sinking or buoyant rising relative to the water surface), or when they are entrained in vertically circulating parcels of water (Litchman 2000; Litchman and Klausmeier 2001; Dimier et al. 2009). Similar variations occur for algae grown for biofuels, such that mixing and turbulence both cause cells to move between high light to low light (or darkness) within a photobioreactor or a raceway pond; the frequency of oscillation

depends on the mixing or aeration regime (Grobelaar 1994; Janssen et al. 2000) and the density and depth of the culture (Ritchie and Larkum 2012).

Understanding how fluctuating light interacts with the photosynthetic machinery was first critically examined by Emerson and Arnold (1932a, b), who simplified the fluctuations to short, high-irradiance flashes separated by dark intervals of varying length. Each flash provided sufficient integrated photon energy to excite all the open (reduced donor side and oxidised acceptor side) reaction centres, while the dark period allowed the non-photochemical reactions to proceed. With a long enough dark period, all reaction centres became 'open', and allowed the estimation of the one measure of the photosynthetic unit size as oxygen produced per flash per unit chlorophyll, while the relation of (saturating) flash frequency allowed the estimation of the effective rate constant for the downstream reactions (Falkowski and Raven 2007). Using the flash yield and the effective rate constants allowed reconstruction of the steady-state rate of light-saturated photosynthesis, although the comparison can be complicated by photoinhibition (Falkowski and Raven 2007). Subsequent improvements in technology used to produce very short high-irradiance flashes, mainly from lasers, has allowed flash photolysis to make, with absorption and fluorescence spectroscopy, major contributions to our understanding of redox reactions in thylakoid membranes (e.g. Witt et al. 1963).

Starting by considering the effects of fluctuating light at the cell or organism level rather than at the population or biotechnological scale, it is important to point out that increased photosynthesis and productivity cannot result from fluctuations applied to PAR-limited organisms already operating at their maximum energetic efficiency. Such highly efficient photosynthesis has been observed for the diatoms *Thalassiosira pseudonana* (Welschmeyer and Lorenzen 1981) and *Phaeodactylum tricornutum* (Geider et al. 1985, 1986) among microalgae, and for several marine macroalgae (Lüning and Dring 1985; Markager 1993), see Raven et al. (2014) for a comparison of the measurements with mechanistic predictions. Increased yield under fluctuating light, when PAR is limiting, could only come about if photosynthesis under non-fluctuating PAR was operating at less than the theoretical maximum efficiency, as in the case of Frada et al. (2013; contrast Geider et al. 1985, 1986). Accordingly, it is with mean PAR values that are at or very near saturating for photosynthesis that benefits of fluctuating light are to be sought.

Raven (1994) examined the possibility of the fluctuating light effect working at the scale of a single cell as a result of the Brownian movements of suspended cells combined with the package effects (self-shading). The argument was that self-shading would create a PAR gradient across the cell and that an appropriate frequency of rotation would swap the more- and less-illuminated sides such that use could be made of the fluctuating light effect. However, the appropriate frequency of

alternation of more and less illuminated sides would only occur in picoplankton cells with a very small package effect, and hence a minimal difference in PAR incident on the two side. Cells with a sufficiently large package effect (such as large diatoms) would alternate high- and low-illumination sides at too low a frequency to derive any benefit (Raven 1994). Further illumination problems would arise from the stochastic nature of Brownian forces, meaning that there would not be regular rotation (therefore exposure to a regular light field), as well as the increasingly diffuse nature of the PAR with increasing depth in cultures or natural environments would further limit this effect (Falkowski and Raven 2007).

More generally, a number of experiments have been carried out on the effects of fluctuating light on microalgal cultures growing at or close to PAR saturation with a constant total daily light dose ($\text{mol photons m}^{-2} \text{ day}^{-1}$). These experiments (Table 2) compare PAR supplied at a constant rate during the photoperiod, or with a sinusoidal variation which mimics the change in solar photon input throughout the photoperiod, with PAR fluctuations of varying frequency imposed on the same pattern (constant or sinusoidally varying) as in the controls. The importance of studying the same total daily light dose in the controls and in the fluctuating light treatments is that it is then possible to distinguish the effects of fluctuations in PAR independently of variation in total light dose per day, such as the complicated interpretation of the otherwise exemplary work of Wagner et al. (2006). These experiments, using the same total daily light dose (Table 2), include a number of measurements other than maximum photosynthetic rate or daily biomass production rate which aid interpretation of the effects of fluctuating light.

The outcome of the experiments shown in Table 2 can be summarised as follows. The growth rate and maximum photosynthetic rate can be increased or decreased by fluctuating PAR, depending on the organism and the frequency of fluctuation used. Fluctuating PAR gives a greater release of dissolved organic carbon by the cells; this loss is generally considered to have no benefit to the organism except in cases where the excreted compound aids the acquisition of resources, e.g. siderophores or reducing agents aiding iron acquisition, and carbonic anhydrases and alkaline phosphatases aiding inorganic carbon and inorganic phosphate acquisition respectively, or in deterring grazers (Raven 2010). Such potential benefits are of less importance in biofuel production than in the natural environment, so dissolved organic carbon loss is a process to be minimised in algal biofuel production. Another loss factor is dark respiration, which is vital for biosynthesis in growth and in maintenance, so decreasing respiration rates is only beneficial when there is more respiration than is needed for essential processes. Respiration in the dark is either unaffected or decreased by fluctuating PAR, and a smaller fraction of dark respiration occurs in the photoperiod (Table 2). The interpretation of these results in terms of their

influence on overall productivity requires more detailed mechanistic analysis.

Fluctuating PAR can also change the number of photosynthetic units or photosystem II per cells, and/or alter their size (antenna chromophores per reaction centre). The direction of these changes in fluctuating PAR relative to controls is more likely to resemble acclimation to a higher constant PAR than to a lower constant PAR (Table 2), i.e. in the direction which is considered to be useful in decreasing resource input to antenna pigments without decreasing productivity, including not increasing the potential for photoinhibition, under relatively controlled culture conditions. Following on from this, Table 2 shows that there may be less photoinhibition under fluctuating PAR; however, there was no obvious change in the number of xanthophyll cycle pigment molecules per chlorophyll *a*.

Two critical aspects of fluctuating light need to be considered to fully understand the impact on photosynthetic efficiency; the frequency and intensity of the flash and the relative period of darkness compared with light (e.g. Phillips and Myers 1954; Greene and Gerard 1990; Grobelaar 1991, 1994; Grobelaar et al. 1996; Kübler and Raven 1996a, b; Dimier et al. 2009; Iluz et al. 2012). Using solar radiation for the light periods, the optimal dark period is around 10–50 ms (Williams and Laurens 2010; Iluz et al. 2012; Sforza et al. 2012) with about a 10:1 ratio dark to light, while flash periods shorter than 10 ms are less photosynthetically effective, presumably because not all of the open reaction centres are excited under the prevailing irradiance. This allocation to illumination and darkness is called the duty cycle; in the instance given it would be characterised as 0.1, i.e. 10 % light and 90 % dark. The net effect of intermittent light dark cycles is that high-irradiance light is averaged over the dark period, so cells can be exposed to sub-second duration flashes of $2,000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a duty cycle of 0.1 and would be equivalent to $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of continuous irradiance. Therefore, cells can be exposed to full solar irradiance without suffering photoinhibition if the duty cycle is short enough. This pulse frequency roughly corresponds to the PSII electron transfer rates; such that the PSII reaction centres have passed the electron to the PQ pool and are then able to receive the next photon, without excess reductant accumulating and leading to photoinhibition which will ultimately decrease the energy transformation rate per PSII reaction centre and reduce the efficiency. PSII electron turnover rates are generally limited by the rate of electron flow through the PQH_2 cytochrome b_6-f complex, or sometimes the reaction rate of Rubisco (Falkowski and Raven 2007). Fluctuating light also requires flexibility in the photosynthetic apparatus—as occurs in many lacustrine and oceanic phytoplankton (Dimier et al. 2009). Response to fluctuating light varies widely between species; such that *P. tricornutum* made more effective use of a fluctuating light field than did *Chlorella*

Table 2 Effect of fluctuating light on microalgal physiology in data sets in which all treatments have an identical (or closely similar) total daily light dose of PAR

Alga	TDLD PAR (mol photons m ⁻²)	PAR regime	Response to fluctuation	Reference
<i>Lauderia borealis</i>	8–0, 10 and 5–6 in different experiments at 12:12 L/D	Sinusoidal vs. fluctuating, $T=3$ h	Higher $P_{\max}$ and less afternoon depression of photosynthesis	Marra (1978)
<i>Scenedesmus protuberans</i>	1.83 at 10:14 L/D	Sinusoidal vs. fluctuating, $T=1.25$ or 2.5 h	Higher $P_{\max}$, smaller PSU, i.e. similar to acclimation to high continuous PAR	Flamelling and Krompkamp (1997)
<i>Skeletonema costatum</i>	0.86 (LL) and 1.72 (HL) at 8:16 L/D	Sinusoidal vs. fluctuating, $T=80$ min	More but smaller PSU at high fluctuating light	Krompkamp and Limbeek (1993)
<i>Chlorella vulgaris</i>	8.2 (CL), 10.73 (sinusoidal) and 11.83 (FL) at 8:14 L/D (check)	Fluctuations with $T=1$ h	Lower $P_{\max}$, lower in FL	Kroon et al. (1982)
<i>S. costatum</i>	About 5.6 at 12:12 L/D	Constant vs. fluctuation, $T=1$ h in 12:12 L/D	Similar or slightly higher growth under rapidly fluctuating light; greater release of DOC in FL	Cosper (1982)
<i>Scenedesmus acuminatus</i> , <i>Scenedesmus armatus</i> , <i>Stephanodiscus minutulus</i> , <i>Synedra acus</i> , <i>Limnithrix redekei</i> and <i>Planktothrix agardhii</i>	About 3 or about 7	Continuous vs. LD cycle (12:12 L/D, 8:16 L/D and 4:20 L/D) or LD cycles with fluctuations ($T=30$ or 60 min)	Growth rate decreased under LD cycles and LD cycles plus fluctuations. Smallest effect for diatoms, largest for cyanobacteria. Shortest growth periods decreased growth rate. Least growth suppression with longer fluctuations	Nicklish (1998)
<i>P. agardhii</i> and <i>Stephanodiscus neoastraea</i>	6.3 (<i>Planktothrix</i>) 4.8 (<i>Stephanodiscus</i>)	Continuous vs. FL, $T=30$ min in 12:12 L/D	No effect of chlorophyll per cell or on PSU of PSI or PSII per cell for <i>Planktothrix</i> ; twice as many PSII PSU with half the antenna size, no effect on PSI PSU, in FL in <i>Stephanodiscus</i> . Slightly lower $P_{\max}$, halved R , in <i>Planktothrix</i> . Slightly higher $P_{\max}$. No effect on R , in <i>Stephanodiscus</i>	Fietz and Nicklisch (2002)
<i>Pelagomonas calceolata</i>	9.67	Sinusoidal vs. FL with $T=4$ h or HFL with $T=2$ h	Tendency to decreasing growth rate in the order FL>sinusoidal>HFL became significant at end of batch culture. HFL has more chlorophyll per cell with no change in other light-harvesting pigments per chlorophyll a indicating more PSU per cell with constant PSU size (equivalent to acclimation to low constant PAR) than in FL or sinusoidal. No effect of treatment on xanthophyll cycle pigments per chlorophyll a . Similar absolute $ETR_{\max}$ on a cell basis for all treatments	Dimier et al. (2009)

In all cases, the daily dose is said (or at least seems) to be saturating for growth. Based on Litchman (2000)

CL continuous light, DOC dissolved organic carbon, FL fluctuating light, HFL highly fluctuating light, HL high light, LL low light, $P_{\max}$ rate of photosynthesis at light saturation, PAR photosynthetically active radiation, R respiration (excluding photorespiration), T period of fluctuation, TDLD total daily light dose

vulgaris (Wagner et al. 2006), although as indicated above, the fluctuating treatment has a lower total daily photon dose than the sinusoidal treatment, complicating mechanistic comparisons. Nevertheless, the comparison of the two species in fluctuating PAR by Wagner et al. (2006) is very useful.

Photobioreactor design can utilise the benefits of fluctuating light when the cultures are optically dense in tubular bioreactors,

such that as cells move from the high light exposed outer surface of the reactor into the optically dark inner region of the reactor, this movement simulates a fluctuating light field (Molina Grima et al. 2000). In flat-bed photobioreactors, the culture is optically translucent and therefore fluctuations cannot be used to enhance production. The interaction between RuBP, ATP and the fluctuating light effect has been modelled by Yoshimoto et al. (2005);

however, like some other experiments and models discussed by Iluz et al. (2012), this relationship should be further considered with respect to C limitation (and N limitation) and its impact on lipid production under fluctuating light.

Fluctuating light has been used in closed photobioreactors where the light source (LED) has been pulsed to achieve this effect; as with any artificial light source efficiency of concentration of energy input into photosynthetically useful radiation is important, as is the nature (preferably from renewables) of the energy input (Greenwell et al. 2010). The efficiency of conversion of the electrical energy used by the red emitting LED (659 nm) into production of *Chlorella pyrenoidosa* was highest at a frequency of 20 kHz (50 μ s duty cycle) with 5 μ s flashes and a 45- μ s dark period; less algal growth per unit electricity used was found when using lower LED flash frequencies or with LEDs operating continuously (Matthijs et al. 1996).

A role for proteorhodopsin?

A further possibility for light harvesting and energy transduction is the use of energy-transducing rhodopsins. These share a phylogeny with the sensory rhodopsins but use light energy for the active transport of protons out of the cytosol or, less commonly, chloride ions into the cytosol (Raven 2009a). They were first found in the Archaea, and are now known to be widespread in Archaea and Bacteria and are also known from some fungi and from archaeophyte (=Plantae) and chromalveolate algae (Raven 2009a; Slamovits et al. 2011; Marchetti et al. 2012). The only member of the archaeophytes known to have an energy-transducing rhodopsin is the acellular marine benthic ulvophycean *Acetabularia* where, perversely, protons are moved into the cytosol from the seawater medium rather than from cytosol to seawater (Raven 2009a). Among the chromalveolates, gene sequences corresponding to the opsins of energy-transducing rhodopsins have been determined in marine dinoflagellates (the phagotrophic, non-photosynthetic *Oxyrrhis*, the mixotrophic *Alexandrium* and the large-celled vertically migrating photosynthetic *Pyrocystis*), in two open-ocean marine diatoms (*Fragilariopsis cylindricum* and *Pseudonitzschia granii*) and in the marine haptophyte *Phaeocystis globosa* (Slamovits et al. 2011; Marchetti et al. 2012). In *Oxyrrhis*, the gene product is localised to the endomembrane system, so the energy-transducing rhodopsin could be involved in acidification of food vacuoles of this phagotroph. The oceanic diatoms show up-regulation of proteorhodopsin under iron deficiency (Marchetti et al. 2012), consistent with the high iron requirement for the thylakoid reactions of photosynthesis, and especially of PSI, and the absence of iron involvement in operating the energy-transducing rhodopsins. However, it is by no means clear that energy-transducing rhodopsins can substitute for photosynthetic proton transport into the thylakoid lumen. The available evidence on localisations shows that they are in the plasmalemma

of *Acetabularia* and the endomembrane system in *Oxyrrhis*; neither of these membranes house an ATP synthetase, so that the proton free energy gradient could not be used in ATP synthesis, but only in energising the transport of other solutes across the membrane by an electrically driven uniport, or by proton cotransport and, in the phagotrophic *Oxyrrhis*, acidification of the food vacuoles. As the only known product of the energy-transducing rhodopsin is a trans-membrane ion electrochemical gradient, any redox products would have to be generated by ion-driven uphill electron transport such as occurs in chemolithotrophs (Kiesov 1967; Elbehti et al. 2000) and in the photosynthetic proteobacteria (Falkowski and Raven 2007).

Raven (2009a) points out that the photon yield of rhodopsin-driven proton transport (1 proton per photon) is less than that of proton pumping by non-cyclic electron flow (1.5 protons per photon). The fraction of the energy from the 550-nm photons absorbed by rhodopsins which is found in the proton-free energy difference (8 %) is less than the fraction of the energy in photons at 680 and 700 nm used by PSII and PSI respectively, which is found in the oxidised and reduced products, as well as proton pumping are considered (47 %) (Raven 2009a). For cyclic electron flow, with no net redox products and up to 3.5 protons pumped per photon absorbed (see above), the difference is up to 3.5-fold, as only one proton is moved per photon absorbed by rhodopsins. With the assumptions of Raven (2009a) and Raven et al. (2014), the difference is up to 4-fold.

Raven (2009a) also considers the protein cost of proton pumping by rhodopsin and by chlorophyll-based machinery. Raven (2009a) cites Raven (1984a, b) for the protein to chromophore ratio (kDa protein per mol chromophore) in a range of pigment protein complexes. For chlorophyll–protein complexes, the range is 2.0–63, and for phycobilins, it is 5.8–15.7, while for rhodopsin it is 26. These values cannot be directly compared, as each rhodopsin operates independently, while the chlorophyll–protein and the phycobilin antennae transfer energy to a photochemical reaction centre. The reaction centre is itself a chlorophyll-(carotenoid)-protein complex, and a number of other chlorophyll-free (or almost chlorophyll-free—one chlorophyll per cytochrome *b₆-f*-non-haem iron complex has one chlorophyll per complex) protein complex associated with them which are required for the overall protein pumping and redox activities. Raven (2009a) shows that consideration of these additional protein complexes does not cause the overall protein per unit chromophore in oxygenic photosynthesis to equal that for opsin and retinal in rhodopsin. This is because each chlorophyll-carotenoid-protein or phycobilin is present in a ratio of 300:1–600:1 for each of the three major complexes (PSII, PSI, cytochrome *b₆-f*-non-haem iron complex), and the much smaller proteins (cytochrome *c₆* and/or plastocyanin, ferredoxin or flavodoxin) (Raven et al. 1999; Falkowski and Raven 2007; Raven 2009a).

The conclusion on retinal, as a supplement to the usual redox and proton-pumping mechanisms of oxygenic photosynthesis, is that it would not justify adding rhodopsin and displacing some of the present protein complexes in the already crowded thylakoid membrane on the basis of photon yield of proton pumping, light-saturated rate of protein pumping, or the rate of proton pumping per unit protein at any photon flux density. Only on the basis of iron use efficiency do energy-transducing rhodopsins improve upon chlorophyll-based proton pumps (Raven 2009a), and it has been shown that iron deficiency increases expression of these rhodopsins in oceanic diatoms (Marchetti et al. 2012). Even there, it is not certain, or even likely, that the rhodopsin occurs in the thylakoid membrane: they are more likely to be in the plasmalemma or the endomembrane system sparing the use of other energy sources for bringing about trans-membrane solute transport. Accordingly, rhodopsin is not likely to be useful in filling the absorption gap between the chlorophyll absorption peaks in small-celled algae with a small spectral range of antenna pigments (Raven 1996). It is of interest that *Gloeobacter*, a small-celled phylogenetically basal cyanobacterium (Blank and Sanchez-Baracaldo 2010), with no thylakoids and some with the membrane-associated photosynthetic, respiratory and solute transport reaction housed in the plasmalemma, also has an energy-transducing rhodopsin in the plasmalemma (Raven 2009a; Rexroth et al. 2011). In this case, unlike in the best-characterised eukaryotes, the proton gradient generated by proteorhodopsin could be used in ATP synthesis, as proteorhodopsin occurs in a membrane containing a proton-coupled ATP synthetase.

For biofuel applications, the light absorption properties of industrial-scale cultures with a large optical thickness clearly present an important challenge (Scott et al. 2010; Weyer et al. 2010; Williams and Laurens 2010; Beardall and Raven 2013). High PAR irradiances will only pass through a few centimetres of dense algae where the package effect limits the photon use efficiency, while the surface cells will also suffer photoinhibition (further reducing efficiency); weaker light will be used more efficiently but penetrate less far. These constraints can be improved by devices such as EvenGold™ (www.uts.edu.au/research-and-teaching/industry-partnerships/ip-and-commercialisation/uts-ip-portfolio/sustainability-2) and earlier light diffusers (Myers and Graham 1961) that can distribute photons more evenly throughout the depth of the pond or raceway. This means that full sunlight would not be photoinhibitory near the surface, and it would be less limiting deeper in the culture, therefore decreasing the need for mixing as a means of distributing photons more evenly between cells, as opposed to gas equilibration and restriction of sedimentation in a raceway pond. Whereas, tubular PBRs can be optimised to provide sufficient light to penetrate the entire cross-sectional area of the tube and mixing helps homogenise the light dose provided to the cells.

Decreasing the antenna size of photochemical reaction centres

Another solution would be a more equal distribution of PAR within an algal culture allowing, even under natural irradiances, the possibility of increased photosynthesis by decreasing the size of the photosynthetic units, defined as the number of antenna pigment molecules serving each PSI and each PSII reaction centre (Durnford and Falkowski 1997; Scott et al. 2010; Blankenship et al. 2011). Such a decrease has been achieved for PSII but not for PSI, through the selection of mutant strains (Beckmann et al. 2009; Scott et al. 2010; Williams and Laurens 2010; Blankenship et al. 2011) and could result in an increased growth at near-saturating PAR as a result of the smaller resource allocation to antenna pigment–protein complexes, which is a significant fraction of cell proteins (Table 2) (Raven 1984a, b). This would also decrease the potential for photoinhibition as there is less chance of unusable excitation energy reaching the reaction centre of PSII in a given radiation environment. This has the additional possibility of down-regulating photoprotection and excitation energy quenching (photochemical other than photosynthesis; non-photochemical) mechanisms, with a corresponding decrease in resource allocation to these processes. However, decreased antenna size would decrease the capacity for growth at low, strictly limiting, PAR values, such as would be encountered for a short period of time near dawn and dusk. While the importance of decreasing the antenna size of PSI is less in terms of avoiding photoinhibition, such a reduction could further decrease the resource cost of photon absorption at high irradiances and thus further increase the rate of photosynthesis on the basis of the resources used to produce light harvesting (Fig. 1). However, algal biofuel strains with such a decrease in the antenna size of PSI have not yet been isolated (Williams and Laurens 2010), presumably because there are more dedicated PSI antenna pigment–protein complexes than is the case for PSII, where the reported decreases in antenna size relates to the peripheral rather than the core antenna pigment–protein complexes. Nutrient starvation has also been shown to reduce the antenna size (Stephenson et al. 2010); this could have the additional benefit of triggering greater lipid production along with altering light capture.

With devices such as EvenGold™ which give all cells in the culture approximately equal exposure to PAR, decreased photosynthetic unit size should increase overall conversion of PAR into energy stored chemically in cells; increased photosynthesis during the periods of high PAR would offset the decreased rate of photosynthesis when PAR is low. Decreased potential for photoinhibition is also found in cells with large package effects (Raven 2011), although the larger package effect means that relatively more resources are allocated to antenna pigments than is the case for cells with a smaller package effect (Raven 2011; Raven and Hurd 2012).

Increasing the ATP/proton ratio

A further possible means of increasing the yield of cells per absorbed photon of PAR is by decreasing the number of protons needed to phosphorylate an ADP to form ATP. The F_0F_1 ATP synthetase family found in Archaea, Bacteria, mitochondria and plastids has a F_0 stator component which is an integral protein complex in the coupling membrane. The cylinder of 8–15 c subunits of F_0 (Watt et al. 2010; Pogoryelov et al. 2012) rotate relative to the other F_0 subunits, along with the γ subunit of F_1 which occurs within the c subunit ring. Each of the c subunits acts as a proton channel, each apparently moving one proton per catalytic cycle. The $\alpha\beta$ subunits are on the stromal (plastids), cytosolic (Archaea and Bacteria, including cyanobacteria) or matrix (mitochondria) side of the coupling membrane, and phosphorylates 3 ADP to ATP per catalytic cycle (=one rotation). Depending on the number (8–15) of c subunits per complex, the proton:ATP stoichiometry should be 8:3–15:3, i.e. 2.67–5. The cyanobacterial and plastids CF_0CF_1 ATP synthetases of cyanobacteria and plastids have 13–15 c subunits per complex (Pogoryelov et al. 2012), and so, on structural biological grounds, should have proton:ATP ratios of 13:3–15:3 or 4.33:1–5:1.

Direct measurements on the commonest CF_0CF_1 variant with 14 c subunits, have found the proton:ATP ratio is 3.9 ± 0.3 rather than that predicted from structural biology of 4.67 (=14/3) (Petersen et al. 2012). Similarly, for the *Saccharomyces* mitochondrial F_0F_1 , the prediction from structural biology is 3.33 (=10/3), while the measured value is 2.9 ± 0.2 . In each case, the measured value is significantly less than that predicted by 0.84–0.87 protons per ATP. If the measured value was higher than the predicted value, this discrepancy could be attributed to damage to the ATP synthetase, so that some protons moving through the c subunit channels are not coupled to ADP phosphorylation; however, a lower proton:ATP ratio than that predicted should be taken more seriously. Assuming that the proton:ATP ratio is not more than the predicted value, but is proportional to the predicted value, then more ATP could be made per proton transported through the ATP synthetase if mutation or genetic engineering could decrease the number of c subunits in CF_0 . The success of such an operation depends not only on whether the free energy (electrochemical potential) of protons across the thylakoid membrane is sufficient to produce ATP at the in vivo free energy of hydrolysis of up to 55 kJ/mol, despite the lower proton/ATP stoichiometry (from data in Falkowski and Raven 2007). A larger proton electrochemical potential difference in turn depends on the free energy available from the redox reactions in the thylakoid membrane which transport 3 protons to the thylakoid lumen per electron moving from water to $NADP^+$ and up to 3.5 H^+ per electron undergoing a photochemically powered cycle round PSI. Depending

on the allocation of the electrochemical potential for protons between the electrical and the pH difference components (Raven 1984b; Tang and Wei 2001; Cruz et al. 2005; Falkowski and Raven 2007; Checchetto et al. 2012), a higher proton electrochemical potential difference could mean a lower intra-thylakoid pH, with implications for both the expression of non-photochemical quenching and the activation of proteases involved in the D1 protein repair cycle following photodamage to D1. An increase in either or both of the electrical and pH difference components of the electrochemical potential difference across the thylakoid membrane would not have a detrimental effect of CCMs involving the carbonic anhydrase *Cah3* functioning in the thylakoid lumen (Raven 1997; Raven et al. 2014). However, combined with a decrease in the H^+ :ATP ratio, it would mean that the CCM would be a relatively larger competitor with ATP synthesis. Biofuel strain selection should consider the benefit of assessing the allocation of energy and whether it is near to optimal. This is considered below under waste minimisation.

Algal growth involves mitochondrial, as well as plastidic ATP synthesis (Falkowski and Raven 2007), and similar considerations apply to mitochondrial as to plastidic ATP synthetases. Here, the proton electrochemical potential difference is almost as the electrical potential difference, and the number of c subunits in the ATP synthetase is at or near the bottom of the known range, i.e. 8 (bovine mitochondria: Watt et al. 2010) and 10 (yeast mitochondria: Petersen et al. 2012). There is no information on the number of c subunits in the mitochondria of algae; this must be known before any suggestions are made as to the possibility of increased energetic efficiency might be achieved. In any such considerations two points should be remembered. One is that the number of functioning c subunits is usually less than the measured number (Petersen et al. 2012). The other is that most of the ATP generated in the mitochondria is used in the cytosol, and transport of an ATP out of the mitochondrial matrix, and of an ADP and an inorganic phosphate into the matrix, involves the exergonic transfer of a proton into the matrix, so that for cytosolic ATP the theoretical (from structural data) proton/ATP ratio is 3.67–4.33, and there is an increase in the free energy of hydrolysis of ATP from about 45 kJ/mol in the matrix to 55 kJ/mol in the cytosol (Falkowski and Raven 2007; Watt et al. 2010; Petersen et al. 2012; Nichols and Ferguson 2013).

Improved resource use efficiency of growth by changing the pathway of autotrophic inorganic carbon assimilation

The Rubisco–Benson–Calvin cycle in the present atmosphere

All oxygenic photosynthetic organisms have the Rubisco–Benson–Calvin cycle as the core autotrophic CO_2 assimilation

pathway. The perceived ‘inefficiencies’ of the Rubisco–Benson–Calvin cycle in photosynthesis in today’s atmosphere have been much discussed (e.g. Tcherkez et al. 2006; Raven 2009b; Bar-Even et al. 2010, 2011, 2012; Savir et al. 2010; Fast and Papoutsakis 2012; Young et al. 2012), especially in relation to the increase in the number of autotrophic inorganic carbon assimilation pathways which have become known in the last few years and the possibility that one of these pathways, or a combination of components from such pathways, could replace the Rubisco–Benson–Calvin cycle in biotechnological applications of algae (Raven 2009b; Bar-Even et al. 2010, 2011, 2012; Berg 2011; Fast and Papoutsakis 2012; Raven et al. 2012). The Benson–Calvin cycle (photosynthetic carbon reduction cycle), with Rubisco as the carboxylase, is the core autotrophic CO₂ assimilation mechanism in all oxygenic photosynthetic organisms which account for about 50 Pg (marine) and 60 Pg (terrestrial and inland waters) of carbon per year of primary productivity (Raven 2009b; Raven and Donnelly 2013). The remaining autotrophic inorganic carbon assimilation involves anoxygenic autotrophic photosynthetic bacteria and chemolithotrophs, and contributes about 0.4 Pg of carbon year⁻¹ in (mainly) marine and soil environments (Johnston et al. 2009; Raven 2009b; Raven and Donnelly 2013). Some of the autotrophic inorganic carbon assimilation by anoxygenic autotrophic photosynthetic bacteria and chemolithotrophs involves the Benson–Calvin cycle, so Rubisco accounts for at least $((50+60)/(50+60+0.4)) \times 100 = 99.6\%$ of global autotrophic inorganic carbon assimilation today. The remaining autotrophic inorganic carbon assimilation occurs through the five other well-characterised pathways, with possible minor contributions from other pathways (Raven 2009b; Bar-Even et al. 2010, 2011, 2012; Berg 2011; Erb 2011; Raven et al. 2012; Raven and Donnelly 2013).

This overwhelming dominance of Rubisco as the carboxylase in autotrophic organisms is achieved in spite of its perceived shortcomings. Rubisco has a low specific reaction rate per unit enzyme protein at CO₂ saturation, while O₂ is an alternative substrate that is competitive with CO₂. Even in the absence of O₂, few if any of the known Rubisco variants are saturated with CO₂ at the current atmospheric CO₂ concentration and at the temperature at which the Rubisco variant usually functions in vivo (Tcherkez et al. 2006; Savir et al. 2010; Young et al. 2012; Studer et al. 2014). The detailed mechanistic analysis by Tcherkez et al. (2006) shows that there are mechanistic trade-offs among the specific reaction rates at CO₂ saturation, the CO₂/O₂ selectivity and the CO₂ affinity. Accordingly, it is not possible to combine the highest specific reaction rate at CO₂ saturation with the highest known CO₂/O₂ selectivity and the highest known CO₂ affinity known among the Rubisco variants into a single enzyme molecule. This situation is aptly epitomised in the title of the paper by Tcherkez et al. (2006): ‘Despite slow catalysis and confused

specificity, all ribulose biphosphate carboxylases may be nearly perfectly optimized’. Studer et al. (2014) come to similar conclusions based on stability-activity tradeoffs. As will be discussed below, it is of interest that the enzyme with the lowest specific reaction rate in the pathway, whose characteristics make it the best potential replacement for the Rubisco–Benson–Calvin cycle, i.e. the 3-hydroxypropionate pathway, is also a carboxylase, i.e. propionyl CoA carboxylase (Bar-Even et al. 2010, 2012).

Rubisco evolved well before the global oxygenation event, some 2.35 billion years ago (Blank and Sanchez-Baracaldo 2010) when higher than today’s greenhouse gases levels, such as atmospheric CO₂, occurred or higher atmospheric pressures were present where broadening of the absorption by N₂, was required to explain the continued occurrence of liquid water over the last 3.8 billion years despite solar energy output being only about 80 % of the present level 2.7 billion years ago (Tcherkez et al. 2006; Raven et al. 2012; Som et al. 2012). The atmospheric pressure 2.7 billion years ago is constrained by estimates of air density based on fossil raindrop imprints (a method suggested by Lyell 1851) to less than twice the present value (Som et al. 2012). These measurements constrain the upper limit on the CO₂ content of the atmosphere to less than the very high levels (in excess of two atmospheres partial pressure of CO₂) allowed by some interpretations of Archaean geochemistry (Som et al. 2012) but allow values that would be well above the level needed to saturate even the lowest-affinity examples of present-day Rubiscos. Thus, Rubisco evolved in a very different environment to today’s air-equilibrated solutions.

Rubiscos in extant oxygenic photolithotrophs cope with CO₂ and O₂ levels which they encounter in air, or air-equilibrated natural waters, by a range of ‘fixes’ occurring downstream or upstream of Rubisco in the continuum of the transport of environmental inorganic carbon to Rubisco, the reaction of Rubisco with CO₂ and O₂ and then dealing with the products of the reaction of Rubisco with O₂ (Raven et al. 2012, 2014). Photorespiratory carbon oxidation cycle is a downstream ‘fix’ used by all of the oxygenic photolithotrophs. The well-characterised examples of those cycles metabolise 2-phosphoglycolate, the product of the oxygenase reaction of Rubisco, to 2-phosphoglycerate and triose phosphates which can be used in biosynthesis, with the additional benefit of removing 2-phosphoglycerate which is inhibitory to metabolism at high concentrations, as well as sequestering phosphorus (Raven et al. 2012, 2014). The upstream ‘fixes’ are the use of one of the CO₂ concentrating mechanisms (CCMs) which increase the CO₂ concentration around Rubisco during steady-state photosynthesis to concentrations higher than those in the atmospheric, or air-equilibrated natural water (Raven et al. 2012). A minority of extant terrestrial species of photolithotrophs have CCMs, mainly with C₄ photosynthesis or crassulacean acid metabolism (CAM), while a majority

of aquatic species of photolithotrophs have CCMs, mainly involving active transport of an inorganic species or H^+ across a biological membrane (Giordano et al. 2005; Raven 2010, 2013a, b, c; Raven et al. 2012, 2014). This difference between aquatic and terrestrial habitats apparently relates to the greater diffusive limitation imposed on inorganic carbon species by the diffusion boundary layer round the organisms in aquatic rather than in terrestrial habitats. The aquatic species lacking CCMs generally live in habitats with CO_2 above the air-equilibrium values as a result of a CO_2 subsidy from terrestrial primary productivity in the catchment of inland waters, or light limitation in some deep-growing marine benthic macroalgae (Raven et al. 2005, 2012; Raven 2010). The organisms with CCMs account for at least half of the global gross primary productivity (Raven 2013a).

These 'fixes' have their energy costs; Raven et al. (2014) calculate and tabulate minimum costs of photorespiration and of CCMs, while the values cited in this paper are mean values. Taking into account the energy cost (as NADPH and ATP) of making the RuBP substrate of the oxygenase function of Rubisco, and using the variant of conversion of 2-phosphoglycolate to triose phosphate using glycolate dehydrogenase, glycine decarboxylase and serine hydroxymethyl transferase, the conversion of one CO_2 into 1.3 of a triose P is 4 NADPH and 5.7 ATP, as compared with the 2 NADPH and 3 ATP used in the Benson–Calvin cycle under CO_2 -saturating conditions (Table 1). If Rubisco kinetics give an oxygenase:carboxylase ratio of 0.25 for an air-equilibrated solution at the growth temperature of the organism, the energy cost per net CO_2 fixed assimilated to produce 1/3 triose P is 2.5 NADPH and 3.7 ATP rather than the 2 NADPH and 3 ATP used in the Benson–Calvin cycle under CO_2 -saturating conditions (Table 1). The minimum additional energy cost per CO_2 assimilated using a CCM is 1 ATP, and more generally 2 ATP (Giordano et al. 2005; Falkowski and Raven 2007; Hopkinson et al. 2011) (Table 1). Table 3 also gives data for ATP and NADPH costs for the other autotrophic CO_2 assimilation pathways which might be engineered into oxygenic photolithotrophs. Bar-Even et al. (2012) point out that there are also glycine synthase pathways of inorganic carbon assimilation using either glycine reductase or serine hydroxymethyl transferase, which occur only in anaerobic bacterial chemo-organotrophs (heterotrophs); a very poorly characterised glyoxylate synthase pathway has also been identified in green tubers of *Solanum tuberosum*. These pathways will not be considered further.

Comparison of autotrophic inorganic carbon assimilation pathways in relation to replacement of Rubisco–Benson–Calvin cycle

It will be seen from Table 1 that the Rubisco–Benson–Calvin cycle is more energy-costly to operate than all but one of the

other pathways (3-hydroxypropionate-4-hydroxybutyrate pathway) at CO_2 saturation for Rubisco and significantly more energy expensive than any of the other pathways in air-equilibrium solutions. However, Table 1 also gives information on the half-saturation value for the lowest-affinity carboxylase for the various pathways, expressed in terms of CO_2 (many of the carboxylases have HCO_3^- as their immediate substrate, so the cited values are for CO_2 at equilibrium with HCO_3^- at the in vivo pH, temperature and ionic strength). Although the value cited for the Wood–Ljungdahl pathway (Table 1) is very high relative to the CO_2 concentration in the habitats occupied by some of the organisms which use the pathway (Raven et al. 2012), it is clear that some of the pathways would need a very large cell quota of the lowest-affinity carboxylases. This would incur the associated additional resource costs of synthesis of the carboxylase, to permit growth in an air-equilibrium medium unless a CCM was used, again with an additional energy cost of operation and resource cost for its production. The energetic and carboxylase affinity arguments mean that only the 3-hydroxypropionate pathway is clearly superior to the Rubisco–Benson–Calvin cycle. The case for focusing on the 3-hydroxypropionate pathway is strengthened by considering O_2 sensitivity (Table 1); only the 3-hydroxypropionate,4-hydroxybutyrate pathway shares with the 3-hydroxypropionate and Rubisco–Benson–Calvin pathway the absence of irreversible O_2 damage. The lack of inhibition by O_2 of the 3-hydroxypropionate pathway (Zarzycki et al. 2009; Bar-Even et al. 2010, 2012) have apparently not been tested up to the highest O_2 concentrations generated locally by some extant by oxygenic photolithotrophs in their cells, i.e. four times air-equilibrium values—equilibrium with an atmosphere with 84 kPa O_2 at sea level (Raven and Larkum 2007). However, the capacity of the organisms to grow at such high O_2 concentrations (Raven and Larkum 2007) means that they must be able to synthesise fatty acids and other lipids which requires the activity of acetyl CoA carboxylase; this is the carboxylase which with the closely related propionyl CoA carboxylase, is used in the 3-hydroxypropionate pathway (Bar-Even et al. 2010, 2011, 2012). Finally, while irreversible damage by O_2 (including reactive oxygen species) does not rule out the occurrence of a biological process in an oxygen-containing environment, as is shown by the occurrence in such environments of PSII of photosynthesis and nitrogenase (Falkowski and Godfrey 2008), it seems an unnecessary complication to use an oxygen-damageable pathway, with its corresponding resource cost of protection and/or repair, when oxygen-tolerant alternatives (not the case for nitrogenase or PSII) are available. This problem can occur in closed PBRs where O_2 can be significantly above the air-equilibrium concentration, with variable inhibitory effects depending on the algae and growth conditions (Raven and Larkum 2007).

Table 3 Fraction of total protein in leaves and cyanobacterial cells occupied by Rubisco and, for comparison with other highly expressed proteins, the fraction of total protein in cyanobacteria and microalgae occupied by apoproteins of light-harvesting and reaction centre pigment–protein complexes and ribosomal protein

Protein	Fraction of total protein	References
Rubisco in leaves of C ₃ terrestrial plants	0.13–0.30: lowest value for the extreme shade plant <i>Alocasia</i> . Values for Rubisco are expressed as the fraction of total leaf N present in Rubisco; as there are leaf N pools other than protein, the values cited are under-estimates	Evans (1989)
Rubisco in cells of cyanobacteria and microalgae	0.03–0.16: most values are for organism expressing CCMs; some values are for organisms potentially capable of expressing CCMs but grown at high CO ₂ concentrations which repress the CCM with diffusive CO ₂ entry providing CO ₂ concentrations at the Rubisco site at least as high as are maintained by the CCM 0.02–0.06: 7 marine, 1 freshwater phytoplankton in exponential resource-saturated growth; the 8 species grown under nutrient limitation, and field samples, have values of not more than 0.025. Values are more reliable than the earlier values cited by Raven (1991), largely because of assumptions made in converting from a chlorophyll to a total protein basis in many cases	Raven (1991) and Losh et al. (2013)
Apoproteins of light-harvesting and reaction centre pigment–protein complexes	0.04–0.4: highest values are for extreme shade organisms using phycobiliproteins as their major light-harvesting complexes	Raven (1984a, b), Geider and La Roche (2002) and Beardall and Raven (2013)
Ribosomal protein in microalgal cells	0.09–0.21: based on ranges of RNA and protein content as fraction of cell dry matter, assuming that 90 % of RNA is rRNA and half the dry matter in ribosomes is RNA and the other half is protein	Geider and La Roche (2002)

Modified from Table 4 of Raven et al. (2013a; see also Raven 2013b, c)

In addition to the high energetic costs of operating of the Rubisco–Benson–Calvin cycle relative to other pathways in the present atmosphere, Rubisco is perceived as having high resource costs of synthesis on the enzyme (Ellis 1979; Evans 1989; Bar-Even et al. 2010, 2012). Even the Rubiscos with the highest CO₂-saturated rates of photosynthesis per unit protein have lower specific reaction rates than many other carboxylases which have been examined, and the fact that Rubisco is the most abundant single protein (Ellis 1979) in the world relates to its low specific activity, as well as the global biogeochemical importance of the reaction it catalyses. Table 3 shows the fraction of cell protein attributable to Rubisco in C₃ land plants (those relying on a diffusive supply of CO₂ from the atmosphere to Rubisco) and in a range of cyanobacteria and microalgae. For comparison, the range of values for the fraction of cell protein taken up by other highly expressed categories of proteins, i.e. light-harvesting and photochemical reaction centre pigment–protein complexes, and ribosomal proteins, are also provided in Table 2. The fraction of total protein contributed by the three categories of microalgal proteins overlap and are each substantial. An exception is the recent work of Losh et al. (2013), whose Rubisco values do not overlap with the range of values for ribosomes, and are each substantial, and the fraction devoted to autotrophic inorganic carbon assimilation, i.e. Rubisco, plus the other enzymes of the Rubisco–Benson–Calvin cycle, the photorespiratory carbon oxidation cycle and the CCM, still does not exceed that of the pigment–protein

complexes and ribosomal proteins (Raven 1991, 2013a; Bar-Even et al. 2010, 2012; Losh et al. 2013).

How do the other CO₂ fixation pathways compare and could their properties be used to enhance algal biofuel production? Bar-Even et al. (2010, 2012) have calculated, as far as is possible using the available information, the requirement for enzyme proteins for various CO₂ fixation pathways to catalyse a certain rate of CO₂ assimilation into carbohydrate. Within a particular pathway, Bar-Even et al. (2010, 2012) calculated the quantity of each protein required, based on each enzyme's specific reaction rate at substrate saturation adjusted for stoichiometry of the C atoms in the enzyme substrates for their stoichiometry of CO₂ assimilation (see also Raven 1984b, 1988), and summed these values to give the total protein cost of the pathway related to a given rate of CO₂ fixation. Attention was restricted to the 3-hydroxypropionate pathway, the CO₂ assimilation pathway with the CO₂ affinity and lack of O₂ inactivation properties which are most appropriate to replace the Rubisco–Benson–Calvin cycle, the construction costs in terms of energy, carbon and nitrogen are similar to those of Rubisco–Benson–Calvin cycle when photorespiration is ignored. When photorespiration (for diffusive CO₂ entry) is considered the Rubisco–Benson–Calvin cycle costs 1.5 times the 3-hydroxypropionate pathway (Bar-Even et al. 2010, 2012), and a smaller increment of cost for the Rubisco–Benson–Calvin cycle results from the production of a CCM which minimises photorespiration (and the protein costs it entails) in the current atmosphere and significantly

decreases the content of Rubisco protein, while requiring some additional protein in the components of the CCM (Raven 1991, 2013a; Losh et al. 2013). Such analyses suggest that there are lower capital costs of producing the autotrophic carbon assimilation mechanism from replacing the Rubisco–Benson–Calvin cycle by the 3-hydroxypropionate pathway (Bar-Even et al. 2010, 2012). Interestingly, the enzyme with the lowest specific reaction rate (mole substrate transformed), adjusted for stoichiometry with the total CO₂ fixation rate (Bar-Even et al. 2010), per unit protein per unit time in the 3-hydroxypropionate pathway is propionyl CoA carboxylase. It is a carboxylase which imposes the largest protein cost for the overall cycle for both the Rubisco–Benson–Calvin cycle and the 3-hydroxypropionate pathway.

A restriction on the replacement of the Rubisco–Benson–Calvin cycle is the requirement for at least some enzymes of the photosynthetic carbon reduction pathway (Rubisco–Benson–Calvin cycle) or the oxidative pentose phosphate pathway for the synthesis of particular metabolites, e.g. erythrose-4-phosphate which is required for the shikimic acid pathway of phenolic synthesis (Srinivasan et al. 1955; Maeda and Dudareva 2012). Although Archaea have other metabolic routes to erythrose-4-phosphate and ribose-5-phosphate used in nucleotide and hence nucleic acid synthesis (Soderberg 2005), using these enzymes would mean that even more gene replacement events would be needed. However, the flux of metabolites through these pathways for biosynthesis expressed in the basis of the CO₂ assimilation rate in gross photosynthesis is relatively small, with a corresponding small demand for enzymic machinery, although a full analysis of the enzymic protein requirement of the pathway of the kind done by Bar-Even et al. (2010, 2012) for carboxylation pathways does not seem to have been performed.

Waste minimisation in algal growth by decreasing unnecessary losses of inorganic carbon in dark respiration and of organic carbon by decreasing excretion of dissolved organic carbon

Role of respiration in growth of microalgae

In chemoorganotrophic growth of microalgae involves the loss of up to 0.5 mol C as CO₂/mol exogenous organic C incorporated into cell material (Raven 1976a, b, 1984b). This loss of CO₂ is related to the roles of the Emden–Meyerhof–Parnas (EMP) glycolytic pathway, the oxidative pentose phosphate pathway, the mitochondrial Krebs tricarboxylic acid cycle, the mitochondrial electron transport chain, and the mitochondrial F₀F₁ ATP synthase. These pathways are the sources of the ATP, NADPH and C skeletons used in biosynthesis for growth and also in maintenance processes (including protein turnover which is considered in the next section).

Estimation of the minimum quantitative requirement for dark respiration for growth and maintenance in photolithotrophs is more complex. One problem is the estimation of the CO₂ and O₂ fluxes found in illuminated photosynthetic cells. Even with the use of isotopic labels for O, it is not easy to distinguish the component of O₂ uptake in the light which is attributable to dark respiration rather than to PTOX, Mehler-peroxidase, Rubisco oxygenase or subsequent photorespiratory reactions. The same goes for the use of isotopic C to measure CO₂ production from dark respiratory processes relative to photorespiratory processes. A simplifying assumption is that the dark respiratory processes in steady-state light is equal to the rate found immediately after cessation of illumination; even this is complicated by continuation for a short time of the phosphoglycolate-consuming photorespiratory processes. While the roles of the EMP and oxidative pentose phosphate pathways in producing C skeletons, and the dark respiratory roles in ATP and NADPH production, can be replaced by these substrates produced in photosynthetic partial reactions (Raven 1976a, b), this is not the case for the C skeletons generated by the TCAC (Raven 1976a, b). This required rate of synthesis for growth processes in the light defines the minimum CO₂ production rate in the light. There is a considerable variation in the rate of biosynthetic processes in the dark phase of the diel cycle, from a similar rates in the light and the dark to the occurrence of essentially all of the biosynthesis in the photoperiod (Raven 1976a, b, 2013b, c; Cuhel et al. 1984; Needoba and Harrison 2004) with the only energisation role of dark respiration is for maintenance processes.

Raven (1976a, b, 1984b) consider the overall energetics of growth with varying extents of biosynthesis in the dark phase and of the use of partial reactions of photosynthesis to synthesise ATP and NADPH that would otherwise be generated from respiration of photosynthesized organic carbon. The conclusion is that the smallest energy cost of biosynthesis starting from inorganic materials is for the direct use of the partial reactions of photosynthesis. However there is a need, in this case for synthetic machinery that is only functional in the photoperiod, whereas for continuous functioning in light and dark means that only half as much synthetic machinery would be needed by a cell. The limitation of synthesis of additional copies of the catalytic machinery (e.g. ribosomal components), as well as the functioning of the machinery in producing proteins and hence the rest of the cell, to about half of the diel cycle, which has the possibility to partly offset the energetic advantage of the concentrating ATP and NADPH use in the light phase with direct use of photo-produced cofactors (Raven 1976a, b, 1984b, 2013b, c).

Turning from the temporal aspects of biosynthesis in algae to various reasons for decreased efficiency in microalgal dark respiration, there are a number of causes of decreased efficiencies. Diatoms have the Entner–Doudoroff (ED) pathway

as well as the EMP pathway; the ED pathway has a lower ATP yield (Fabris et al. 2012; Flamholz et al. 2013). *Euglena gracilis* (Shigeoka et al. 1996) and many cyanobacteria (Zhang and Bryant 2011), lack 2-oxoglutarate dehydrogenase, and the enzymes that replace it which decreases the energy yield of this variant of the TCAC relative to that of the typical TCAC. Moving to the mitochondrial redox chain and its proton pumps, dinoflagellates lack mitochondrial complex I which oxidises NADH in the matrix and reduces UQ, with pumping of up to 1.5 protons per electron (Wikström and Hummer 2012) and possible up to 2 protons per electron (Raven et al. 2014) and is replaced by an electron transfer from matrix NADH to UQ which does not pump protons (Danne et al. 2012). This replacement can decrease the number of protons pumped per electron transferred from NADH in the matrix to O₂ (and hence the ATP yield per electron moved to O₂) by up to 40 %. This may help to explain the high respiration rate (on several bases) of dinoflagellates relative to other algal higher taxa (Geider and Osborne 1989). Another source of decreased proton:electron ratio is the expression of the mitochondrial alternate oxidase, widespread among algae (Prihoda et al. 2012), which transfers electrons from UQH₂ to O₂. If the UQ is reduced by a redox sequences which does not pump protons, there are no protons pumped in that sequence from external NADH, matrix NADH other than by complex I, or matrix FMNH₂, and UQH₂ is oxidised by the alternate oxidase, no protons are pumped in transfer of electrons to O₂. As mentioned above, where Complex I is the source of electrons to UQ, the proton per electron (and hence ATP per electron) is up to 40 % of that found with operation of mitochondrial complexes I, II and III. The mitochondrial uncoupling protein, which functions as a proton channel not coupled to ADP phosphorylation, is another means of decreasing the ATP per electron transferred down mitochondrial electron transport chains to O₂ (Jarmuszkiewicz et al. 2010). However, little is known of the occurrence of this uncoupling protein in algae (Jarmuszkiewicz et al. 2010). Either of these means of decreasing the ATP per electron ratio in mitochondrial respiration can help to balance the role of respiration in C skeleton production and in NADPH and ATP production, but there can be cases where there seems to sometimes be over-engagement of at least the alternate oxidase. The possibilities of manipulating the proton:ATP stoichiometry of Complex V, the F₀F₁ mitochondrial ATP synthase were discussed above under ‘Increasing the ATP/proton ratio’.

Dissolved organic carbon loss

Microalgae lose a diversity of organic compounds with a range of relative molecular masses (Hellebust 1974; Engel et al. 2004; López-Sandorval et al. 2013). There is a large variation (1–10 %) in the fraction of organic carbon produced in photosynthesis that is lost as dissolved organic carbon, with

large interspecific variation so that there is no clear difference among higher algal taxa (López-Sandorval et al. 2013). While some of the extracellular products have known functions (siderophores, toxins, extracellular enzymes such as carbonic anhydrases and alkaline phosphatases), the majority of the mass of dissolved organic carbon generally has no known function. Minimising such ‘wasteful’ losses are made difficult by the large range of compounds, the usually unknown mechanism of efflux and the possibility of as yet unknown functions of the dissolved organic matter lost from the cells.

Waste minimisation in algal growth by decreasing the rate of protein turnover

To enhance the cost effectiveness of biofuel production, we can improve efficiency and/or reduce inefficiencies, such as unnecessary biosynthesis (e.g. pigments and enzymes) that is not needed in controlled PBR conditions or ponds where environmental range precludes certain biochemical cycles, such as hypo-salinity. Protein turnover, i.e. breakdown of proteins and their replacement by the same, or of different, proteins, occurs in all living cells: see Quigg and Beardall (2003) and Raven (2012, 2013c) for consideration of protein turnover in photosynthetic organisms. There is a body of data on the mean turnover rate of thousands of proteins present in algae and other oxygenic photosynthetic organisms measured with isotopic labelling techniques (reviewed by Quigg and Beardall 2003). Previous studies of the turnover of individual proteins in algae have been restricted to one or a few peptides, such as the subunits of Rubisco (Iwanij et al. 1975), as well as components of the PSII reaction centre, especially the D1 protein (reviewed by Raven 2011). Only recently has isotopic labelling coupled with proteomic techniques been used to determine the turnover rates of ten or more individual proteins in the marine picophytoplanktonic prasinophycean alga *Ostreococcus tauri* (Le Bihan et al. 2011; Martin et al. 2012) and the freshwater chlorophycean microalga *C. reinhardtii* (Mastrobuoni et al. 2012). Other recent studies (Piques et al. 2009) used leaves of the model flowering plant *Arabidopsis thaliana* and predicted the turnover for 35 enzyme proteins from the number of ribosomes and the rate of loading of transcripts into polysomes, which gave an estimate of the gross rate of synthesis of the proteins and the content of the proteins in the proteome.

Quigg and Beardall (2003) measured the rate of protein turnover in two marine microalgae, *Dunaliella tertiolecta* and *P. tricornutum*, and, using literature values for the energy cost of protein synthesis per peptide bond, found that protein turnover consumed about a third of the ATP produced by respiration in the dark. Why do organisms turn over proteins, with this large expenditure of energy? Wagner (2005, 2007; see also van Kleunen and Fischer 2005;

Bradshaw 2006; van Kleunen and Fischer 2007) showed that energy costs associated with an increase in the expression of a gene, as happens during protein turnover, is likely to be actively selected against unless there is some counteracting contribution to fitness associated with protein turnover. This is an area where screening for candidate strains for biofuel production can be highly effective.

One obvious reason for protein turnover is acclimatory changes in gene expression in a changing environment. The extent to which such environmental changes are minimised in biofuel cultures relative to what happens in the wild increases the potential for minimising protein turnover related to changes in the proteins represented in the proteome. An aspect of most outdoor culture systems is the use of solar radiation with the natural light–dark cycle which may be associated with protein turnover. Diel protein turnover can be very large, e.g. in iron-deficient cultures of the diazotrophic marine cyanobacterium *Crocospira watsonia* (Saito et al. 2011), with iron shuttled between the photosynthetic apparatus which is broken down at dusk into the nitrogen fixation system which is synthesised at dusk, with the reverse degradations and synthesis occurring at dawn. Even in a completely constant environment there is protein turnover related to regulation of the cell cycle (Reed 2003; Perrot-Rechenmann 2010). Any diel changes in turnover of individual proteins, e.g. involved in photosynthesis and respiration, cannot be synchronous with those of the cell cycle for microalgae growing with more than one cell division per day, such as the marine diatom *T. pseudonana* (Granum et al. 2009). It is important to note that this unavoidable protein turnover involves mainly the re-use of the amino acids released in protein breakdown in the synthesis of other proteins (Feller et al. 2008), so there is no scope for decreasing resource allocation, and especially energy, used in amino acid synthesis for the new proteins (Raven 1984a, b, 2012, 2013b, c; Bragg and Wagner 2009, 2010; Bragg et al. 2012).

In addition to protein turnover that is intrinsic to cell function under constant conditions and acclimatory qualitative and quantitative changes in the genome in relation to changing conditions, there is also protein turnover related to the removal of proteins which are damaged or otherwise non-functional or even catalysing inappropriate reactions (Raven 2012, 2013b, c). Some of these proteins have either an incorrect primary sequence or incorrect folding as a result of errors in transcription, translation or post-translational modification. Protein turnover is then, in part, presumably part of the cost–benefit trade-off of speed and accuracy in transcription, translation and post-translational protein modification (Raven 2012, 2013b, c). Very stringent, and hence slow and probably energy-costly, proof-reading in transcription, translation and post-translational modification means a large investment in ribosomes and other catalysts involved in the net synthesis of perfectly formed proteins (Raven 2012, 2013b, c). Increased

speed of protein synthesis processes, allows higher specific reaction rates of resource-costly catalysts, such as ribosomes and proof-reading machinery, and hence resource savings in protein synthesis, although there then has to be mechanisms for detecting and breaking down the mal-formed proteins and additional resource input to ribosomes and other machinery to replace the mal-formed proteins (Raven 2012). Others non- or mal-functioning proteins are those that have been damaged by environmental conditions (Falkowski and Godfrey 2008; Raven 2012). Falkowski and Godfrey (2008) point out that the global oxygenation event leads to oxidative damage to two key enzymes in biogeochemical cycles, i.e. nitrogenase and the D1 protein of PSII in oxygenic photosynthesis, and competitive inhibition of a third, Rubisco.

Raven (2011, 2012) reviewed the quantitative significance of breaking down oxidatively damaged proteins such as nitrogenase and D1 and of D1 damaged in high light other than by reactive oxygen species, and their resynthesis. His conclusion is that the additional protein synthesis associated with synthesis of new nitrogenase, or the production and operation of protective machinery restricting access of O₂ to nitrogenase, could cause up to a 10 % decrease in specific growth rate relative to growth supported by nitrate or, especially, ammonium, urea or amino acids (Raven 2011, 2012). By contrast, the decrement of growth rate due to resource diversion to provide and operate additional photoprotection processes (e.g. xanthophyll cycles) and/or D1 synthesis to replace photodamaged D1 by synthesis of more D1 under severe photoinhibition conditions is 1 % or less; although the ‘opportunity cost’ of photosynthesis foregone by down-regulation of PSII and/or damage to PSII in photoinhibition could be significantly larger (Raven 2011, 2012). In many ways, improvements in the efficiency of biofuel production will be incremental and relatively small, therefore, careful examination of the cost–benefit (as well as recognising foregone capacity) analysis could present real opportunities for the biofuel industry.

What can be done to decrease the growth rate decrement related to protection of proteins from damage and to replace those proteins that do become damaged? For nitrogenase, the obvious answer is to use a nitrogen source other than N₂, i.e. combined nitrogen such as ammonium, urea, amino acids, nitrite or nitrate. This is what happens in all biotechnological uses of algae. For photoinhibition, the problem is more complex, as natural solar radiation is usually used for pond growth, and this includes UV, as well as PAR. The quantum yield for photodamage to PSII in the terrestrial vascular plant *A. thaliana* is much higher in the UV than in the PAR part of the solar spectrum (Takahashi et al. 2010). The extent to which inhibition of algal growth by high solar irradiances, as moderated by varying depths of natural waters that attenuate irradiance, relates more to UV than PAR, and this has not yet been clarified (Franklin and Forster 1997; Dring et al. 2001;

Bischof et al. 2006), although investigation of wavelength dependence of photodamage in the PAR range in the trebouxiophycean green alga *Chlorella* is much greater in the blue regions than at wavelengths longer than 540 nm (Schreiber and Klüghammer 2013). It is likely that the UV damage to PSII involves a different mechanism to that caused by PAR (Takahashi et al. 2010), including the UVA component of absorption by photosynthetic pigments, so that changing the photoprotective machinery for PAR also affects PSII.

Scattering of solar radiation by appropriately designed light guides could direct solar radiation into the algal pond cultures and, in principle, provide moderate PAR (and UV) fluxes throughout the culture, rather than photoinhibitory PAR (and UV) fluxes at the surface and light-limiting PAR lower in the cultures. This should enable the use of strains of algae with down-regulated PAR photoprotection mechanisms, or of genetically modified algae with partially disabled photoprotective machinery with a potential increase in energy conversion into usable biomass, as less energy is diverted to photoprotection by changes to optimal allocation (Rosen 1967) and on the possibility of survival in the natural environment as an energetic function of trade-offs (Wagner 2005). The capacity for repair (breakdown and resynthesis) of photodamaged PSII reaction centres would have to be retained, but down-regulated. It is likely that in all such breakdown and resynthesis events that the recycling of amino acids, other than those which were damaged, could occur. This means almost zero cost of synthesis of amino acids in protein turnover, avoiding the resource costs of amino-acid synthesis and the differential resource costs depending on the nitrogen or sulphur sources (Raven 1984a, b; Bragg and Wagner 2009, 2010; Bragg et al. 2012). Cost optimisation of biofuels requires that the necessary repair capacity is available without wasting resources on redundant repair systems.

The savings in producing and operating a reduced photoprotective mechanism could be in terms of decreased capacity for state transition, which can decrease the allocation of absorbed photons to PSII (Falkowski and Raven 2007; Cardol et al. 2009) or a decreased capacity for photochemical quenching using cyclic electron flow round PSII, the Mehler-peroxidase 'water–water cycle' using both PSII and PSI, or the PTOX-catalysed 'water–water cycle' involving only PSII (Cardol et al. 2008; Raven 2011), or a decreased capacity for state transitions (Cardol et al. 2009). All of these have resource, including energy costs, so down-regulation should increase the potential for growth per photon absorbed, with biotechnological importance.

The other possibility for down-regulating photoprotection is by limiting non-photochemical quenching. The discussion by Goss and Jakob (2010) and Depauw et al. (2012) shows that non-photochemical quenching, even in green algae, differs from non-photochemical quenching in flowering plants. An important example is the absence of the PsbS gene and its replacement

in the green algae *C. reinhardtii* and *Ostreococcus tauri* by LHCSR proteins such as LhcSR3; both PsbS and LHCSR occur in the moss *Physcomitrella patens* (Li et al. 2004; Bonente et al. 2010; Depauw et al. 2012). Low pH in the thylakoid lumen, indicating a greater rate of proton pumping into the thylakoid lumen that can be used in proton backflow to the stroma using the CF₀CF₁ ATP synthetase, is used in the algae examined to initiate non-photochemical quenching even if (unlike the situation in flowering plants) the low intraluminal pH is not needed for continuation of non-photochemical quenching (Goss and Jakob 2010; Depauw et al. 2012). While in flowering plants the luminal pH is sensed by PsbS (Li et al. 2004; Bonente et al. 2008), in the green alga *C. reinhardtii*, the pH sensing involves LhcSR3, which also performs the role of an excitation energy quencher, which in flowering plants is a function of other pigment–protein complexes, the Lhcbs. A further role of light-driven thylakoid acidification in photoinhibition is in activating a protease (Deg1) involved in D1 protein breakdown in the light in the PSII repair cycle (Kley et al. 2011).

In most algae tested, one or more xanthophyll cycles play a significant or major role in non-photochemical quenching, although all algae also have energy-dependent quenching (qE) which works on a more rapid time-scale for both initiation and termination than do the xanthophyll cycles (Goss and Jakob 2010; Depauw et al. 2012). The major means of non-photochemical quenching in the cyanobacteria and Rhodophyta is presumably qE, as these organisms generally lack a conventional xanthophyll cycle. The water solubility of phycobilins, which are the major antenna pigments for PSII in these organisms (except for the cyanobacteria with chlorophyll *b* or allo-chlorophyll *b*), would pose problems for the usual xanthophyll cycle related to integral membrane proteins in bringing about non-photochemical quenching in the pigment bed (Goss and Jakob 2010). The xanthophyll cycle(s) independent qE component of non-photochemical quenching occurs in a number of the algae examined (Goss and Jakob 2010). An interesting example is that of the Cryptophyta whose antenna pigments include chlorophyll *a–c* pigment–protein complexes in the thylakoid membrane, as well as phycobiliproteins in the thylakoid lumen (Kaňa et al. 2012). In *Rhodomonas salina* examined by Kaňa et al. (2012), there was no involvement of xanthophyll cycles in non-photochemical quenching which resembled qE in being fast and flexible fluorescence quenching with rapid recovery, and it occurred in the chlorophyll *a–c* antenna rather than in the phycobiliproteins. Raven (2011) has attempted, with limited success, to model the costs relative to the benefits of xanthophyll cycle-based non-photochemical quenching, while Zaks et al. (2012) have produced a kinetic model of qE. Considering that the model of Zaks et al. (2012) does not explicitly describe the mechanism of qE, and concentrates rather on the outcomes, it is still an excellent contribution to more comprehensive models of non-photochemical quenching (Harbinson 2012).

For some photoprotective mechanisms, it is possible to make reasonably accurate estimates of the resource savings as a result of down-regulation, while in other cases no such estimates are possible. For photochemical quenching, the energy and material costs of producing components specific to the water–water cycles involving the Mehler-Peroxidase reaction and the PTOX (chloroplast oxidase) systems can be calculated, although their down-regulation has no impact other than on photochemical quenching, for example on chlororespiration in the case of PTOX (Cardol et al. 2008; Houille-Vernes et al. 2011). It is not clear what components would be specific to any cyclic electron flow around PSII, with cytochrome b_{559} as a very plausible component. However, mutations (random and site-directed) in the *psbE* and *psbF* genes encoding the subunits of cytochrome b_{559} have effects on PSII, which extend well beyond any role for cytochrome b_{559} in cyclic electron flow around PSII (see Shukla et al. 2006 for recent work and for references). Among the roles for cytochrome b_{559} is the reduction of the two β -carotenes in PSII, which have been oxidised by PSII; the oxidised forms of the carotenoids cannot carry out what is believed to be their major function, i.e. the quenching of singlet oxygen produced from the triplet state of P_{680} (Telfer 2005). Cytochrome b_{559} can also act to reduce superoxide to peroxide, or oxidise it to oxygen, thereby removing superoxide from the neighbourhood of PSII, supplementing superoxide dismutase (Tiwari and Pospisil 2009).

For non-photochemical quenching involving xanthophyll cycles, Raven (1984a, b, 2011) has shown that estimates can be made of the energy and material costs of synthesis of the xanthophyll components and of the epoxidase and de-epoxidase genes, as well as of the energy cost of producing the NADPH used by both the epoxidase and de-epoxidase reactions. However, little is known of the energy costs of the qE mechanism of non-photochemical quenching which does not involve xanthophyll cycles.

If it was possible to down-regulate the photoregulation of excitation energy flux into the PSII, then this would depend on having a very low pH in the thylakoid lumen. This might allow the use of a CF_0CF_1 ATP synthetase in the thylakoid membrane, which has a lower H^+/ATP stoichiometry than occurs in plastids and many cyanobacteria. Thylakoid CF_0CF_1 ATP synthases have a stoichiometry of 4.67 H^+ per ATP found in plastids and many cyanobacteria based on the structural biology of the ATP synthase complex, and rather lower values based on more direct biochemical measurements (Petersen et al. 2012; Pogoryelov et al. 2012), which have been discussed above. The argument here is that, for a given free energy of hydrolysis of ATP in vivo (approximately 55 kJ/mol, Raven 1984b), the larger the free energy difference per mole of photons across the thylakoid membrane, the fewer proteins are needed to phosphorylate one mole of ADP to ATP. It must be remembered that the proton activity gradient is

only one component of the proton free gradient used in ATP synthesis; the other is the electrical potential difference (Raven 1984b; Tang and Wei 2001; Cruz et al. 2005; Falkowski and Raven 2007; Checchetto et al. 2012; Carraretto et al. 2013) and that there must be a sufficient decrease in free energy in the thylakoid reaction generating the proton free energy gradient to allow the production of a larger steady-state proton free energy difference than occurs in photosynthesising plastids with the present H^+/ATP stoichiometry of the thylakoid ATP synthetase.

The discussion above describes the means by which algae minimise the photodamage to the D1 protein and other components of the PSII reaction centre, and the possibility that down-regulation of the protective mechanisms could occur, with resource savings, provided the alga was protected from PAR values in excess of about 0.8 of E_k , which is the PAR at which the extrapolation of the gradient of photosynthetic rate, as a function of PAR at strictly rate-limiting PAR values, meets the extrapolation to the photosynthetic rate axis of the light-saturated rate of photosynthesis. The rationale is that PAR values below about 0.8 of E_k have only the background levels of photodamage to PSII. This corresponds to 1 damaged D1 polypeptide for every 10^7 photons absorbed by the photosynthetic apparatus (Tyystjärvi and Aro 1996; Chow et al. 2005), although the action spectrum suggests that the photochemically active agent is not a characterised pigment of PSII (Takahashi et al. 2010). Above about 0.8 E_k , there is likely to be additional photoinhibition as the rate of excitation energy supply to PSII exceeds the capacity of downstream processes.

Turning to the turnover of other proteins and the possibility that their turnover could be decreased with increased resource allocation to growth, we have seen that protein destruction with synthesis of the same protein species relates to replacement of examples of the protein with the incorrect primary, secondary, tertiary or quaternary structure generated when it was synthesised, or which became damaged by free radicals or UV during its life in the cell. Minimising malformation of proteins at their synthesis requires accuracy of peptide chain elongation and subsequent folding which presumably involves slower and/or more resource-expensive protein synthesis (Raven 2012). Minimising damage during the life of the protein involves control of damaging aspects of the environment, as described for restricting photodamage to PSII reaction centres. An assumption that is usually made in considering the breakdown and replacement of faulty proteins is that the organism can detect, and selectively degrade, the faulty proteins (Raven 2012). Progress is being made in determining how proteases identify photodamaged D1 protein in photoinhibition (Adam et al. 2011; Kley et al. 2011; Kato et al. 2012). If detection of mal-functional proteins is not possible (see Raven 2012), then a blanket proteome-wide breakdown and replacement is the only option: this

would replace fully functional, as well as faulty proteins. Unless such turnover of proteins occurs at least as rapidly as the net synthesis of proteins in cell growth, there would be a steady increase in the fraction of mal-functional proteins in the population of a given protein species in the cell. In reality, the replacement rate is only on the order of 10 % of the net rate of protein synthesis (Quigg and Beardall 2003), strongly suggesting that there is generally a means of selectively destroying, and replacing the mal-functional proteins. Even with selective destruction of mal-functional proteins, it is not clear if it would be possible to decrease the rate of protein turnover of this sort under a given set of environmental conditions without increasing, generation by generation, the fraction of mal-functional proteins in the proteome. Protein destruction with synthesis of a different protein related to a changes in the time in the cell cycle cannot be avoided, while protein destruction with synthesis of a different protein related to changes in the environment (other than those entraining the cell cycle to, for example, diel periodicity) can to varying extents be avoided by control of culture conditions. However, the diel light–dark cycle cannot be avoided if only solar energy is used in drive growth.

In conclusion, there are possibilities of decreasing energy costs related to photoinhibition by decreasing antenna size and regulating photon supply to algal cultures which could allow economies in resource allocation to photoprotection and to damage repair, as well as decreasing the opportunity costs of having lower photosynthetic capacity when photochemistry is down-regulated and/or PSII reaction centres are being repaired (Fig. 1). The biofuel industry can benefit from years of fundamental research that has laid the path for a detailed biochemical understanding of algal cell biology.

Summary and conclusions

There are possibilities for increasing the rate of photon absorption per unit resource used in producing the light-harvesting machinery at photon flux densities similar to full sunlight by decreasing the size of the antenna pigment–protein complexes per reaction centre. This decreases the chance of excitation energy reaching reaction centres in high natural illumination at the expense of decreasing the effectiveness with which low irradiance can be used. For a well-mixed pond culture there could be gains in photon use efficiency, which could be further increased by decreasing resource allocation to photoprotection using non-photochemical and photochemical quenching, and the machinery involved in the repair of damage to PSII, as a result of the decreased potential for photoinhibition. Increasing the wavelength range over which energy can be supplied to reaction centres at the long-wavelength end of the solar spectrum is possible using chlorophyll *d* or, especially, bacteriochlorophylls. This will allow

photochemistry to operate at 1,050 nm using three photons (and three distinct photochemical reactions) to move an electron from water to carbon dioxide rather than two, as a result of the lower (by 1/3) energy per photon at 1,050 nm relative to 700 nm. An alternative, suggested by Blankenship et al. (2011), would be to use PSII plus a PSI re-engineered to use photon out to 1,050 nm; this would require careful management of photons at less than 700 nm where excitation energy could be supplied to both PSII and the re-engineered PSI. This would only require two photons per electron moved from water to carbon dioxide, though with tighter energy margins, and with significant absorption of infrared wavelengths of the water in the photobioreactors or raceways.

Complementary possibilities for increasing light harvesting, and hence conversion of the energy in solar radiation into algal biomass, include the addition of antenna pigment–protein complexes which fill in absorption minima in the spectrum of the existing pigments in algae with chlorophylls *a* plus *b* and (except cryptophytes) with chlorophylls *a* plus *c*, playing similar roles to phycobilins in cyanobacteria, glaucocystophytes, rhodophytes and cryptophytes. The extent to which this would increase the productivity of individual light-limited algae would be greater in smaller cells with less package effect. Another way of filling the green absorption gap of chlorophylls and carotenoids is with energy-transducing (proton-pumping) rhodopsins. However, rhodopsins have lower photon yields and a greater protein cost than chlorophyll-based proton pumps, and the latter can also generate redox products. However, the rhodopsin proton pumps may be expressed at membranes other than the thylakoid membrane, and they have no direct iron cost, as opposed to the high iron cost of proton pumping in photosynthesis (although iron deficiency is unlikely to be a problem in algal biofuel production). There are also possibilities in using the ‘flicker effect’, in which temporal variations in PAR availability can, within strict limits of frequency and mean irradiance, increase algal productivity.

Downstream of the light-driven driven proton pumps in the thylakoid membrane is the proton-driven ATP synthetase. The cyanobacterial and chloroplast ATP synthetases have the lowest known ATP:proton stoichiometry of any such proton-coupled ATP synthetase and there seems to be scope for decreasing the energetic (ultimately photon) cost of ATP synthesis by increasing the ATP:proton ratio. However, care must be taken to avoid disturbing processes dependent on the absolute value of the intra-thylakoid pH (such as non-photochemical quenching, PSII repair cycle) by increasing the electrical component of the trans-thylakoid proton electrochemical gradient.

Most of the NADPH and ATP produced by thylakoid reactions are used in autotrophic carbon dioxide assimilation. All oxygenic photosynthetic organisms use the Rubisco–Benson–Calvin cycle. While all six known autotrophic

pathways converting carbon dioxide into carbohydrate use 2 NAD(P)H per carbon dioxide assimilated under carboxylase-saturating carbon dioxide concentrations, the Rubisco–Benson–Calvin cycle has the joint highest ATP cost of 3 ATP per carbon dioxide. Furthermore, there is a widespread and probably true perception that the protein cost of producing sufficient quantities of the Rubisco–Benson–Calvin cycle enzymes to achieve a certain rate of carbon dioxide assimilation, is largely as a result of the properties of Rubisco. This higher protein cost, and also higher ATP and NADPH cost, is clearly seen for the Rubisco–Benson–Calvin cycle when sub-saturating carbon dioxide concentrations are used in the presence of oxygen, regardless of whether a photorespiratory carbon oxidation cycle is used alone, or is suppressed to varying extents by a carbon dioxide concentrating mechanism, the protein capital costs and the NADPH and ATP running costs of the Rubisco–Benson–Calvin cycle are higher than for any of the other five pathways for which sufficient data are available. Thought has been given to engineering one of the other pathways into oxygenic photosynthetic organisms to decrease the protein, ATP and NADPH cost of carbon dioxide assimilation. The 3-hydroxypropionate pathway is the most promising, as it has both a high carbon dioxide affinity and lacks irreversible damage by oxygen. This substitution could decrease the capital and running cost of autotrophic carbon dioxide assimilation by about a third compared with the Rubisco–Benson–Calvin cycle operating in air levels of carbon dioxide and oxygen with small variations depending on whether or not a CCM is operating in series with the Rubisco–Benson–Calvin cycle. There are some possibilities of limiting ‘wasteful’ aspects of dark respiration used in growth and maintenance. Perhaps less readily approached is decreasing that component of the loss of dissolved organic carbon compounds with no known function, largely because the mechanism of loss from the cells is poorly understood.

Protein breakdown and resynthesis in microalgae has an energy cost equivalent to a third of the energy transformation occurring in respiration during the dark phase of a diel cycle. This protein turnover and associated energy costs were determined for cells cultured under constant temperature and with continuous illumination, so cannot be related to changes in the proteome (breakdown of one set of proteins and synthesis of another) as a function of changed environmental conditions. Even under constant conditions there is protein breakdown and the synthesis of another set of proteins related to progress through the cell cycle, and possibly also other endogenously regulated protein changes. Under diel light–dark alternations there are also diel periodicities in the synthesis of particular proteins, although this does not necessarily mean breakdown of other protein species. A further rationale for protein turnover is the replacement of damaged proteins. This can be a major cause of protein turnover in diazotrophs in aerobic environments, but diazotrophs are not of obvious interest as

algal biofuel feedstock. Replacement of photodamaged D1 protein (psbA gene product) may account for up to 5 % of protein turnover in microalgae, and the previously discussed decrease in PSII antenna size should help to decrease the damage, and hence the need for repair. Cell cycle and other endogenous cycles, and repair of photodamage to PSII, do not seem to account for the entire observed protein turnover. More work is needed to determine the nature of this protein turnover and hence the possibility that it could be decreased without having adverse effects on the algae, therefore enhancing the energy yield.

Many of the suggestions for maximising the rate and/or efficiency of individual processes contributing to algal biomass accumulation, and minimising losses from previously produced biomass (minimising waste), are technically very difficult, if not impossible to undertake for eukaryotic algae with our present state of knowledge. Even if the individual processes could be changed in the ways suggested, there is still the question of how the changed processes would operate in the cell. Would optimal allocation of resources among the various processes immediately occur or, in the absence of lengthy (hundreds of generations) natural selection to provide a graded approach to optimal allocation, would a further level of intervention using systems biology approaches (Noble 2006) to direct resource allocation among processes be necessary?

Finally, we reiterate that the interventions suggested here are more readily applied to relative small-scale cultures where more control of the environment is possible than in very large-scale outdoor mass cultures where less control is possible.

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