Anaerobic energy metabolism in unicellular photosynthetic eukaryotes

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1. Introduction: life without oxygen

Life without oxygen is common on Earth. Oxygen shortage in biotopes can result from geochemical or physical circumstances but also from bacterial activities. Anoxia can be transient or protracted, extending from diurnal periods to months or years or millennia or more. Examples of diurnal anoxia include tidal zones [1,2], while marine oxygen minimum zones can remain anoxic for many years [3,4], and seafloor brines [5] can remain anoxic for 50,000 years or more [6]; all of these habitats harbor rich eukaryotic floras. Survival in anoxic habitats requires means of anaerobic energy metabolism, which are far more varied in prokaryotes [7] than in eukaryotes [8]. This chapter will focus mainly on photosynthetic eukaryotes which abound in anoxic environments [4,9], anaerobic energy metabolism in heterotrophic eukaryotes having been reviewed in depth recently [8].

Whether in aerobic or anaerobic environments, the challenge of staying alive means staying far from equilibrium, and that means maintaining metabolic flux and redox balance. In anaerobic energy metabolism, just as in aerobic metabolism, the generation of ATP entails the accumulation of reduced cofactors such as NADH and FADH₂ that are generated by catabolic pathways, usually glycolysis, and that have to be reoxidized in a process that involves the transfer of electrons to suitable acceptors that are then excreted by the organism to sustain metabolic flux. Among eukaryotes, anaerobic photosynthesis has not been described, hence for eukaryotes, only two processes are known to maintain redox balance and conserve energy under anoxia: i) fermentations, which usually entail substrate level phosphorylation (SLP), and ii) anaerobic respiration involving chemiosmotic coupling. In eukaryote respiration, electrons, usually stemming from carbohydrates and lipids but sometimes from sulfide [10,11], enter via NADH and FADH₂, the mitochondrial electron transport chain, which is comprised of membrane proteins, the prosthetic groups of which harbor increasingly positive potentials in the course of electron flux towards the terminal acceptor. Electron flux serves to establish an electrochemical gradient across the inner mitochondrial membrane, resulting in a proton motive force that drives ATP

Abbreviations: ACS, acetate CoA synthetase; ACK, acetate kinase; ADHE, aldehyde/alcohol dehydrogenase; ASCT, acetate: succinate CoA-transferase; ATP, adenosine 5’-triphosphate; FTA, phosphotransacetylase; PFL, pyruvate formate-lyase; PFL-AE, pyruvate formate-lyase activating enzyme; PFO, pyruvate:ferredoxin oxidoreductase; SLP, substrate level phosphorylation

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synthesis. In anaerobic mitochondria, the terminal acceptor is not O₂, but is often fumarate [12]. Fermentations are processes in which the terminal electron acceptor is generated by the cell through metabolism. ATP is generated via SLP, where an intermediate contains a high energy phosphate bond (usually a mixed anhydride) which is used to phosphorylate ADP. SLP occurs with the help of soluble, rather than membrane-associated, enzymes. In eukaryotes, some fermentation pathways entail the harnessing of chemiosmotic energy, for example the reduction of endogenously generated fumarate to succinate in the respiratory chain of some anaerobic mitochondria [8,13]. Overall, respiration and fermentation differ with respect to the enzymes employed for cofactor reoxidation and the energy conservation processes involved.

The biochemical anaerobes has been far more extensively studied in prokaryotes than in eukaryotes, probably because most of what we know about eukaryotic energy metabolism stems from studies of multicellular organisms, the majority of which are specialized towards oxygen as the terminal acceptor. Most multicellular eukaryotes such as animals and plants can only withstand short periods of anoxia. A few metazoans which withstand longer periods of anoxia [1,8,12] or even total anoxia do exist, as the recently reported marine sediment-dwelling animals Loriciferae [6]. Most of the metabolic diversity in eukaryotes is found among unicellular species (protozoa), among them parasites (many of which live in oxygen deprived environments) and photosynthetic organisms (microalgae), which frequently encounter hypoxic or anoxic conditions in their natural environments, e.g. soils, microbial mats, or marine and freshwater sediments.

Much effort has been devoted to the study of parasites with the goal of identifying targets to develop specific treatments, and a comprehensive review on the anaerobic pathways in heterotrophic eukaryotes has been recently published [8]. Compared to medically relevant parasites, our understanding of anaerobic energy metabolism in photosynthetic eukaryotes has always been more limited. This circumstance has changed somewhat over the last ten years, with the growing interest in microalgae for biotechnological applications, for example the production of biofuels such ashydrogen and hydrocarbons [14–18]. Here we provide an update on the knowledge on the anaerobic metabolic routes occurring in unicellular green algae. The significance/relevance of the compartmentalization for some anaerobic pathways is considered as the evolutionary origin of these pathways and the impact of phototrophy origins for anaerobic pathways.

2. Microalgae in anaerobic environments

For photosynthetic algae anoxia is commonplace and often transient. The oxygenation of their habitats, such as soils, fresh and marine waters, is intrinsically linked to light intensity, the depth of the waters, and to biotic activities. Low light can help to bring the cells to hypoxia when O₂ production in plastids is outpaced by O₂ consumption. Algal blooms also destabilize the ecosystems by attenuation of light and oxygen levels.

The metabolic responses of microalgae to anoxia have been the subject of examination for some time. Green algae, such as *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Chlorogonium elongatum*, *Chlorella fusca*, and *Scenedesmus D3*, ferment their plastidic starch to a variety of end products including acetate, ethanol, formate, glycerol, lactate, H₂ and CO₂ [19–23]. The algal heterofermentation patterns observed clearly contrast with the lactate or ethanol homofermentation of yeasts and multicellular organisms (plants, animals), while showing some similarities to the mixed-acid fermentations common to enteric bacteria [24] that lead to the excretion of partially oxidized metabolites [25].

Typical for mixed-acid fermentations, the distribution of excreted end products varies among the green algal species (and sometimes among strains) and furthermore varies with environmental factors, medium composition and carbon source. In *C. reinhardtii*, dark fermentation leads to the production of formate, acetate and ethanol in a 2:1:1 ratio [22,26]. In contrast, *C. moewusii* produced no formate under dark anoxia; the major excreted products in these conditions were acetate, glycerol and ethanol, and their relative amounts appear to diverge between studies [21,27]. During fermentation, some green algae produce hydrogen. H₂ evolution in the dark is low as compared to that measured in the light [19,26]. The amount of H₂ produced appears to be species-specific [22,27,28]. Skjanes et al. [29] have identified three green algal strains from brackish waters and marine habitats (*Chlamydomonas euryale*, *Chlamydomonas vectensis*, cf *Oocystis*) capable of producing H₂ anaerobically, indicating that this ability is not restricted to freshwater species.

Rather than excreting the fermentation end-products of the anaerobic metabolism, some algae accumulate them. Under anaerobiosis, various *Euglena gracilis* species accumulate large amounts of wax esters (C₁₀–C₁₈ fatty acids esterified with C₁₀–C₁₈ aliphatic alcohols), with a simultaneous net production of ATP [30]. The fatty acids are synthesized in the mitochondrion, acetyl-CoA serving as the terminal electron acceptor during carbohydrate oxidation. The wax esters are not excreted but are deposited in the cytosol instead, where they accumulate, constituting up to 65% of the protist’s dry weight in some strains [31]. Upon return to oxic conditions, the aliphatic chains can be converted once again to acetyl-CoA, which can be oxidized to CO₂ in mitochondria or used to form paramylon reserves. Wax ester fermentation occurs in the mitochondria as a malonyl-CoA independent synthesis of fatty acids [32]. In acid mine drainage biofilms mainly constituted by *Euglena mutabilis*, large amounts of wax esters have been detected [33].

Diatoms and dinoflagellates can survive in dark anoxic marine sediments after sinking [34]. The sedimented diatoms were found to contain high concentrations of nitrate that they accumulated from sea water [35]. Recently, Kamp et al. [36] showed that the levels of accumulated nitrate and the diatom survival under dark/anoxic conditions are correlated. The consumption of the intracellular nitrate pool was furthermore correlated with excretion of ammonium, in both benthic and pelagic species, suggesting that diatoms might obtain the energy for cell survival by ammonium fermentation, as described for the fungus *Fusarium oxysporum* [37].

Importantly, environmental sequencing has uncovered vast eukaryotic communities in anaerobic environments. Sequence analysis of both nuclear (18S) and plastid (16S) ribosomal RNA clones revealed a great variety of phytoplankton in marine (oxic and anoxic) samples [4,9,38–42] and in freshwaters [43]. Most microalgae newly identified in coastal and open-sea samples belong to known phylogenetic groups, such as prasinophytes, haptophytes, dinoflagellates, cryptophytes, and diatoms [4,42,44–46]. Photosynthetic algae have also been discovered in a variety of chemically extreme environments. In natural rock acid drainages and acid mine drainages, characterized by extreme acidity (pH –2–3) and high heavy metal concentrations, large eukaryote communities have been discovered in biofilms. Among these eukaryotes, a surprising assortment of phototrophic species was found, including chlorophytes (*Chlamydomonas*, *Chlorella*, *Dunalieila*), rhodophytes (*Galeria*), euglenozoans and diatoms (*Navicula, Nitschzia*) [47,48].

3. Enzymes of fermentation and anaerobic respiration in eukaryotes

Especially among heterotrophs, glycolysis is the backbone of eukaryote carbon and energy metabolism, leading to the production of pyruvate, ATP and NADH. The further fate of pyruvate can take place not only in the cytosol and the mitochondria, but also in plastids. In eukaryotes, the most common cytosolic fermentation processes to regenerate NAD⁺ emanate from pyruvate, involving either
ethanol fermentations via pyruvate decarboxylase (PDC, EC 4.1.1.1) and alcohol dehydrogenase (ADH, EC 1.1.1.1) (typical of plants, certain animals and fungi), or lactic fermentations via lactate dehydrogenase (LDH, EC 1.1.1.27) (typical of many eukaryotes, including mammalian muscle). Alcohol and lactate fermentations are typical pathways for dealing with short term anoxia, but some fish, carp for example, can survive weeks in anoxia through ethanol fermentation [49,50]. Among protists, pyruvate metabolism is typically more diversified, including additional anaerobic pathways and enzymes that are not present in higher animals. These pathways often localize to mitochondria or hydrogenosomes, anaerobic forms of mitochondria that lack cytochromes and produce ATP fermentatively [8]. In algae, a plastidic location for fermentative metabolism is also clearly invoked, to which several recent studies bear witness [51,52].

In addition to simple fermentation processes, some facultative anaerobic eukaryotes exhibit fermentation processes linked to the mitochondrial electron transport chain, thereby generating a proton gradient across the membrane [8]. This process is often referred to as anaerobic respiration although the electron acceptor (fumarate) is not obtained from the environment but from the cells' metabolism.

The range of ATP yields from glucose breakdown in eukaryotes is modest [8]. The overall energetic differences between mitochondria using oxygen as a terminal acceptor, or not, boil down to about a factor of six. Taking into account the costs of ADP–ATP exchange and phosphate transport across the mitochondrial inner membrane, it can be calculated that the complete oxidation of glucose via mammalian mitochondria yields ~30 molecules ATP [53,54]. Eukaryotes with anaerobic mitochondria gain, in addition to 2 ATP from glycolysis, 2 ATP per glucose through substrate level phosphorylations leading to acetate and propionate production and about 1 additional ATP per glucose from proton pumping at complex I using fumarate as the terminal acceptor, making their overall energy yield about 5 ATP/glucose [8,12,55]. Protists that contain hydrogenosomes, anaerobic forms of mitochondria [56] best studied in the human parasite Trichomonas vaginalis [57], obtain about 4 ATP per glucose: 2 from glycolysis and 1 ATP per pyruvate, which is fermented to H2, CO2, and acetate [8,58].

Eukaryotes such as Giardia intestinalis [59] that possess mitosomes – highly reduced forms of mitochondria with no direct role in ATP synthesis [60,61] – satisfy their energy needs from cytosolic fermentations that deliver 2 ATP per glucose from glycolysis and somewhere between 0 and 1 additional ATP per pyruvate, depending upon environmental conditions. Although Giardia does not grow in the presence of atmospheric oxygen levels, low levels of oxygen allows Giardia to maintain redox balance with the help of cytosolic NADH oxidases that do not conserve energy but allow it to metabolize pyruvate to acetate, which yields one ATP per pyruvate [8]. If no oxygen is available, they rely on pyruvate metabolism to maintain redox balance, excreting ethanol with no additional ATP gain. Thus, eukaryotes with mitosomes obtain 2–4 ATP/glucose, depending on the environmental conditions [8]. Pure ethanol fermentations that entail no energy conservation from pyruvate metabolism, such as in yeast, which can ferment indefinitely, or carp, which can survive months of complete anoxia [50], deliver 2 ATP/glucose. From this, it is evident that differences among eukaryotes with respect to energy yield, end products and underlying enzymes concern the metabolic fate of pyruvate. It should also be mentioned that some eukaryotes have no bona fide energy metabolism at all, namely the energy parasites, such as some microsporidians, that steal ATP from their host cells via ADP/ATP carriers in their plasma membrane [62].

3.1. Conversion of pyruvate into acetyl-CoA by PFO and PFL

In typical aerobic mitochondria, the conversion of pyruvate into acetyl-CoA is catalyzed by pyruvate dehydrogenase (PDH), a multi-subunit complex, and the resulting acetyl-CoA is typically oxidized to CO2 through the citric acid (TCA) cycle, yielding ATP, NADH and FADH2. PDH can also function in some anaerobic mitochondria, for example during malate dismutation in Fasciola (liver fluke) mitochondria [12,13] or during wax ester fermentation in Euglena [31,63]. More typical of many prokaryotic and eukaryotic anaerobes is pyruvate oxidation via pyruvate:ferredoxin oxidoreductase (abbreviated PFO or PFOR, sometimes PFR; E.C. 1.2.7.1) generating CO2, acetyl-CoA and two electrons. These two electrons are then transferred via three [4Fe4S] clusters to the final electron acceptor, either a ferredoxin (FDX) or a flavodoxin [64]. Like PDH, PFO belongs to a large family of enzymes which depend on the cofactor thiamine pyrophosphate (TPP) for the cleavage of the carbon–carbon bond linking the carbonyl and carboxyl groups of pyruvate [65]. In some anaerobic autotrophic prokaryotes though PFO functions in the reverse direction, i.e. as pyruvate synthase [66]. Except for the PFOs from Desulfovibrio species [67], all the other PFOs studied so far are readily inactivated by oxygen. PFO activity in eukaryotes was first discovered in the hydrogenosomes – H2-producing forms of mitochondria – of trichomonads [68]. Subsequent studies have found PFO in anaerobic parasites from distinct lineages [69,70]. PFO has been extensively studied because it is the target for metronidazole, the current treatment against many anaerobic protistan parasites of humans [71].

An alternative form of PFO has been described in eukaryotes which is a fusion protein consisting of a PFO domain at its N-terminus and an NADPH–cytochrome P450 reductase domain at its C-terminus [72,73]. This pyruvate:NADP+ oxidoreductase (PNO) de-carboxylates pyruvate to acetyl-CoA while transferring electrons to the flavoprotein domain to reduce NADP+. PNO was first described in E. gracilis [74] and has since been found in apicomplexan parasites [72] and various eukaryotic heterotrophic lineages [70,73,75]. In Euglena, the enzyme localizes to mitochondria [74] whereas in the heterotrophic stramenopile Blastocystis, PNO is found in the organism’s anaerobic mitochondrion [73].

In addition to PFO, there is another route of pyruvate breakdown to acetyl-CoA that operates in various prokaryotes and a few eukaryotic lineages, that is pyruvate formate-lyase (PFL; EC 2.3.1.54) [76–78]. PFL uses a radical-based homolytic mechanism to convert pyruvate into acetyl-CoA and formate [79]. PFL is activated post-translationally by the introduction of a free-radical, a reaction catalyzed by an iron-dependent activating enzyme (PFL-AE; EC 1.97.1.4) which requires the cofactor S-adenosyl methionine (SAM) as well as a reduced ferredoxin (or flavodoxin) [79–81]. PFL is irreversibly inactivated by hypophosphite, a formate analogue [82]. Like PFO, PFL is extremely sensitive to oxygen. The radical–containing enzyme is irreversibly inactivated by oxygen via oxygenolytic cleavage at the glycine radical [79]. In some bacteria such as Escherichia coli species the iron-dependent aldehyde/alcohol dehydrogenase ADHE plays the role of PFL-deactivase by removing the radical on PFL [83]. Based upon current sampling, it appears that PFL is more widespread than PFO/PNO in photosynthetic protists, in contrast to heterotrophic protist in which PFL is less common than PFO [8].

3.2. Iron-only hydrogenase

Hydrogenases function to dispose of excess electrons accumulated during fermentation or to extract electrons from hydrogen for the reduction of substrates in energy-yielding processes. Different classes of hydrogenases have been identified based on their metal content (FeFe, NiFe, NiFeSe, FeS-free) [84–86]. Hydrogenases are very widespread among prokaryotes while among eukaryotes, they have so far only been found in unicellular species, including parasites and photosynthetic algae [8,28]. All eukaryotic hydrogenases characterized so far belong to the class of Fe-only ([FeFe]) hydrogenases in which the [4Fe4S] cluster is linked through a cysteine residue to a [2Fe2S] cluster [87,88]. In most H2-producing heterotrophic eukaryotes studied to date, the electrons arising from pyruvate oxidation
by PFO are transferred to a ferredoxin, and subsequently to hydrogenase [8].

Even though it is not known yet to occur among eukaryotes, there is a new and exciting aspect to [FeFe] hydrogenase function in prokaryotes that deserves mention: electron bifurcation. Schut and Adams [89] found that the [FeFe]-hydrogenase of Thermotoga maritima generates H₂ with one electron coming from reduced ferredoxin via a [2Fe2S] cluster containing subunit and one electron coming from NADH via a flavoprotein subunit. This allows Thermotoga to generate H₂ in part from NADH, which is a thermodynamically unfavorable reaction; but by coupling it to the oxidation of a low potential reduced ferredoxin, the overall reaction (H₂ production) becomes thermodynamically favorable and goes forward. This is potentially relevant for eukaryotic [FeFe] hydrogenase, because the corresponding [2Fe2S] cluster-containing and flavoprotein subunits are found in eukaryotes and have furthermore been found in association with the [FeFe] hydrogenase catalytic subunit [8]. Electron bifurcation in eukaryotes and have furthermore been found in association with the [FeFe] hydrogenase catalytic subunit [8]. Electron bifurcation is being found in many prokaryotic anaerobes (reviewed by Buckel and Thauer in this volume), and it is possible that it occurs in eukaryotes as well, with the reaction catalyzed by [FeFe] hydrogenase being a candidate.

3.3. Aldehyde/alcohol dehydrogenase (ADHE)

Acetyl-CoA produced from pyruvate (either by PDH, PFL or PFO) can be used to generate ATP from ADP by conversion to acetate (see below) or to maintain redox balance by conversion to ethanol. In bacterial mixed-acid fermentations, ethanol is typically produced via bifunctional aldehyde/alcohol dehydrogenase (ADHE or ADH1) [25]. This enzyme combines a coenzyme A-dependent acetaldehyde dehydrogenase (N-terminal half) and a Fe-dependent alcohol dehydrogenase (C-terminal half). ADHE is a common enzyme among bacteria where it plays a key role under anaerobic conditions by regenerating reducing power and CoASH [83,91,92]. Among eukaryotes, the protein has been found in anaerobic parasites such as Giardia [93], Entamoeba [94] and in a non-parasitic rumen inhabitant, the chytridiomycete Promyces sp. E2 [95] where it participates in core carbon metabolism. An ADHE has also been identified in two evolutionary related unicellular algae, the green alga C. reinhardtii and its colorless relative Polytomella sp. [96,97].

3.4. Substrate-level phosphorylation (SLP)

In substrate level phosphorylations, a phosphate group in a high energy bond is harnessed to phosphorylate ADP via soluble enzymes without the help of electrochemical gradients. In eukaryotes, acetyl-CoA produced by either PFL or PFO (see above) can be converted into acetate to produce ATP via substrate level phosphorylation; at present, three different routes for this SLP are known [98]. The first route is the ASCT cycle, which involves two enzymes: an acetate:succinate CoA-transferase (ASCT; EC 2.8.3.8) which transfers the CoA moiety of acetyl-CoA to succinate, and a succinyl-CoA synthetase (SCS or STK; EC 6.2.1.5) which converts succinyl-CoA back into succinate. The ASCT reaction converts one thioester into another. In the SCS reaction, the energy in the thioester bond of succinyl-CoA is conserved via phosphorylation to generate succinyl phosphate as a reaction intermediate, the mixed anhydride bond of which directly phosphorylates ADP to ATP [99]. The ACST reaction can be catalyzed by three different types of ASCT proteins (ASCT subfamilies 1A, 1B, 1C) which share little sequence homology [98]. The ASCT cycle is present in some hydrogenosome-containing eukaryotes and in anaerobic mitochondria [8]. In the second route, concomitant production of acetate and ATP from anaerobically produced acetyl-CoA is catalyzed by a single enzyme, the ADP-forming acetyl-CoA synthetase (ADP)-ACS. Among eukaryotes, this enzyme has been characterized in Entamoeba [100] and in Giardia [101]. A third route of anaerobic production of ATP from acetyl-CoA involves the sequential action of phosphotransacylase (PTA or PAT; EC 2.3.1.8) and acetate kinase (ACK or AK; EC 2.7.2.15). The ACK-PTA pathway, well-known among prokaryotes [25], has so far only been found in the photosynthetic alga C. reinhardtii [97,102]. Of these three SLP pathways, which conserve the energy in the thioester bond of acetyl-CoA, the ASCT cycle involving the Krebs cycle enzyme succinyl-CoA synthetase, appears to be the most common among heterotrophic eukaryotes studied so far.

3.5. Anaerobic respiration

Respiration is the use of a terminal electron acceptor that is obtained from the environment. The respiratory process involves the passage of electrons through an electron transport chain, creating an ion gradient (usually a proton gradient) across the membrane that is further used for ATP synthesis and transport processes. Anaerobic respiration is common place among prokaryotes, which can use a great diversity of environmentally available terminal acceptors for ATP synthesis and which respire across their plasma membrane, eukaryotes across their inner mitochondrial membrane [103]. Anaerobic respiration appears to be comparatively rare in eukaryotes, based on present sampling [12]. Malate dmutation is a process that allows some multicellular eukaryotes such as lower marine animals (mussels, oysters) and parasitic helminths (Ascaris suum, Fasciola hepatica) to survive in anaerobic environments [12]. In this process, glucose is degraded to phosphoenolpyruvate which is subsequently reduced to malate by cytosolic malate dehydrogenase, thereby reoxidizing the glycolytic NADH. The produced malate is further metabolized inside mitochondria. To maintain redox balance, some of the malate is oxidized and some is reduced (dismutation). Malate oxidation to acetyl-CoA occurs via malic enzyme (ME) and PDH. The CoA group from the acetyl-CoA is then transferred to succinate by an acetate:succinate CoA transferase, yielding acetate and succinyl-CoA. Regeneration of CoASH allows ATP production through SLP via the ASCT cycle (see above). Malate reduction to succinate occurs in two steps that reverse part of the Krebs cycle, with fumarate as intermediate. Fumarate reduction which involves a membrane-bound fumarate reductase and a quinone – typically a rhodoquinone – is also linked to the mitochondrial electron transport chain via complex I and Fₒ-F₁-ATP synthase [104]. Rhodoquinone is a quinone essential for fumarate reduction as it occurs in some bacteria (such as Rhodospirillum rubrum), and it is also essential for fumarate reduction during malate dmutation in eukaryotic species that possess anaerobic mitochondria. The pathway of rhodoquinone synthesis, however, appears to be different in R. rubrum compared to eukaryotes as in R. rubrum rhodoquinone synthesis proceeds from ubiquinone, whereas this is not the case in eukaryotes [105–108], and A.G.M.T., unpublished observations]. Compared to fermentation, malate dmutation conserves more energy out of the same substrate (phosphoenolpyruvate).

4. Chlamydomonas is the most flexible eukaryotic fermenter so far

C. reinhardtii is a unicellular green alga found in freshwaters and wet soils around the globe. This alga has become a model organism to study photosynthesis and chloroplast biogenesis [109,110], nutrient deprivation [111–115] as well as assembly and function of flagella [116–118]. The ability of C. reinhardtii to survive under anoxia has long been known [22,26], with interest renewed following the release of its nuclear genome sequence in 2007 [119]. Metabolic pathways predicted from physiological studies [22] could be confirmed while unexpected routes were also uncovered [97,120,121]. Among the surprises was the extended array of routes for pyruvate stemming from starch breakdown (Fig. 1). In Chlamydomonas, pyruvate can be
metabolized to ethanol via a PDC–ADH pathway or to lactate via LDH. But pyruvate can also be converted into acetyl-CoA by a pyruvate formate-lyase or a pyruvate:ferredoxin oxidoreductase. The existence of a PFL in C. reinhardtii was proposed earlier based on the inhibition of its formate production by sodium hypophosphite [22]. In contrast, the occurrence of a PFO had not been predicted from experimental approaches.

Microarray studies and RT-PCR have been largely used to unravel the metabolic routes used by the alga to adapt to anoxic atmosphere [122–124]. Although these studies have pinpointed various genes, among them genes for fermentative enzymes, it quickly became clear that there was no strict correlation between transcript levels and protein levels or enzyme activity [123,125]. Furthermore, most of these RNA analyses were carried out on cells experiencing anaoxia in a medium which contains solely potassium phosphate and magnesium chloride (AIB medium) [126]. The cells resuspended in this medium also face nutrient and carbon source deprivation.

Current approaches to understanding the anaerobic metabolism of *Chlamydomonas* entail a multi-faceted approach: i) the study of cells exposed to different anoxic conditions (light vs. darkness, cell resuspension medium), ii) the production of mutant strains deficient in fermentative enzymes, iii) enzymatic characterization of these enzymes, iv) mass spectrometry analyses and v) studies to determine the intracellular localization of the fermentative enzymes, as most of them exhibit an extended N-terminus as compared to their bacterial counterparts, suggesting organellar (chloroplast or mitochondrial) localization.

cDNAs for PFL and its activating enzyme PFL–AE have been sequenced, revealing the relationship to bacterial enzymes, including to the well-studied enzyme of *E. coli* [97,123]. When complemented with the *C. reinhardtii* PFL, *E. coli* cells deficient in pfl were shown to produce formate, demonstrating that the algal enzyme was synthesized and post-translationally activated in *E. coli* [123]. Algal strains deficient in PFLs have been recently produced by insertional mutagenesis in two different research laboratories [52,127]. The lack of PFL activity in these mutants led to a rerouting of dark anaerobic metabolism, with an increased production of lactate, ethanol and CO2 and a lowered production of acetate [52,127]. Immunoblot experiments and mass spectrometry data have provided evidence for the dual localization of the PFL in the chloroplast and mitochondrion [97,102] (Fig. 1). It remains to be ascertained that the enzyme is indeed active in both cell compartments, as the intracellular localization of PFL–AE is currently unknown.

A gene encoding a PFO that is homologous to PFO from other eukaryotes was detected in the *Chlamydomonas* genome [97,121]. *C. reinhardtii* PFO expressed in *E. coli* catalyzes the oxidative decarboxylation of pyruvate, in a CoASH- and TPP-dependent manner; this activity is highly sensitive to oxygen (van Lis et al. in preparation) (Fig. 1). The multiplicity of the metabolic routes that can be used when exposed to dark anaoxia has been revealed by physiological and biochemical studies on wild-type and mutant strains. For most enzymes, subcellular localization was based on previous studies [51,97,102,124,169]. The mitochondrial localization of the fumarate reductase (FRD) is based on targeting prediction by PredAlgo [170]. Although the existence of a PFL in the alga seems to be redox balance in the absence of O2, *C. reinhardtii* possesses two [FeFe] hydrogenases, HYDA1 and HYDA2, which both exhibit the minimal structure for [FeFe] hydrogenases [131–134]. HYDA1 and HYDA2 localize to the chloroplast, where they can independently catalyze H2 production in the light and in the dark [135]. In light, HYDA1 is coupled to the reducing site of the photosynthetic electron transport chain (PSII or NADH:Q oxidoreductase) and accepts electrons from FDX1 (perF) [136–138]. From the analysis of the interaction between FDX and HYDA1, Winkler et al. [139] concluded that among the six known chloroplast [2Fe2S] ferredoxins (FDX1–FDX6), FDX1 appears as the most efficient electron mediator to hydrogenase(s). It is thought that in *C. reinhardtii* PFO is the source of electrons to hydrogenases in the dark, as in the case of heterotrophic unicellular eukaryotes [8]. Studies of the dark fermentation products excreted by a *C. reinhardtii* mutant strain deficient in hydrogenase activity, obtained by disruption of the HydEF gene that encodes a hydrogenase maturation protein, revealed an increased production of succinate [124]. In the algal several metabolic routes can potentially lead to the anaerobic production of this organic acid [124], including a pathway common among heterotrophic eukaryotes and that involves a phosphoenolpyruvate carboxykinase (PCK), a malate dehydrogenase (MDH), a fumarate hydratase (FUM) and a soluble fumarate reductase (FRD) [8] (Fig. 1).

Acetyl-CoA produced by PFO or PFL can be used by either ADHE, allowing the reoxidation of glycolytic NADH, or by the consecutive action of a PTA and an ACK, to generate ATP. Together with formate and acetate, ethanol is one of the major products excreted by *C. reinhardtii* exposed to dark anaoxia [22,23,26,122]. The existence of a Coa-acetylating aldehyde dehydrogenase activity in the photosynthetic alga, similar to that found in facultative bacteria or parasites, was suggested by earlier studies [129] and subsequently confirmed by the isolation of its cDNA [97]. When exposed to dark anaerobiosis in AIB medium, an *Adhe* insertion mutation does not excrete ethanol [140], suggesting that in these conditions, ADHE is the main enzyme involved in ethanol production, as in bacterial mixed-acid fermentations. Comparative proteomic studies [102] and immunoblot experiments [R. van Lis, unpublished results] support a chloroplast localization of this enzyme.

*Chlamydomonas* possesses two sets of genes for phosphotransacetylase and acetate kinase, likely resulting from gene duplication events. These PTA–ACK pathways could participate in anaerobic ATP synthesis when cellular energy demands increase, but they could also be involved in acetate assimilation. From proteomic analysis of organelle-enriched samples, it was inferred that PTA1–ACK2 constitutes a mitochondrial route while PTA2–ACK1 constitutes a chloroplast pathway [97,102] (Fig. 1).
These alternative routes are depicted in Fig. 1. Alanine remained as a metabolite, which is very common in anaerobic metabolism among animals [12], while the other fermentation products are excreted. Progress on *Chlamydomonas* fermentative pathways and metabolic plasticity continues, but the metabolic switches and the interplay between the multiple metabolic routes are yet obscure.

5. Genome survey/metabolic data of the anaerobic metabolic routes found in photosynthetic algae

Eukaryotic photosynthesis arose more than one billion years ago when a heterotrophic host engulfed a cyanobacterial ancestor [141,142]. Following this primary endosymbiotic event, the
cyanobacterium underwent genome reduction and genetic integration within the host genome via gene transfer to the nucleus [143]. Once the endosymbiont was integrated within its host, three major lineages diverged, the green algae (and their land plant relatives), red algae, and glaucophytes [144]. Subsequent rounds of secondary endosymbioses took place, in which red or green algae were engulfed and retained by eukaryote hosts thereby transferring photosynthesis to other eukaryotic lineages. Many algal lineages have acquired their plastids thus through secondary and tertiary endosymbioses [141,145]. At present seven distinct eukaryotic groups that harbor plastids derived from secondary endosymbiosis have been identified: euglenophytes, chlorarachniophytes, haptoophytes, stramenopiles, cryptophytes, dinoflagellates and apicomplexans [145] (Fig. 2).

Experimental studies conducted to reveal the anaerobic potentialities of unicellular eukaryotic phototrophs are so far limited, mainly carried out on green algae (chlorophytes), such as *Chlamydomonas* and *Chlorella*. Another approach to learn about the anaerobic metabolic abilities of these algae is via surveys of genome sequences. At the time of writing, 17 algal genome sequences were publicly available (see Table 1). These genomes are of species that can be cultured in the laboratory, but which are not necessarily the most representative, especially with regard to anaerobic metabolism. Nevertheless, as detailed below, this algal genome sample brings a new light on the extent and diversity of the anaerobic energy metabolism among photosynthetic algae.

5.1. PFL/PFL-AE system

Hyophosphite inhibition of anaerobic formate production, indicating PFO pathways, had been reported earlier for various green species such as *C. reinhardtii*, *Chlorella vulgaris*, *Chlorogonium* and *Scenedesmus* [22,146]. The genome survey indicates that the PFL/PFL-AE system is widespread among the chlorophytes, with the identification of PFL/PFL-AE genes in *Chlamydomonas*, *Trebouxia* (Chlorella NC64) and in Prasinophyceae (*Ostreococcus, Micromonas*), marine algae characterized by a compact genome and a minimal cellular organization [147–149] (Table 1; Fig. 2). The PFL/PFL-AE system is also encoded in the glaucophyte *Cyanophora paradoxa*.

Among the algae that possess secondary plastids, genes for a PFL/PFL-AE system are present in the chlorarachniophyte *Bigelowiella natans*, where PFL is 63% identical to *C. reinhardtii* PFL. In the marine centric diatom *Thalassiosira pseudonana* genes for two sets of PFL/PFL-AE are present (Fig. 3). These genes likely result from a duplication event as the PFLs share 94% sequence identity, and the PFL-AE enzymes share 49% sequence identity with their closest bacterial counterparts. The predicted proteins share 64% sequence identity with *C. reinhardtii* ADHE. Genes for ADHE are found in several lineages with secondary plastids. In *G. theta*, two isoforms of ADHE are predicted which differ mostly in their N-terminal sequence. These enzymes share 49% sequence identity with their closest bacterial counterparts *Thermosynechococcus* and *Clastridiun acetobutylicum*, and 45% with their counterpart in *Chlamydomonas*. In *N. nautam*, the ADHE exhibits 42% amino acid sequence identity to its counterpart in *Chlamydomonas*, and it is predicted to be organellar as it has an extended N-terminus. The presence of an ADHE in *Chromera veia*, a photosynthetic alveolate closely related to apicomplexan parasites [156], is also inferred from ESTs [157].

5.2. PFO/PNO

The ability to perform oxidative decarboxylation by PFO, or its alternative form PNO, is encoded in diverse algal lineages (Table 1; Fig. 2). PFO genes are present in the chlorophytes *Chlamydomonas* and *Chlorella*, and in the diatom *Thalassiosira*. The predicted *Chlorella* PFO exhibits 57% sequence identity with *C. reinhardtii* PFO, and is 55% identical to *C. vulgaris* PFO. In the marine diatom *Thalassiosira pseudonana*, two enzymatic systems capable of the anaerobic conversion of pyruvate into acetyl-CoA, namely PFO and PFL, are present. The co-occurrence of both routes has not been reported in parasitic eukaryotes so far.

5.3. Hydrogenases

Genes for iron-only hydrogenases have been identified in many algal species. The first sequences of algal HYD obtained were from the green algae *Scenedesmus obliquus* [153] and *Chlamydomonas* [131]. These enzymes were unique as they represented the smallest form of [FeFe]-HYD known, comprising only the cluster H, in contrast to bacterial enzymes which exhibit additional clusters on the N-terminal side of the protein. With the release of the genome sequence of *Chlorella variabilis* NC64A and the identification of two hydrogenases (HYDA1, HYDA2), a novel structure for algal [FeFe]-hydrogenases was uncovered as they exhibited an N-terminal F-cluster domain [28]. *Chlorella* HYDAs are as sensitive to O2 as the *Chlamydomonas* HYDs [28], therefore ruling out the hypothesis that the F-cluster could protect the enzyme from oxygen inactivation. All the chlorophyte HYD sequences exhibit a trans- 

5.4. ADHE

Aldehyde/alcohol dehydrogenase is found in several algal lineages (Table 1; Fig. 2), including two chlorophytes *Chlamydomonas* and *C. variabilis* NC64. While *Chlamydomonas* possesses one gene, *Chlorella* exhibits two distinct Adhe genes which encode enzymes sharing 65% sequence identity. The predicted proteins share 64% sequence identity with *C. reinhardtii* ADHE. Genes for ADHE are found in several lineages with secondary plastids. In *G. theta*, two isoforms of ADHE are predicted which differ mostly in their N-terminal sequence. These enzymes share 49% sequence identity with their closest bacterial counterparts *Thermosynechococcus* and *Clostridium acetobutylicum*, and 45% with their counterpart in *Chlamydomonas*. In *N. nautam*, the ADHE exhibits 42% amino acid sequence identity to its counterpart in *Chlamydomonas*, and it is predicted to be organellar as it has an extended N-terminus. The presence of an ADHE in *Chromera veia*, a photosynthetic alveolate closely related to apicomplexan parasites [156], is also inferred from ESTs [157].

5.5. Energy conserving acetate metabolism

The majority of the algae surveyed here exhibit at least one of the three systems for substrate-level phosphorylations involving acetate known in eukaryotes [98]: see Section 3.4). Therefore it seems that these algae possess a metabolic route for ATP synthesis to contribute to the cell’s viability in anaerobic environments. *Chlorella* possesses a gene encoding a putative acetate:succinate CoA-transferase which shares 40% sequence identity with that of *Fasciola hepatica* (ASCT- subfamily 1B). Genes for an ASCT are also present in the genome of the diatoms *Thalassiosira* and *Phaeodactylum*. Predicted diatom ASCTs share up to 47% sequence identity with the mitochondrial ASCT from kinetoplastids (*Trypanosoma* species), which belong to the subfamily 1A [98].

The *Thalassiosira* genome also encodes a gene for a putative acetyl-CoA synthetase (ADP forming) (Table 1; Fig. 3). The predicted protein exhibits 36% amino acid sequence identity with the enzyme from *Giardia lamblia* which has been studied in detail [101]. Unlike
its parasite counterpart, the diatom ACS exhibits an N-terminal extension, suggestive of an organellar localization (Fig. 3). In three other diatoms, incomplete DNA sequences suggest that an ACS (ADP-forming) might also be present.

Ack and Pta genes have also been identified in several algal lineages, including the crytophytes, the haptophytes, and the chlorarachniophytes (see Table 1), suggesting that a PTA–ACK route for anaerobic ATP production (or assimilation of acetate) might occur in these algae as in Chlamydomonas.

5.6. Glycerol as an end product

Glycerol is usually a minor product of dark fermentation of green algae [22,26]. However, Chlamydomonas can excrete significant amounts of glycerol; this was observed in conditions where the ADHE route is blocked [140]. The typical route to glycerol formation is a two-step reaction involving the reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol-3-phosphate, by an NAD+-dependent glycerol-3-phosphate dehydrogenase (GDPH), followed by the hydrolysis of the glycerol-3-P by a glycerol-3-phosphatase (GPP) (Fig. 1). This pathway which allows the reoxidation of glycolytic NADH is known in some heterotrophic anaerobic eukaryotes such as Paramecium (8). The potential for glycerol production is expected to be widespread among algae as genes for GDPH and GPP are found in various algal lineages, such as Cyanidioschyzon merolae, Thalassiosira and G. theta.

5.7. Anaerobic respiration

The anaerobic respiratory pathways that have been found among eukaryotes so far occur in heterotrophic species [12]. An exception is Euglena, whose wax ester fermentation is thought to involve the respiratory chain [158] with the rhodoquinone-dependent reduction of fumarate to succinate being coupled to the mitochondrial electron transport chain [159]. Benthic or pelagic diatoms species can accumulate high concentrations of nitrate. Kamp et al. [36] recently showed that under dark anaerobic conditions, nitrate consumption followed ammonium production. They proposed that nitrate might be respired to ammonium via an anaerobic biochemical route known as ammonium fermentation. In Fusarium, nitrate reduction to nitrite and the subsequent reduction of nitrite to ammonium are thought to entail ATP synthesis via substrate-level phosphorylation only [37] or possibly via respiration and electron transport phosphorylation in addition
Table 1
Distribution of genes involved in anaerobic energy metabolisms among unicellular photosynthetic eukaryotes as inferred from genome survey. BLASTp and TblastN searches on NCBI whole genome databases and/or on the website of each given alga were performed to identify the genes encoding fermentative enzymes. Homology to the different enzymes was determined using an E value cutoff of less than 10^-10 using either the C. reinhardtii sequences or other eukaryotic ones and at least 80% coverage.

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 gene was found; –, not found; ?, unknown.

Abbreviations are as follows: DOE JGI, Department of Energy Joint Genome Institute; NCBI, National Center for Biotechnology Information; F/B/M, freshwater, brackish water, marine water.

a ASCT, acetyl-CoA:acetate-CoA transferase; 1A, homolog to members of subfamily ASCT-1A (Trypanosoma cruzi, EAN79240); 1B, homolog to members of subfamily ASCT-1B (Fusocita hepatica, ACTF06126).
b Homolog to Giardia lamblia acetyl-CoA synthetase (ADP forming) (XM_001705692).
c Protein is predicted or known to be compartmentalized, likely in the chloroplast or in the mitochondrion.
d At least two distinct genes were detected.

f Cyanophora paradoxa Genome project http://cyanophora.rutgers.edu/cyanophora/home.php.

[160]. Though the enzymatic partners of the fungal pathways are not fully identified, nitrite reductase (NirK) from Fusarium has been characterized, it is homologous to copper-containing nitrite reductases from proteobacteria, likely has a mitochondrial origin and furthermore has conserved homologues in Chlamydomonas [161]. In diatoms, the pathway could provide energy to prepare for the resting stage or long-term survival under dark anoxic conditions, but none of the enzymatic components have been characterized so far.

6. Evolutionary insights

During evolution, enzymes and pathways can readily undergo recompartmentation between mitochondrial, cytosolic, and in the case of algae, plastidic compartments, as the example of ADHE localization shift for Polytomella (mitochondrial) vs. Chlamydomonas (chloroplast) underscores [51,97]. The movement of whole pathways from one compartment to another became evident through studies of chloroplast-lysyl oxidase enzymes [162], studies of differential localization of isoprenoid biosynthesis to plastids and the cytosol of algae [163], starch metabolism in algae [164], peroxisomal pathways [165], and the glycosome in trypanosome evolution [166]. The problem of how to transfer whole pathways (or major segments thereof) from one compartment to another has been a longstanding evolutionary puzzle because a single enzyme in a new compartment is of no use and hence hardly selectable in evolution, the whole functional unit has to move. One possible solution to this problem

Fig. 3. Tentative reconstruction of the anaerobic energy metabolism in the diatom Thalassiosira pseudonana. Highlighted in blue are the different metabolites, redox sinks and/or fermentation products that are typically identified in other unicellular algae but are currently not known for T. pseudonana. Although little is known about intracellular locations of the different enzyme pathways, the cytosolic presence of OPPP and glycolytic enzymes was reported by Kroth et al. [172]. Also, these authors depicted a plastidic location of PPIK and PYC, and probably of [ADP]-ACS. The nitrogen cycle enzymes, soluble nitrate reductase (NR) and nitrite reductase (NIR) are represented as proposed previously [173]. Reducing power in the form of reduced FDX for nitrite reduction in the chloroplast may be obtained via ferredoxin:NADP reductase (NFR), using NAPDH derived from the oxidative pentose phosphate pathway, as seen in maize roots [174]. Although the depicted compartmentalization of the different enzymes clearly has to be provisional, as indicated by the dotted lines of the organelles, predictions can be made based upon knowledge on Chlamydomonas and other (photosynthetic) eukaryotes. The metabolic routes involving succinate are usually mitochondrial. In C. reinhardtii, PFL is dually targeted to both chloroplast and mitochondria, in the diatom two PFL isoforms with N-terminal extensions are found that could be sent to different cellular compartments. Note that no genes for FeFe-hydrogenase maturation factors HydEFG have been found in the diatom genomes so far, and no hydrogen production has so far been reported for T. pseudonana, but can be predicted; note in addition that Giardia intestinalis also lacks detectable genes for FeFe-hydrogenase maturation factors but does produce H2 [8]. Enzyme abbreviations are as in Fig. 1.

Image: Courtesy of Nils Kröger (Georgia Tech’s School of Chemistry and Biochemistry and the School of Materials Science and Engineering).
involves the concept of minor mistargeting [167]. If protein targeting in eukaryotes is not 100% specific (and many examples of dual targeting of enzymes are known), then minor amounts of whole pathways could conceivably end up in the wrong compartment. Such minor mistargeting is more likely for highly expressed proteins, such as those involved in core carbon and energy metabolism. If a
small amount of a whole pathway is present, it could readily become a unit of function, albeit at a low initial activity, and hence a unit upon which natural selection could efficiently act to generate more or less of a new compartmentation variant. Early in eukaryotic evolution, before the origin of the mitochondrial protein import machinery of the inner and outer mitochondrial membranes, the situation was simpler, because gene transfers from mitochondria to the nucleus would have given rise either to pseudogenes (junk DNA) or to enzymes expressed in the cytosol [168] and similar considerations apply for algal genes acquired from plastids before the origin of the plastid protein import machinery [143]. In that way, pathways ancestrally encoded by organelar genomes can be readily transferred to the eukaryotic cytosol.

With regard to more general evolutionary issues concerning the origin of anaerobic energy metabolism in eukaryotes, it is often assumed that ancestral eukaryotes were aerobes and that in order to inhabit anaerobic habitats, eukaryotes had to acquire, via some kind of lateral gene transfer, enzymes that would enable them to generate ATP without the help of oxygen respiration in mitochondria [70]. But that can hardly be true, as the study of anaerobic enzymes and pathways in algae indicates, through three lines of evidence. First, algae are photosynthetic and produce oxygen, hence they are under no evolutionary pressures to inhabit anoxic environments in the first place (they can survive with light); accordingly, no compelling selective pressure for them to specifically acquire genes for that purpose can be construed. Second, algal fermentation pathways consist of the same enzymes of anaerobic energy metabolism as found in eukaryotic heterotrophs; were eukaryotes acquiring genes in order to access anaerobic environments, one would hardly expect different lineages of eukaryotes to acquire the same genes and enzymes (and in the case of [FeFe] hydrogenase and PFL, even the same accessory proteins needed for maturation). In other words, if eukaryotes had to acquire genes in order to access anaerobic environments, eukaryotes would harbor as many different anaerobic energy-producing pathways as prokaryotes do, which is hardly the case, as the present study and other recent surveys [8] indicate. Third, no enzymes of anaerobic energy metabolism have yet been found in eukaryotes that are specific to any particular lineage (with the so far nagging exception of trans-2-enoyl-CoA reductase of Euglena mitochondria [63] that has so far failed to uncover homologues in any other eukaryote); rather they all trace to the eukaryote common ancestor and as more lineages become sampled, the more pronounced this trend becomes. The first real surprise in that respect came from the Chlamydomonas genome, which uncovered all of the major enzymes of anaerobic energy metabolism currently known among eukaryotes [97]. The algae (oxygen producers) turn out to be the most flexible eukaryotic anaerobes known. That insight is quite new and helps to change our views of the phylogenetic distribution and ecological significance of anaerobic energy metabolism in eukaryotes.

7. Conclusions

To date, C. reinhardtii is the alga that expresses the most complete repertoire of anaerobic enzymes. It has a flexible mixed-acid fermentation in which aspects of bacterial-, plant- and yeast-type fermentation can be found. Its fermentation capabilities included hydrogen production, which is currently of considerable interest in the context of biofuels. Chlamydomonas is so far unique among eukaryotes in that it possesses two enzymatic systems to convert pyruvate into acetyl-CoA under anaerobic conditions: PFL and PFO. The interplay between these two routes, their regulation and their ecological significance warrant further study.

Among the photosynthetic algae, literature and genome surveys show a broad panel of fermentative enzymes and a few cases of anaerobic respiration. On the basis of evidence surveyed here, it appears that anaerobic respiration among eukaryotic algae is comparatively rare and that anaerobic fermentation is widespread. Anaerobic respiration is far more common among prokaryotes, where it is significant at a global scale and influences the global cycling of elements, sulfur, nitrogen and carbon. The diversity of metabolic routes in algal groups shows marked variation among species (Micromonas, diatoms) and strains, for example Chlamydomonas [27] and Euglena [31] in those cases that have been studied. Further investigations are needed to assess the scope and ecological significance, if any, of such variation.

Most of the enzymes for anaerobic pathways that are inferred from genomic studies have not been characterized biochemically. For anaerobic metabolism in eukaryotes, there is still intense reliance upon a handful of well-studied organisms [8], for photosynthetic eukaryotes the anaerobic paradigm currently centers on Chlamydomonas. The expression and biochemical properties of these enzymes and pathways need further study and in a broader spectrum of model systems. In addition, the spectrum of end products produced by various algae during anaerobic fermentations is still mostly unknown. This would be important information for understanding metabolic diversity among algae. Finally, fermentation of algae appears to represent a significant ecological component of carbon flux in soil that influences the content in organic acids, alcohols and hydrogen, this poses a challenge for future ecological studies.

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