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Published in:
Nature Geoscience

Publication date:
2016

Published in final edited form as:
Nat Geosci. 2017 January ; 10(1): 30–35. doi:10.1038/ngeo2843.

Metabolism in anoxic permeable sediments is dominated by eukaryotic dark fermentation

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Abstract

Permeable sediments are common across continental shelves and are critical contributors to marine biogeochemical cycling. Organic matter in permeable sediments is dominated by microalgae, which as eukaryotes have different anaerobic metabolic pathways to prokaryotes such as bacteria and archaea. Here we present analyses of flow-through reactor experiments showing that dissolved inorganic carbon is produced predominantly as a result of anaerobic eukaryotic metabolic activity. In our experiments, anaerobic production of dissolved inorganic carbon was consistently accompanied by large dissolved H_2 production rates, suggesting the presence of fermentation. The production of both dissolved inorganic carbon and H_2 persisted following administration of broad spectrum bactericidal antibiotics, but ceased following treatment with metronidazole. Metronidazole inhibits the ferredoxin/hydrogenase pathway of fermentative eukaryotic H_2 production, suggesting that pathway as the source of H_2 and dissolved inorganic carbon production. Metabolomic analysis showed large increases in lipid production at the onset of anoxia, consistent with documented pathways of anoxic dark fermentation in microalgae. Cell

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Author contributions

All FTR experiments were performed by MFB and supervised by PLMC. MFB and PLMC were responsible for experimental design and research direction with significant input from RNG and JB. Effluent volatile fatty acid analysis was performed by MFB and supervised by PJM. Metabolomics analysis was performed by HHS. Algal culture experiments were performed by MK and MFB and supervised by JB and PLMC. Manuscript was written by MFB and PLMC. All authors contributed to discussion and editing.

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counts revealed a predominance of microalgae in the sediments. H_2 production was observed in dark anoxic cultures of diatoms (*Fragilariopsis* sp.) and a chlorophyte (*Pyramimonas*) isolated from the study site, substantiating the hypothesis that microalgae undertake fermentation. We conclude that microalgal dark fermentation could be an important energy-conserving pathway in permeable sediments.

Keywords

H_2 production; Dark fermentation; Diatom; Sand

Permeable sediments and organic matter metabolism

Microalgae are globally ubiquitous in photic sediments and often have a biomass exceeding phytoplankton in overlying waters1. In permeable (sandy) sediments, their biomass may comprise 10 – 40% of the organic carbon pool based on previously reported carbon to chlorophyll *a* ratios2,3 and a high proportion of functional chlorophyll4 . In contrast, bacterial biomass has previously been reported to comprise <10% of the organic carbon pool in sandy sediments5. This is also consistent with the dominance of microalgae in mediating carbon flows in such sediments6. Given their high biomass, it is reasonable to expect that microalgae will undertake a large fraction of the carbon mineralization and energy generation pathways in comparison to bacteria. Despite this, it remains widely assumed that bacterial fermentation and sulfate reduction are the main mechanisms of dissolved inorganic carbon (DIC) production in anoxic permeable sediments7. Surprisingly, there have been no systematic studies of electron acceptor utilisation in freshly collected sands and, despite their dominance, the role of microalgae in these processes8.

The dynamic nature of permeable sediments means that microalgae are often observed to be evenly distributed to ≥ 15 cm within sands where dark anoxic conditions will prevail1,6. It has been shown that microalgae mixed into the dark sediment can remain viable for long periods of time (half-lives of 6-22 days), highlighting their adaptation to this dynamic environment9. However, the metabolic basis of how they survive light and oxygen fluctuations is unclear. Nitrate respiration has previously been shown to sustain the survival of axenic cultures of phototrophic eukaryotes in dark anoxic conditions10; however, it remains to be determined whether nitrate respiration is a relevant strategy in the environment and it is unlikely that it is sufficient to support microalgal populations in oligotrophic systems with low nitrate concentrations. Fermentation is an alternative strategy that microorganisms use to couple carbon mineralization to energy-generation. While some microalgae have been shown to undertake fermentation under dark anoxic conditions, for example chlorophytes11,12, there have been no studies to date to quantify the importance of this in the environment.

In this work, we present the first study of the importance of anoxic microalgal metabolism in permeable sediments. We combined flow through reactor experiments with microbiological approaches to determine the dominant contributors and pathways of dissolved inorganic carbon (DIC) production in permeable sediments. We show that microalgal dark

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fermentation is the dominant metabolic pathway, which is the first time this has been documented in an environmental setting.

Phototrophic eukaryotes dominate metabolism

We initially compared the rates of DIC production and electron acceptor utilization using sediments collected at Port Philip Bay (Australia) and Kerteminde (Denmark). We used flow through reactors (FTRs) to compare solute concentrations at the inlet and outlet of a sand column in order to calculate volumetric rates. Under anoxic conditions with the addition of 50 μM $^{15}NO_3^-$, the FTR experiments showed that rates of nitrate reduction to nitrite and N_2 were low compared to DIC production; they were only able to account for less than 10% of DIC production in Australian and Danish samples (Fig 1a & 1b). This gap between the rate of DIC production and nitrate reduction is consistent with previous observations by Evrard *et al* 201313 and Marchant *et al* 201614, suggesting a common, although not ubiquitous15, phenomenon. Rates of iron (II), sulfide and methane production in the presence of nitrate were less than 0.6, 0.2 and 0.05 nmol mL^{-1} h^{-1}, respectively, which cannot account for the DIC production rates observed. We rule out significant rates of iron reduction and subsequent trapping of Fe^{2+} as FeS or sorption onto FeOOH on the basis that no alkalinity production (<-10 nmol mL^{-1} h^{-1}) was observed and also the low reactive iron pool present in permeable sediments16. Likewise, measurements of intracellular nitrate were negligible (<1.5 nmol mL^{-1}), ruling out the use of an accumulated intracellular nitrate pool by diatoms17. We also dismiss abiotic processes such as carbonate dissolution driving DIC production given the absence of alkalinity production. There was no significant difference in DIC production between oxic and anoxic conditions ($p = 0.76$) and in the presence or absence of nitrate (ANOVA, two factor with replication, $p = 0.44$) (Fig 1c); this suggests that the organisms responsible for DIC production under oxic conditions were able to use an energy-conservation pathway to maintain their metabolism under anoxia, and that nitrate, iron or sulfate reduction were not significantly contributing to this, consistent with Figures 1a and 1b.

To elucidate which group of organisms were involved in the DIC production under anoxic conditions, FTRs containing the Australian sediments were exposed to three seawater reservoir treatments: a control treatment, one spiked with 50 mg L^{-1} of amoxicillin, and one spiked with 2 mmol L^{-1} $HgCl_2$. The production of DIC was shown to be halted by the mercuric chloride treatment (Fig 1d), indicating that carbon mineralization was driven by biotic processes. However, there was no significant difference between the DIC production rates of the control and amoxicillin treatments (ANOVA, single factor, $p = 0.80$), indicating that amoxicillin did not inhibit the dominant microbial community responsible for carbon mineralization. Amoxicillin is a broad spectrum antibiotic that has been shown to exhibit bactericidal action at concentrations in the range of 0.1 to 2 mg L^{-1} depending on the strain of bacteria18. If bacteria were responsible for producing DIC, we would expect to see a decrease in the DIC production rate in the amoxicillin treatment compared to the control. This suggests that eukaryotes, not bacteria, are responsible for the vast majority of DIC production in permeable sediments. The possibility of bacteria being resistant to amoxicillin was investigated by quantifying the rate of denitrification (primarily a bacterial process) in the presence and absence of amoxicillin (see Supplementary Information Figure S1). As

expected, amoxicillin inhibited denitrification by factors of 2 to 5, compared to the control, over the course of the experiment, suggesting that antibiotic resistant bacteria do not explain our observations.

Possible eukaryotic organisms responsible for DIC production include meiofauna, macrofauna and microalgae. We ruled out macrofauna and meiofauna on the basis that DIC production was maintained for over 10 days after anoxia (data not shown), which would kill these organisms, and that no macrofauna were observed in the sediment. We therefore hypothesized that the source of the DIC production is microalgae, such as diatoms, chlorophytes, and other eukaryotic microalgae. Consistent with this, cell counting revealed a diverse community of microalgae at the study site (see Supplementary Information, Table S2). Diatoms were particularly abundant, exceeding 10^5 cells per mL, with species from the genus *Amphora*, *Cocconeis*, and *Fragilariopsis* most numerous. Scaling the sediment chlorophyll *a* content to total microalgae using a carbon to chlorophyll *a* ratio of 40[3] gives a sediment microphytobenthos carbon content of 850-860 μg g^{-1} of dry sediment, which comprises approximately 57% of the total organic content of the sediment, highlighting the likely dominance of these organisms in DIC production.

Eukaryotic dark fermentation pathways are the major source of DIC production under anoxic conditions

Having demonstrated that the major respiratory electron acceptors in marine systems cannot account for DIC production, we looked for other potential sources that may be responsible for carbon mineralization. Intriguingly, we observed through microsensor experiments that H_2 production began ~36 hours after the commencement of anoxia. H_2 concentrations in the FTR reached 44 ± 10 μmol L^{-1} and 23 ± 4 μmol L^{-1} (data not shown) in the presence and absence of nitrate, respectively (Figure 2a). These concentrations are almost an order of magnitude higher than previously reported maximum transient concentrations of H_2 by bacterial fermentation of 2 to 6 μM19,20. These values correspond to production rates of 26 ± 7 nmol mL^{-1} h^{-1} and 13 ± 2 nmol mL^{-1} h^{-1} whereas DIC production rates in these columns were 81 ± 36 and 117 ± 46 nmol ml^{-1} h^{-1} for anoxic treatments with and without nitrate, respectively (data not shown). The addition of the antibiotic ciprofloxacin at a concentration of 150 μmol L^{-1} to the columns had no effect on H_2 production (Figure 2a). Single species assays have shown ciprofloxacin to have an EC-50 value of between 15 and 51 nmol L^{-1} for typical gram positive bacteria21,22, and 241 nmol L^{-1} for the gram negative bacteria of *Pseudomonas putida*23. Eukaryotes have been shown to be far less sensitive to ciprofloxacin, as the EC-50 values for green algal species *Selenastrum capricornutum* and *Pseudokirchneriella subcaptitata* range between 9000 nmol L^{-1} and 56,000 nmol L^{-1}24. Despite the presence of ciprofloxacin, H_2 production persisted in both control and nitrate treatments, and indeed continued to increase over the course of the experiment. This strongly suggests that, as with DIC production, H_2 production cannot be attributed to a bacterial source and is therefore likely being produced by eukaryotes which are known to be capable of H_2 production through fermentation under anoxia25.

There are two well-studied eukaryotic metabolic processes that produce H_2: photobiological production and dark fermentative metabolism25. In photobiological production, light-excited electrons derived from water (direct biophotolysis) or organic compounds (indirect biophotolysis) are transferred to ferredoxin and then [FeFe]-hydrogenase resulting in H_2 production26. By contrast, dark fermentative metabolism involves the glycolytic breakdown of organic compounds (e.g. intracellularly stored starch). The resulting pyruvate is then oxidized to acetyl-CoA by pyruvate ferredoxin oxidoreductase (PFR) resulting in carbon dioxide25. Acetyl-CoA is then converted to stored lipids. The ferredoxin reduced by PFR is in turn reoxidized by [FeFe]-hydrogenase resulting in H_2 production27. Photobiological H_2 production can be excluded as these pathways require light yet all experiments were conducted in darkness. Therefore, the most likely pathway to be responsible for the observed H_2 production is dark fermentative metabolism. The transfer of electrons from the reduced form of ferredoxin to hydrogenase, which is required for dark fermentation to proceed, can be effectively inhibited by low concentrations of metronidazole28. Consistent with this H_2 production pathway, we observed administration of 5 mg/L metronidazole effectively inhibited H_2 production compared to a control treatment (Fig 2b).

We subsequently investigated the fate of the organic end products produced through dark fermentation. Dark fermentation in microalgae results in the production of a wide variety of organic end products in addition to H_2 and CO_2 depending on the microalgal species, pathway and environmental conditions12,29. For example, *Chlamydomonas* excrete acetate, ethanol, and formate or glycerol at ratios that vary depending on the species30,. Additionally, some algae do not release fermentative products from the cell at all and instead they accumulate them intracellularly. For instance, Inui et al (1982)31 have found that *Euglena gracilis* generates large concentrations of wax esters which are stored in the cytosol32 that are reoxidized to generate ATP upon return to oxic conditions. We were unable to detect any alcohols or volatile fatty acids in the column effluent using solid phase micro extraction (SPME) followed by GC/MS analysis with a detection limit of <1 μM, suggesting that that fermentation products are predominantly stored intracellularly. We therefore performed metabolomic analysis of sediment collected from dark anoxic FTRs under H_2 production to detect possible storage products. This showed a 3 fold increase in phosphoglycerolipids and ceramides and a 5 fold increase in oleate (Fig 2c) as compared with oxic conditions. This is consistent with the synthesis of lipids from the fermentation product acetyl-CoA as previously documented for green algae (Fig 3)25. Reactions 1 and 2 indicate possible stoichiometries for the breakdown of glucose to produce oleic acid and other fermentative products and are spontaneous under standard conditions. Under non-standard conditions, both reactions remain spontaneous regardless of high partial pressures of H_2 and CO_2 (0.8 atm), however, reaction 2 becomes most favourable at pCO_2 and pH_2 < 0.3 atm (see Supplementary Information Figure S2).

$$4.25\,C_6H_{12}O_6 \rightarrow C_{18}H_{34}O_2 + 7.5\,CO_2 + 8.5\,H_2O \quad \Delta G^\circ = -280\,\mathrm{kJ\,mol^{-1}} \qquad (1)$$

$$5\,C_6H_{12}O_6 \rightarrow C_{18}H_{34}O_2 + 12\,CO_2 + 4\,H_2O + 9\,H_2\;\Delta G^\circ = -243\,kJ\,mol^{-1} \quad (2)$$

Using a combination of reactions 1 and 2, a wide range of CO_2:H_2 production stoichiometries are possible. No H_2 production was observed until ~48 h after anoxia, suggesting a reaction analogous to 1 before a reaction analogous to 2 commenced or net H_2 consumption. Once net H_2 production commenced, it was produced at 30-50% of the rate of DIC production, however, we note that the gross H_2 production rate is most likely higher than this. By analogy with other sediment ecosystems, it is more likely that much of the H_2 produced is immediately recycled by respiratory hydrogenotrophic bacteria inhabiting the sediments33. Microbial ecology studies have shown that there is an abundance of both aerobic and anaerobic bacteria in these sediments33,34,35 with phylotypes similar to known hydrogenotrophs26,34,35. If we assume that denitrification was the dominant electron sink and is driven by hydrogenotrophy, with a $2H_2$:NO_3^- stoichiometry, then the total H_2 production rates could be double the release measured here. It remains unclear why any release of H_2 occurs because bacteria normally rapidly consume it. We speculate that in highly dynamic sand environments, bacterial biomass is unable to accumulate and that there is a lag time of days to weeks before their growth allows them to utilize all the H_2 once the environment becomes more static. In support of this hypothesis, we observe that sands become highly sulfidic ~1 week after collection from the field.

Diatoms and chlorophytes isolated from the permeable sediments fermentatively produce H_2

To further substantiate that microalgae produce H_2, we isolated and cultured representatives of two of the most dominant microalgal genera from the study sites (see Supplementary Information, Table S2). Axenic cultures of five diatom species (all *Fragilariopsis* sp.) and a chlorophyte (*Pyramimonas* sp.) were isolated from Port Phillip Bay. Incubations under dark anoxic conditions confirmed that all six cultures rapidly produced H_2 resulting in concentrations of 800 ± 450 nM after 118 hours of anoxic incubation (Fig 2d). While it is well-established that green algae can fermentatively evolve $H_2$36, this to our knowledge is the first observation of H_2 production by diatoms. It has previously been observed that such organisms harbor the genes required to fermentatively produce $H_2$12, i.e. PFR and [FeFe]-hydrogenase, thereby supporting our observations. It has long been recognized that diatoms can survive for many weeks in darkness37 and that they may do this through dissimilatory nitrate reduction in dark anoxic conditions38. Whilst this mechanism is viable in relatively eutrophic habitats such as the Wadden Sea, which have high nitrate concentrations over the winter months, this mechanism is unlikely to be viable in relatively oligotrophic habits such as Port Phillip Bay, where nitrate concentrations are very low in the water column (typically < 1 μM) and there is intense competition for the little nitrate that exists. We therefore propose that fermentation is the principle mechanism that these dominant organisms use to persist during anoxia.

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Summary

The results presented here challenge the conventional formulation of anoxic metabolic pathways using the redox cascade in high energy permeable sediments39,40. We demonstrated here that the previously-identified carbon mineralization pathways (e.g. nitrate respiration, sulfate and iron reduction) and organisms (i.e. respiratory bacteria) in permeable sediments do not account for DIC production during anoxia. Instead, dark fermentation mediated by microalgae may be the dominant metabolic pathway, resulting in H_2 release and DIC production. Given that light penetration is sufficient to support positive net benthic community production can occur over 33% of the coastal ocean41, this may be a globally important metabolic pathway. It is also plausible that the H_2 produced is recycled by aerobic and anaerobic respiratory bacteria, thereby shaping ecology and biogeochemistry within the temporally and spatially variable ecosystem of the permeable sediment. In light of these findings, we are presently investigating the molecular pathways of carbon mineralization and H_2 metabolism in this ecosystem. Further studies are also required in realistic flow settings such as *in situ* or in flumes to investigate the dynamics of H_2 cycling in this ecosystem.

Materials and Methods

Flow through reactor experiments

Flow through reactor experiments (FTRs) were packed using approximately the top 15 cm layer of sediment from Port Phillip Bay, Victoria, Australia and Kerteminde, Denmark as described in Table 1. The FTRs used were acrylic cylinders with a diameter of 4.6 cm and a length of 3 cm sediment. PVC caps were placed at either end of the cylinder, and were machined with grooves converging to a central outlet port overlaid with 0.1 mm nylon mesh to allow even plug flow through the column. Plug flow within these columns has been verified during breakthrough curve experiments performed by Evrard *et al*42 and Bourke *et al*43. Freshly collected site water was pumped through the FTRs using a peristaltic pump located upstream of the FTR. Reservoirs were maintained in oxic/anoxic states by continuous purging with air or argon respectively. The system was confirmed to have negligible leak rate of oxygen by running deoxygenated water through the system, which was then observed to have a concentration of <1 μM at the outlet. For H_2, a loss of 3.6% was observed when a solution containing 50 μmol L^{-1} H_2 was pumped through the FTR set up. Water samples at the column outlet were collected by directly connecting glass syringes to the outlet tubing and ensuring no bubbles were present. Reaction rates were calculated based on the difference between the relevant solute concentration in the reservoir and the outlet of the column, the reactor volume and the flow rate through the reaction.

Solute and gas measurement

Oxygen was monitored at the inlet and outlet of the columns using Pyroscience Firesting flow through dissolved oxygen sensors. Denitrification rates were calculated using the isotope pairing technique44 with the addition of 50 μM $15NO_3^-$ and we report total denitrification rates here (denitrification of $^{14}NO_3^-(D_{14})$ and $^{15}NO_3^-(D_{15})$). Samples for analysis of $^{15}N\text{-}N_2$ were transferred from glass syringes into 12 mL Exetainers (Labco, High Wycombe, UK) and preserved with 250 μL of 50% w/v $ZnCl_2$. Samples for dissolved

inorganic carbon were sampled into 3 mL Exetainers and preserved with 30 μL of 6% $HgCl_2$ before analysis using flow injection analysis45 with a precision <1%. H_2 analyses were performed using a calibrated Unisense H_2-100 sensor (Unisense A/S, Aarhus, Denmark) fitted with a glass flow through cell connected directly to the column outlet. Nitrate and nitrite concentrations were determined using a Lachat Quickchem 8000 flow injection analyser fitted with a spectrophotometric detector. A UV-Visible spectrophotometer (GBC) was used to determine iron and sulfide concentrations in FTR effluent following the Ferrozine46,47 and Fonselius48 methods, respectively. Iron and sulfide samples were filtered using MicroAnalytix 0.2 μm cellulose-acetate filters and were preserved using 0.5 mL of Ferrozine and 100 μL of Zn acetate per mL of sample, respectively. Alkalinity production was quantified using a modified Gran titration49. Following the addition of 100 μL aliquots of 0.01 mol L^{-1} HCl, changes in pH were recorded using an NBS buffer calibrated pH electrode attached to a portable HACH HQ40d meter. Chlorophyll *a* content was analysed using a UV-Visible spectrophotometer (GBC), following a methanol extraction step50. Sediment organic carbon and isotope ratios were analysed using a Sercon 20-22 Isotope-Ratio Mass Spectrometer. Methane samples were preserved using 100 μL 50% w/v $ZnCl_2$ and given a 4 mL He headspace, prior to analysis. Methane concentration in samples was determined using a Varian 3700 Gas Chromatograph with a C-18 Poracil column equipped with a flame ionization detector. Intracellular nitrate pools were measured on freshly collected samples before and after exposure to nitrate concentrations of 50 μM. Samples were extracted according to Stief, et al. 17 before analysis for nitrate, as described previously.

Metabolome analysis

For metabolome analysis, cells were lysed and metabolites were extracted from sediments in the FTRs in a methanol:chloroform mixture (1:2, v:v; -20C°) for 30 min under sonication. The metabolites in the supernatants were analysed by GC-QTOF-MS and LC-QTOF-MS (both Agilent Technologies) following Godzien, et al. 51 and Kind, et al. 52 with slight modifications. Volatile fatty acid concentration in FTR effluent was determined using SPME GC-MS. A HP-5MS non polar column (Agilent Technologies, Mount Waverley, Australia) of dimensions 30 m x 0.25 mm ID x 0.25 μm film thickness was used for separation in conjunction with splitless injection mode and H_2 carrier gas. The method was optimized by exposing Carboxen/Polydimethylsiloxane SPME fibre to 10 mL of sample seawater headspace at 50°C for 60 min with 2g NaCl and acid addition prior to injection.

Microbiological analysis

For microalgal counts, sediment and culture samples were preserved with Lugols iodine solution and identified and quantified at MicroAlgal Services, (Ormond, Victoria, Australia) using a Zeiss Standard compound microscope equipped with Phase Contrast Optics with up to 400x magnification used in a Sedgewick-Rafter counting chamber. The identification of MPB present in the sediment samples was carried out using reference material from Grethe 53. Microalgae were isolated from sediment collected at the study site (but not used for flow through reactor experiments) and grown in F/2 medium under continuous illumination at 60 μmol photons m^{-2} s^{-1} at a temperature of 18 °C. To ensure no bacterial growth, the cultures were treated with a dilute antibiotic cocktail containing ampicillin, streptomycin and

tetracycline with final concentrations of 40, 20 and 8 mg L^{-1}, respectively. Cultures were incubated in 3 mL gastight exetainers under dark anoxic conditions and supernatant H_2 concentrations were determined using the H_2-100 sensor.

Data Availability

The data that supports the findings of this study are available from the corresponding authors upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Australian Research Council grant DP1096457 awarded to PLMC and RNG. RNG was additionally supported by Danish Council for independent Research, Natural Sciences, FNU, (0602-02276B) and the European Research Council through an Advanced Grant (ERC-2010-AdG20100224). The data reported in this paper can be made available by contacting the primary author. We thank Jack Middelburg and 3 anonymous reviewers for thoughtful comments on this work.

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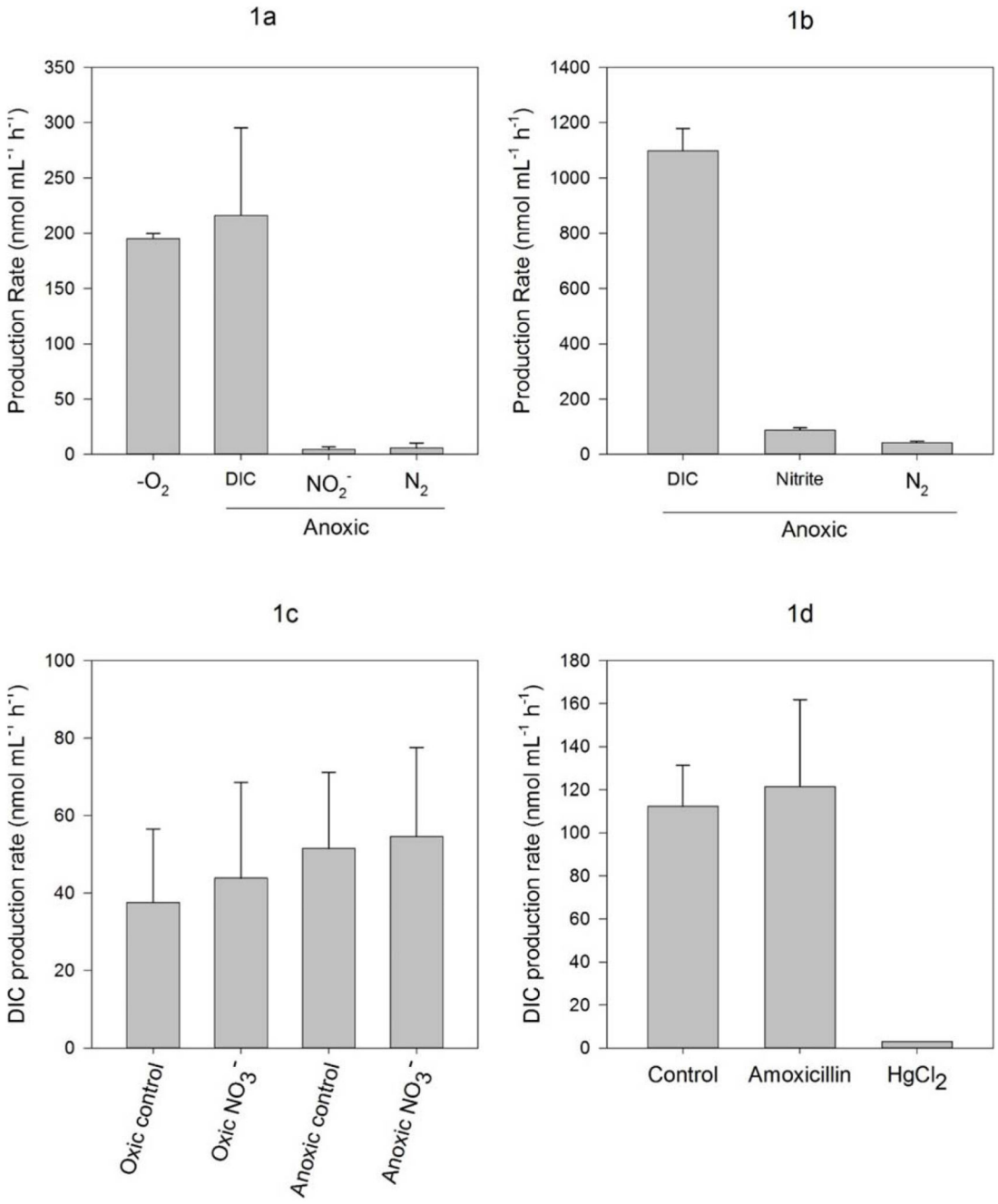

Figure 1.

Metabolism measured in FTR experiments. Sediments collected from Port Phillip Bay, Australia (a,c,d) and Kerteminde, Denmark (b). (a), (b) Oxygen consumption, dissolved inorganic carbon (DIC), nitrite and dinitrogen (as N) production in experiments switched anoxic after O_2 consumption was measured n=4. c) DIC production rates under oxic and anoxic conditions and in the presence and absence of nitrate, n=3 for each treatment. d) DIC production under anoxic conditions: control (n=2), in presence of 50 mg L^{-1} amoxicillin (n=2) and 2 mmol L^{-1} $HgCl_2$ (n=1). All error bars are standard deviation.

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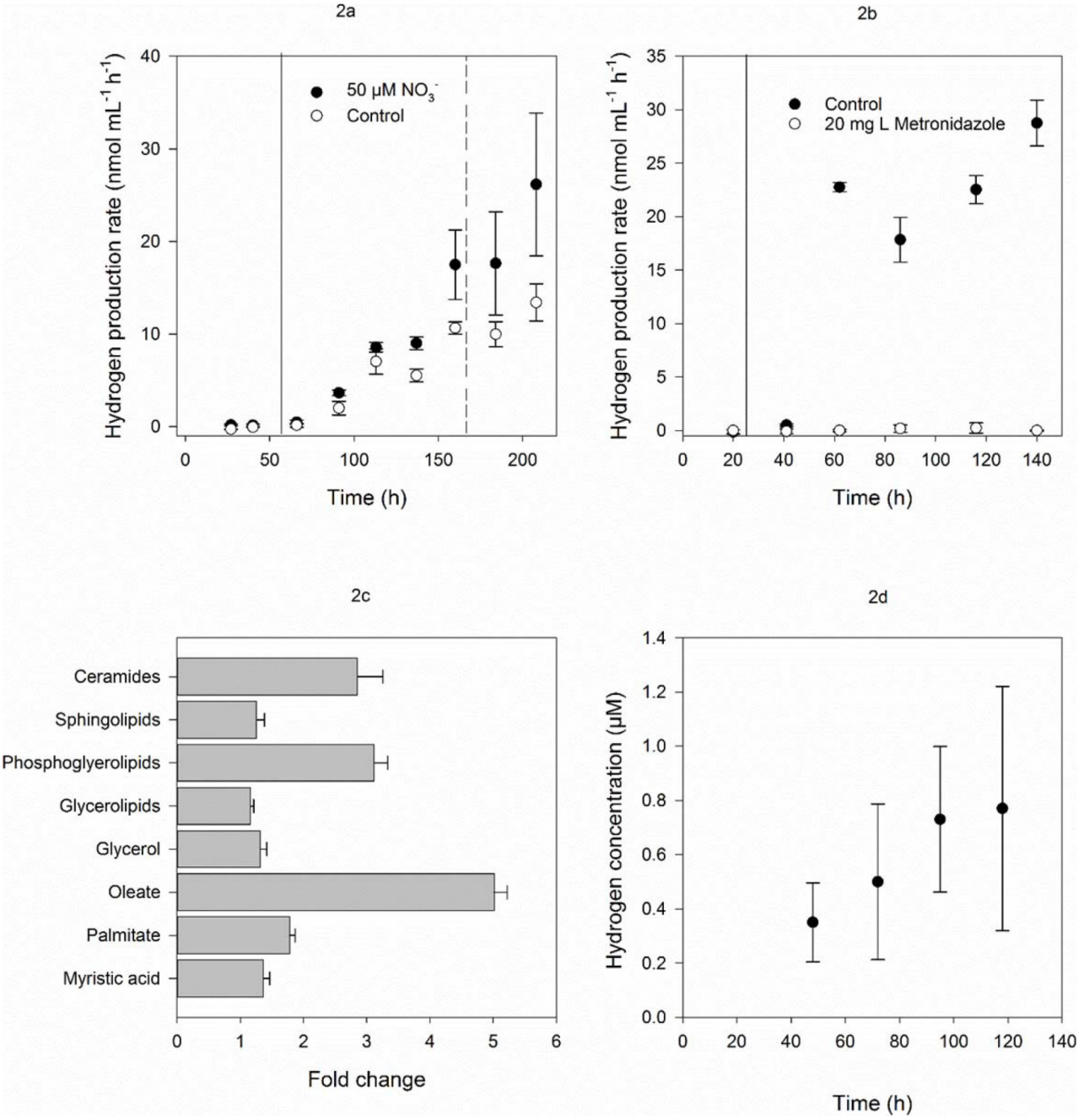

Figure 2.

H_2 and metabolite production in permeable sediments from flow through reactor experiments (a-c) and cultures (d). (a) H_2 production in the presence and absence of 50 µM nitrate. The solid line and broken lines represent a change from oxic to anoxic conditions and the addition of 150 µmol L^{-1} ciprofloxacin respectively, n=3. (b) H_2 production in a control and 20 mg L^{-1} metronidazole, n=3. (c) The relative concentration of metabolites during H_2 production compared to oxic conditions, n=3. (d) H_2 production in cultures of five

diatom species and a chlorophyte incubated anoxically in the dark, n=6. **E**rror bars represent standard deviations.

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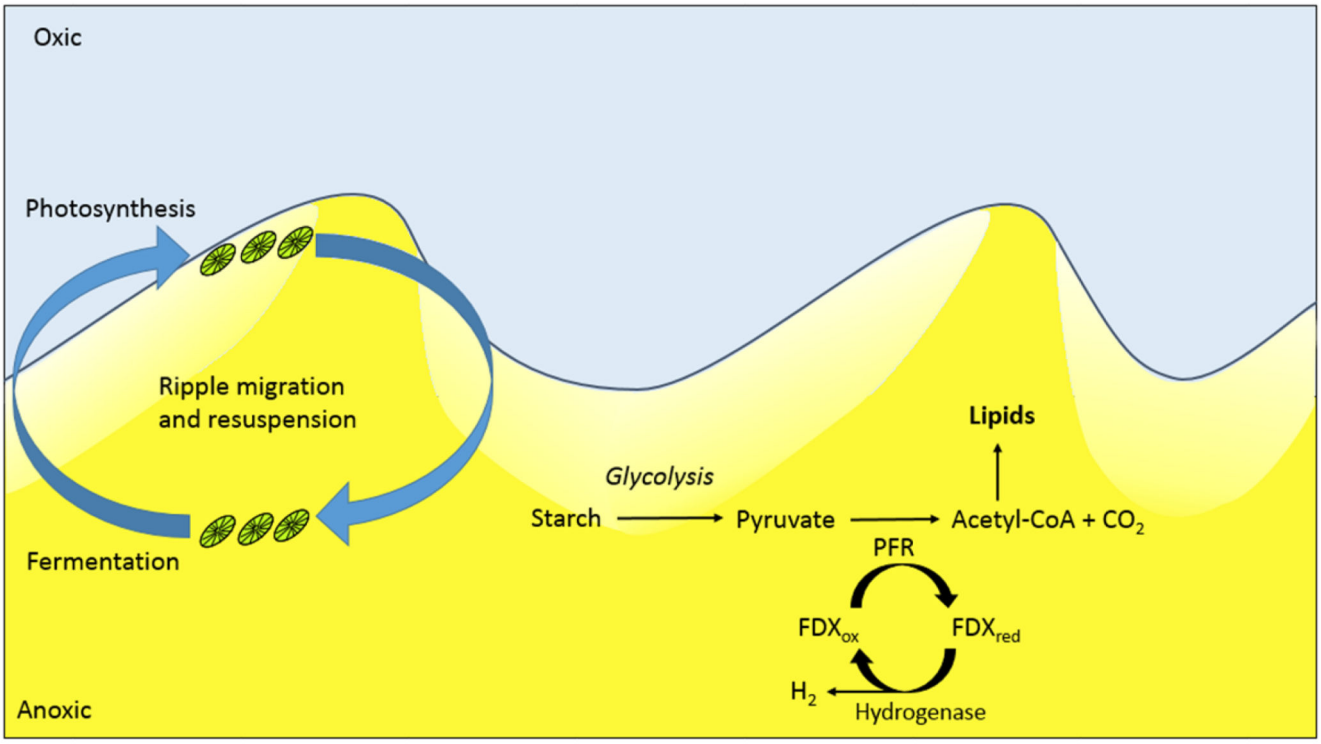

Figure 3.

Conceptual model of benthic algal metabolism in sand sediments. In this energetic environment, ripple migration and sediment resuspension regularly move algal cells many centimetres into the sediment where it is dark and anoxic. Under these conditions, microalgae undertake dark fermentation associated with high rates of H_2 and lipid production 25. Enzyme designations are pyruvate ferredoxin oxidoreductase (PFR) and ferredoxin (FDX). Lightly shaded areas represent declining oxygen concentration within the sediment. Yellow shaded area represents anoxic permeable sediment.

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Table 1

List of experiments performed, location, date of sediment collection and products measured. Coordinates for the sites are 55°27’20.65”, 10°39’56.14”E for Kerteminde and 37°51’8.73”S, 144°57’27.07”E for Port Phillip Bay. Note sampling was carried out in all seasons and so temporal variability explains the variability of DIC production rates presented in Figure 1.

Figure	Location	Date	Flow Rate (mL min^{-1})	Products measured.
1a	Port Phillip Bay , Victoria, Australia	16/09/2014	1.0	DIC, $^{28}N_2$, $^{29}N_2$, $^{30}N_2$, Nitrite, Iron and Sulfide.
1b	Kerteminde, Denmark.	7/11/2012	0.38	DIC, $^{28}N_2$, $^{29}N_2$, $^{30}N_2$, Nitrite.
1c, 2a, 2c	Port Phillip Bay , Victoria, Australia	15/08/2015	0.39	DIC, Dissolved H_2, metabolomic analysis.
1d	Port Phillip Bay , Victoria, Australia	6/03/2015	0.72	DIC, $^{28}N_2$, $^{29}N_2$, $^{30}N_2$
-	Port Phillip Bay , Victoria, Australia	23/01/2014	-	Intracellular nitrate accumulation
2b	Port Phillip Bay , Victoria, Australia	31/10/2015	0.87	Dissolved H_2
2d	Port Phillip Bay , Victoria, Australia *	12/12/2015		Dissolved H_2

* Sediment collected in this instance was used to grow algal cultures that are presented in Figure 2d.