**Chemical Science**

EDGE ARTICLE

Fluoro-electrochemical microscopy reveals group specific differential susceptibility of phytoplankton towards oxidative damage†

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In the vicinity of an electrode creating a highly oxidising environment the fluorescence – arising from the presence of chlorophyll-a – of single cellular phytoplankton becomes inhibited. Even for phytoplankton that are very comparable in size (ca. 2–20 μm) the rate of this (electro)chemically induced fluorescence inhibition differs significantly between phytoplankton species; the fluorescence signal of the freshwater algae Stichococcus bacillaris turns off ~70 times faster than that of the marine coccolithophore Emiliania huxleyi. The varying behaviour reflects the differing susceptibility of these globally important phytoplankton species towards extreme levels of radical induced oxidative stress, indicating the physical and chemical properties of the plankton cell wall and membrane are very different between species, and are important in determining their susceptibility. These results have potential implications for the analytical detection and characterisation of phytoplankton cells in the natural environment.

Introduction

The denomination phytoplankton is a broad catch-all term encompassing a range of single cellular microorganisms spanning twelve taxonomic divisions and includes both Eukaryotes and Bacteria. Over 5000 species of phytoplankton1 have been identified with sizes ranging from 1 μm to over 100 μm. However, all phytoplankton cells contain chlorophyll-a (chl-a) which absorbs light and is used for the conversion of dissolved inorganic carbon (e.g. CO2(aq)) to organic molecules such as carbohydrates, lipids and proteins.2 Although individual phytoplankton are microscopic in size, they are responsible for approximately half of the photosynthesis on Earth, and are the conduit through which energy enters aquatic ecosystems. There is currently active debate regarding the extent to which global phytoplankton biomass may have declined over the previous century;3,4 this has serious implications for the global marine ecosystem. Phytoplankton cells can achieve high densities in the natural environment that alter the colour of the surface ocean which can be viewed in satellite images.5 These images can be used to remotely measure the scale and density of a phytoplankton bloom.6,8

Beyond the use of chl-a fluorescence to estimate phytoplankton biomass, the time variation of the cellular chl-a fluorescence intensity may also be used to yield insight into the photosynthetic performance of phytoplankton.7,10 Fast repetition rate fluorometry11 allows, amongst other parameters, the photochemical conversion efficiency and the photosystem II functional absorption cross section to be analysed. Such cell physiology measurements, in combination with the assessments of cell growth are a common route by which the effects of cellular oxidative stress are investigated.12 In terms of species identification and cell density estimation, both at sea and in the laboratory, flow cytometry is a primary technique;13 where the relationship between side scatter versus fluorescence intensity is used to identify the presence of different plankton species. This procedure is however not without limitations; problems arise when species have similar optical properties or when single species display a wide size range due to cell agglomeration and chain formation.14

Reflecting the fact that phytoplankton occur in a number of taxonomic divisions, their composition, shape and size varies widely between species. For example Emiliania huxleyi is a representative species of calcifying phytoplankton; such plankton produce plates of calcium carbonate inside their cells which are extruded onto their external cell walls. These calcifying plankton species play a critical role in the oceanic carbon cycle.15–18 Despite being energetically costly,19 it has been proposed that calcification offers coccolithophores physical
electrodes.

occurs at significant potentials above +1.4 V (see ESI† Section 1 for further information).

This work starts by studying the response of the diatom H. coffeaeformis in a fluoro-electrochemical cell. Fig. 1 depicts a series of fluorescent microscope images for H. coffeaeformis. The bright features present in the optical field are the phytoplankton present either as individuals or in larger aggregates. These diatoms are held in a thin layer cell (ca. 50 μm depth) which also contains a carbon fibre electrode (electrode radius = 3.5 μm). The cell also contains counter and reference electrodes. A schematic of the cell is shown in the ESI† Section 2 along with full experimental details. These carbon fibre electrodes are non-fluorescent and hence not observable under the epi-fluorescent illumination conditions. In Fig. 1 the position of the carbon fibre wire is indicated by the blue line across the microscope image. Initially a potential of 0.0 V was initially applied to the electrode for 60 s and a series of microscope images recorded at 10 frames per second. At this potential (0.0 V) no Faradaic reactions occur at the carbon fibre electrode. After 60 seconds the applied potential is stepped to an oxidative potential of +2.3 V. Fig. 1 shows a series of images of the thin-layer cell taken at different times after the onset of the higher potential (the full Video can be found in ESI† Section 3). As can be seen from Fig. 1 the fluorescence response of H. coffeaeformis closest to the electrode are ‘switched off’ first and the fluorescence associated with the diatoms further away from the electrode progressively decrease with increasing time. First, a potential above approximately +1.4 V (see ESI† Section 4)

![Fig. 1 Optical images of the chl-α fluorescence of H. coffeaeformis (diatom, CCAP 1001/2) during the fluoro-electrochemical experiment, where f_{\text{on}} is the time which a potential step of 2.3 V (vs. pseudo Ag wire) was applied to the working electrode. $\lambda_{\text{exc}} = 475 \pm 35$ nm and $\lambda_{\text{em}} > 590$ nm. The blue line depicts the location of the carbon fibre electrode. A potential of 0 V (vs. pseudo Ag wire) was applied for $t = 0$–60 s, before the potential steps to 2.3 V for $t = 60$–90 s.](image-url)
needs to be applied to the electrode in order for the fluorescence of the phytoplankton to be affected over the timescale of the experiment (ca. tens of seconds). Second, no change in the fluorescence response of the diatoms was observed if a reductive potential was held on the electrode (studied down to −2.0 V). The following section serves to quantify the rate at which this electrochemically induced change in cellular fluorescence occurs.

The fluorescence response of individual phytoplankton were measured over the entire plankton cell via integration of the microscope image to obtain per plankton mean fluorescence intensities. The perpendicular distances between different plankton and the electrode were also measured and found to be in the range of 2 μm to 330 μm. Fig. 2 shows the integrated fluorescence intensity of five individual and representative H. coffeeaformis cells as a function of time, the fluorescence intensity has been normalised against its value recorded at t − t_{pot-on} = 0 s and each trace in Fig. 2 is for a different diatom. The fluorescent transients depicted in Fig. 2 are for diatoms situated in the range of 10–250 μm away from the carbon electrode. As can be seen, the mean phytoplankton fluorescence intensity can be used to quantify the rate at which the electrode inhibits the chl-a fluorescence signal, for the diatoms their fluorescence intensity decreases dramatically over the course of the experiment.

The inlay of Fig. 2 plots the time at which the phytoplankton fluorescence intensity decrease is initiated as plotted against the distance squared; a clear quasi-linear correlation is recorded; such that, the time taken for the diatom fluorescence to be ‘switched off’ correlates directly with the distance of the individual algal cells from the electrode. The results shown in the inlay of Fig. 2 come from three separate experiments highlighting the reproducibility of the system. Note at longer distances there is a further deviation away from this linearity, see ESI† Section 5 and 8 for further discussion and the data analysis method. From this set of experiments it is clear that the chemical products of the electro-oxidation process diffuse away from the electrode and increasingly inhibit the phytoplankton fluorescence at greater distance from the electrode with time.

An important question is; what is the identity of the electro-generated species causing the change in the phytoplankton fluorescence? Previous work in the literature has shown both in vivo and in vitro that strongly oxidising radical species, such as the hydroxyl radical, can cause the oxidative destruction of chl-a. In this work a number of small oxidising molecules can be produced at the electrode potentials used, including, oxygen (1.9–2.1 × 10^{-9} m^2 s^{-1}),^{44} chlorine (1.38 × 10^{-9} m^2 s^{-1}),^{41} hydrogen peroxide (1.43 × 10^{-9} m^2 s^{-1}),^{42} and hydroxyl radicals (1.3–10^{-9} m^2 s^{-1})^{43,44} all of which have comparable diffusion coefficients. In order to try and identify the active species the in vivo chl-a fluorescence spectra of a H. coffeeaformis culture was studied in homogenous solution as a function of the chemical conditions (see ESI† Section 6). The fluorescence emission peak at 680 nm did not change significantly with the addition of either 0.1 M HCl(aq), H_2O_2 (33%) or 100 mM FeSO_4(aq). However, addition of H_2O_2 (33%) together with 100 mM FeSO_4(aq) (known as “Fenton’s reagent”) led to a significant drop in emission intensity at 680 nm. The Fe^{2+} is well known to act as a catalyst for the breakdown of H_2O_2 to generate reactive oxygen radicals in situ^{46}

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{OH}^- \]  

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Although chlorine can also lead to the oxidative destruction of the chl-a phytoplankton fluorescence signal, work provided in ESI† Section 6 demonstrates how the electrochemical inhibition may also be driven to occur in the absence of solution phase chloride. As the presence of chloride is not essential, it is therefore likely that oxidative radical species are the primary cause of the fluorescence inhibition. Furthermore, despite the fact that Fenton’s reagent forms hydroxyl radicals, this species is highly reactive with a sub-micro second lifetime. \cite{44} Consequently, since the phytoplankton are situated away from the electrochemical interface it is possible that it is the generated products from hydroxyl radical reactions in the fluoro-electrochemical experiment that propagated away to react with the phytoplankton and are the primary cause of the observed fluorescence ‘switch off’. However the direct involvement of very low OH\(^-\) concentration cannot be discounted and so in summary it can be concluded, at present, that OH\(^-\) or products derived from it are responsible for the switch off.

Next, we turn to consider how other phytoplankton species are affected by the oxidation process. First, the fluoro-electrochemical experiment was conducted with a cell containing the diatom H. coffeeaformis (as used above), the
cocolithophore *E. huxleyi* and the dinoflagellate *S. trochoidea*; see ESI† Section 3 for example experimental videos. Fig. 3 shows a plot of the normalised fluorescence intensity, for a representative individual *H. coffeeaformis* and *E. huxleyi*, approximately equidistant from the electrode (ca. 20 μm), plotted against time after onset of the oxidising potential. First, the *H. coffeeaformis* fluorescence (yellow line) starts to decrease only a second after the electrode potential has been switched to +2.3 V. Second, in contrast the fluorescence intensity of the *E. huxleyi* (red line) initially increases slightly but then shows a dramatic decrease in fluorescence intensity after 15 s of electrode potential being changed.

As shown in ESI† Section 7 this rapid drop in fluorescence intensity is a general feature of the *E. huxleyi* response towards the oxidative conditions. These two plankton are present in the same electrochemical experiment and hence the differing behaviour is not related to any variability in the electrochemical conditions. The rapid loss of fluorescence for the *E. huxleyi* after an initial delay most likely arises due to the cells outer structure being initially less permeable to the oxidative species followed by the sudden and rapid breaching of the shell, presumably caused by external chemical attack of the oxidative radical species. This conclusion is corroborated by the recorded microscopic response of the *S. trochoidea* (see ESI† Section 3), this phytoplankter is relatively large compared to the others used in this study with a diameter of >20 μm. Consequently, with the use of 40X objective the internal structure of the plankton can be monitored; the experimental video (ESI† Section 3) shows that the major decrease in the phytoplankton fluorescence occurs after an abrupt change in the phytoplankton structure; which is likely associated with the rupturing of the cells external protection layer. These results are strongly suggestive that difference in behaviour of the species relates not to their size but due to their differing biominal covering or outer membrane structures.

We turn to consider to what extent the sensitivity towards oxidative attack differs between phytoplankton groups. The fluoro-electrochemical experiment described above was undertaken with an additional four different species of phytoplankton. Fig. 4a plots the total collated results for the seven different phytoplankton species studied, here the onset of the fluorescence decrease as a function of time has been plotted against the phytoplankton distance from the electrode squared. Fig. 4a shows that, although for a given species there is a strong correlation between the distance between the electrode and the time at which the fluorescence inhibition onsets, there is a very wide difference in behaviour between the different species. For example on the time scale of this experiment, only ca. 24% of the *E. huxleyi* observed within the field of the microscope were affected by the oxidative potential and all of those affected were within 60 μm of the electrode. In contrast, the fluorescence signals of all of the *S. bacillaris*, within a distance of approximately 100 μm of the electrode, were inhibited within 2 seconds of the oxidising potential being applied to the electrode. Note, the dinoflagellate *S. trochoidea* was also studied within the electro-fluorescence cell. *S. trochoidea* are relatively large and present in solution at low cell densities. Hence, the imaging of only three individual *S. trochoidea* was achieved the results of which are presented in Fig. 4a black squares. For all of the species presented in Fig. 4a – excluding *S. trochoidea*, for which insufficient data could be acquired – the scatter plot for each species is quasi-linear; the 95% confidence interval of a line of best fit is overlaid on the data.

This linearity in the plot of the square of the distance versus time predominantly reflects the fact that the reactive species created at the electrode need to diffuse away from the interface prior to reacting with and damaging the phytoplankton. For a molecule freely diffusing in solution, the mean square distance travelled (\(X^2\)) in one dimension is equal to:

\[
\langle X^2 \rangle = 2Dt
\]

where \(D\) (m\(^2\) s\(^{-1}\)) is the diffusion coefficient and \(t\) is time (s). Consequently, after ten seconds a molecule with a diffusion coefficient of \(1 \times 10^{-9}\) m\(^2\) s\(^{-1}\) will have travelled on average a distance of \(\sim 140\) μm. Such distances are comparable to those we observe optically. In Fig. 4a and using the relationship shown in eqn (3), the inverse of the gradient of the line of best fit can be used to characterise the rate at which the phytoplankton respond to the oxidative potential on the electrode. We term this inverse gradient the ‘susceptibility factor’ and it reflects the rate of mass-transport of the radical species, the used experimental conditions and the susceptibility of the plankton species towards attack from the oxidative radicals. ESI† Section 8 presents a simplified theoretical model exploring the dependency of the response time on the diffusional mass-transport rate and phytoplankton susceptibility and gives physical insight into the underlying processes. Ultimately, as the fluorescence response of the plankton are recorded under the same experimental conditions then the measured \(t\) vs. \(X^2\) gradient for the different plankton can be used to give a relative measure of their susceptibility to strongly oxidising environments. Fig. 4b presents the plot of the relative susceptibility factor for the different phytoplankton,
normalised against the measured rate for the phytoplankton most resilient to oxidative environment in this study, *E. huxleyi*. These experimental values differ by almost two orders of magnitude, such that, the least resilient and hence plankton with the highest susceptibility (*C. bacillaris*) reacts 69 ± 2 times faster than the coccolithophore *E. huxleyi*. First, the data presented in Fig. 4b does not reflect either the size or shape of the phytoplankton. Overlaid on Fig. 4b are schematic diagrams that show the approximate size and morphology of the used phytoplankton species, the diatom *H. coffeaeformis* is the largest phytoplankton for which we have measured a relative susceptibility factor; however, its value is similar in magnitude to significantly smaller plankton. Moreover, the plankton *C. singularis* and *C. volutis*, are of the same genus and have very comparable relative susceptibility factor but they are distinctly different in size. Second, broadly the freshwater species (*C. singularis*, *C. volutis* and *S. bacillaris*) appear to be more susceptible to oxidative attack than the marine species (*E. huxleyi*, *S. trochoidea*, *N. oceanica* and *H. coffeaeformis*). These results further corroborate the conclusion that the difference in the observed fluorescence behaviour predominantly reflects the chemical and physical characteristics of the phytoplankton shell and outer membranes. Consequently, the susceptibility of the phytoplankton towards oxidative radical attack is evidenced to be group specific and reflects the diverse structural properties of the exterior of these microscopic single cell organisms.

**Conclusions**

In this work the chl-a fluorescence of phytoplankton was investigated using a fluoro-electrochemical cell. The phytoplankton were found to be highly sensitive to electrochemically oxidising but not reductive conditions. The *in situ* electrochemically generated radical species irreversibly inhibit the phytoplankton fluorescence. This inhibition rate is evidenced to reflect neither the size nor the shape of the phytoplankton but most likely relates to the physical and chemical properties of the shell and outer membrane of the phytoplankton; the rate determining step is the chemical destruction of the outer cellular structure, once the radicals enter the cell the chl-a fluorescence is rapidly extinguished. Profoundly, the rate at which the fluorescence is inhibited varies between species by almost two orders of magnitude. Beyond, possible biological implications of the differing susceptibility of different plankton group towards extreme oxidative stress, on a pragmatic level the ability to differentiate between phytoplankton on the basis of their oxidative susceptibility has potential application in high-throughput methods for phytoplankton identification and bloom monitoring.

**Conflicts of interest**

There are no conflicts to declare.

**Acknowledgements**

This research was partially funded by St. John’s College Oxford. This project has also received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (APPELS project, grant agreement 681746).

**Notes and references**
