Hydroxyl Radical Production by Light Driven Iron Redox Cycling in Natural and Test Systems

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Abstract

Hydroxyl radicals (HO•) formation during the ligand stabilized iron redox cycling was investigated in synthetic media, expended algal growth media (Talaquil) and field collected fresh water. HO• were selectively reacted in situ with terephthalic acid producing hydroxyterephthalic acid, quantified by fluorescence. FeII-EDTA, photoreactive in dim light, was used as a control to compare how media components influence HO• formation rates. Since HO• is highly reactive, transformation depends on to the number of reactive atoms in a media component. Protoporphyrin IX accelerated HO• formation 20-fold, providing a good model for studying rate accelerating components of algae excretion. The results show that HO• production under usual algal test and natural fresh water conditions differ, the latter being more toxic. They enable more reliably comparison between natural waters and artificial test systems, suggesting more realistic conditions for testing toxicity.

Keywords: Hydroxyl radicals; Photoreactive FeII-EDTA; Iron redox cycling; Algal toxicity test conditions

Introduction

Photoreducing ligand stabilized iron III and its fast reoxidation at pH>7 in the presence of O2 produces Reactive Oxygen Species (ROS) in natural and synthetic fresh water. This redox recycling [1] yields hydroxyl (HO•) and other reactive radicals, generating and transforming toxic compounds and impacting exposed organisms. Both toxicants and organisms influence iron redox cycling, e.g., dissolved FeII reduction is mediated by cell surface reductases, an established iron uptake pathway for plants [2,3] and microorganism [4,5]. Appropriate ligands need always be present. Biological systems produce iron binding ligands, they’re environmentally ubiquitous, e.g., by poly-carboxylates [6], siderophores [7] and human activities [8] (e.g., agricultural fertilizers [9], food fortifiers [10], detergent stabilizers, etc.).

Both the diverse factors influencing iron redox recycling and the fact that the absorption and metabolism of iron, an essential nutrient, depends on it [11] create difficulties impacting environmental toxicity testing and risk assessment. Many studies have quantified ROS in diverse matrices. Hydroxyl radicals are most reactive, toxic and important, since they form readily radicals with other matrix components (e.g., carbon [1], nitrogen [12,13] and sulfur [14]), which are more stable and reactive. E.g., HO• forms carbonate radicals contributing significantly to environmental pesticide metabolism [15]. Reactive photo-induced HO•, O3, and triplet DOM have been quantified in fresh and estuarine waters and presented alongside a kinetic model for xenobiotic solar photo-transformation [16].

To recognize and quantify extracellular iron and ROS dependent effects HO• mediates, the photo-reductive behaviour of FeII-EDTA complex was investigated both with field collected fresh water under natural conditions and in expended synthetic algal growth media in laboratory conditions. Iron is used because it’s the most efficient redox active metal producing ROS and EDTA is most widely used as chelator in consumer goods and industry. Since most metal EDTA complexes, including FeII-EDTA, aren’t eliminated by sewage treatment [17], EDTA is the commonest environmental chelator [18]. Only EDTA is used as a biological growth media metal “buffer” [19], maintaining constant free FeII concentration. The light source (PAR: 130 μEm-2s-1, UV-A: 0.055 mWcm-2) used to photo-generate HO• is highly reactive, details of its formation and reaction mechanism, whether a HO• molecule or a higher valency FeOL-ferryl complex is reacting [20], are still debated. Whatever
species is reactive, it’s here named HO\(^\cdot\) and usually quantified by reaction with such aromatic compounds as benzene and benzoate. Least non-fluorescent hydroxylated aromatics such as phenol and benzophenols are produced, GC or HPLC are used for quantification, at lower detection limits fluorescence \[21\] on a plate reader is more convenient for detecting reaction products.

Terephthalic acid (TA) has been used to measure HO\(^\cdot\) in such matrices as cerebrospinal fluid \[22\] or in water sonolysis \[23\], but has only recently been fully assessed for measuring photo-chemically generated HO\(^\cdot\) \[24\]. Being photo-stable, it’s HO\(^\cdot\) specificity and slight darkening effect render it superior to widely used fluorescein based dyes for reporting HO\(^\cdot\).

Materials and Methods

Chemicals and media

Available analytical grade chemicals were used without purification unless otherwise indicated. Merck CaCl\(_2\)•2H\(_2\)O, MgSO\(_4\)•7H\(_2\)O and NaHCO\(_3\), Aldrich terephthalic acid (TA) and 2-bromoterephthalic acid (Br-TA), Sigma NaOH•H\(_2\)O TraceSelect and FeCl\(_3\)•6H\(_2\)O and Fluka ethylenediaminetetraacetic acid (EDTA) and Protoporphyrin IX (PPIX) were used. 2-Hydroxyterephthalic acid (HO-TA) was synthesized according to Yan et al. \[25\]. Fractions precipitated from synthesis solution were 98%, 95% and 92% pure (analyzed by HPLC and UV spectroscopy). Atlantic Research Chemical HO-TA became available during the study.

FeIIIEDTA was synthesized by adding FeCl\(_3\) solution to acidic EDTA–solution (1:1 molar ratio) in the dark and raising the pH to 6-7 with NaOH after a few hours. The FeIIIEDTA-solution was kept strictly in the dark at 4°C. Various reaction media were used to evaluate components’ influence on HO\(^\cdot\) formation rates. Reaction media made from a stock solution and components cited in the text, yielding a medium containing (mM) HCO\(_3\) (1), Cl (0.5), SO\(_4\)\(^{2-}\) (0.075), Na\(^+\) (1), Ca\(^{2+}\) (0.25) and Mg\(^{2+}\) (0.075) at pH 8.0 ± 0.2, corresponding to natural carbonated water. The medium maintained its pH without further buffered additions. pH measurements were performed initially and after irradiation experiments.

Experiments were also conducted with fresh Talaquil \[26\] and expended Talaquil medium from \textit{Clamydomonas reinhardtii} cultures filtered (0.45 µm) after culture for 8-33 days. River water, mainly groundwater discharge (Chriesbach, CH), was used as the final reaction medium and filtered (0.45 µm), containing (mM) alkalinity (5.0 ± 0.3), Ca (2.8 ± 0.1), Mg (0.63), Na (0.84), K (0.11), DOC (0.165 ± 0.02), total Fe <1 µM, pH 7.9 ± 0.2. Media were stored in the dark at 4°C.

Photoreactions

Philips Tanning fluorescence tubes (16, TL 8W) were used as light source. The irradiance as a function of the distance was measured using Sky Instruments PAR and UV-A sensors (Spectro Sense 2). The energy at the solution surface was 130 μEm\(^{-1}\)·PAR and 0.055 mWcm\(^{-2}\) if not otherwise stated. Reaction media samples (10 mL) were irradiated in 50 mL crystallization dishes washed with 3.5% HCl. The low volume enabled complete light penetration through the sample and a loose glass lid allowed air (O\(_2\)) exchange, for algae illumination in an Erlenmeyer flask.

Unwashed dishes gave higher and unstable background fluorescence. Up to 6 dishes were irradiated together on a non-reflecting support. Aliquots (300 µL) were transferred to plate wells at the corresponding reaction time and covered with a black lid. The samples were measured within 10 minutes and kept in the dark until re-measured at the next sampling time point. Repeated measurements of the same reaction solution showed no change over several hours, proving the reaction was light dependent and stopped in the dark.

Detection of HO-TA

HO-TA fluorescence (λ\(_{em}\)=320 nm) was measured in Greiner, 96 well black plates (Huber, Basel) using a TECAN M200 plate reader. Readings were taken at every other wavelength between λ\(_{em}\}=400-460 nm for a total 30 points. Instrument gain was set for optimal signal to noise ratio, background didn’t exceed 10% of the full count scale. H\(_2\)O UV Raman-activity showed plate reader irradiance varied by ± 10%. Average readings between 408 and 446 nm were used for quantification. Non-irradiated reaction solutions kept in the dark and at the same temperature were used as blanks and subtracted. HO-TA (0-8 nM) in the corresponding media was used to calibrate the fluorescence emission counts by a linear function. HO-TA down to 10\(^{-10}\) M was detectable in samples with a low fluorescence background. The HO-TA (1-25 µM) fluorescence was stable during irradiation in the presence of FeIIIEDTA. HO-TA-rates were calculated as the initial linear increase in concentration over time.

Results

Since TA and HO-TA were reported to be stable under higher light energies \[24\] than used in this study, TA alone, TA either in the presence of EDTA or Fe produced no detectable fluorescence. HO-TA was formed at the same rate from both Br-TA and TA (results not shown). Br-TA wasn’t used as a reporter for HO\(^\cdot\) production due to unknown side reactions and reactivity with other ROS but is representative of the reactions of halogenated herbicides.

Hydroxyl radicals react very fast and non-selectively with media components at close to a speed controlled by diffusion \[24\], the TA-probe reaction depends on media composition. The influence of several components present in natural waters and Talaquil media on TA reacting with HO\(^\cdot\) produced during iron photo-redox cycling were investigated.

Adding such inorganic components as NaCl (≤ 500 mM), KNO\(_3\) (≤ 2 mM) and borate (≤ 5 mM) to the reaction medium didn’t alter HO-TA formation rate but carbonate and phosphate reduced the TA-OH\(^\cdot\)rate three-fold for 2 mM carbonate and two-fold for 25 µM phosphate.

FeIIIEDTA/TA ratios

FeIIIEDTA and TA were applied in different ratios to the reaction medium to determine optimal HO-TA yield. HO-

TA was undetectable at ratios >1 but increasing the excess of TA produced enough. A ten-fold excess yielded maximal HO-TA, greater excesses lowered it (Figure 1). The HO-TA-formation rate also depended on FeIIIEDTA- concentration. Under the same irradiance and identical FeIIIEDTA/TA ratios but increasing FeIIIEDTA concentrations (1-5 µM) yielded higher HO-TA-formation rates. The reproducibility was better for 2 µM FeIIIEDTA so this concentration was used for most of the investigations.

Organic components

Organic buffer salts frequently used in algae growth media at 2-50 mM/L concentrations provide carbon reacting readily with HO\(^\cdot\). The radical capturing properties of the commonest buffers, TRIS and MOPS were investigated, showing decreasing HO-TA formation rate with increasing buffer concentration (Figure 2a). Six- and four-fold molar buffer excess reduced the TA reaction by 50% in both cases. The difference between TRIS and MOPS apparent in Figure 2a is due solely to using molar concentrations. Thus allowing for the number of reactive
C and N atoms (5 for TRIS and 8 for MOPS) gave no difference in TRIS and MOPS reactivity with HO•, (Figure 2b), a 50% rate reduction per 625 µM reactive atoms can be deduced when 4 times as many reactive TA atoms are present. The reduction deviates strongly from a linear behaviour at higher concentrations.

Such naturally occurring organic components as porphyrins are known to act as photosensitizers, so PPIX served as model for side-chain degraded chlorophylls. Low amounts of PPIX gave an acceleration factor of 20 in HO-TA formation (Figure 3).

River water

HO•-formation was evaluated in river water with 0.16 mM natural DOC added and 30 times more carbonate than in AAP (US EPA), 8 times more in TG 201 (OECD) and twice as much as in Talaquil growth media. Despite this higher carbon content, HO-TA formation rate in river water was four times higher. Increasing TA concentrations to match carbon content yielded TA values up to 160 µM, and a three-fold rate increase compared to Chriesbach river water with 10 µM TA.

Spent algae Talaquil medium

The high organic buffer concentration (10 mM MOPS) required adjusting TA-concentration to 500 µM counter HO•. Synthetic reaction medium lacking algae, trace elements and nutrients produced the same HO-TA production as Talaquil containing them but the spent and filtered Talaquil medium gave a 6-times higher HO•-formation rate, this rate increased by a factor of 2.5 when 0.1 µM PPIX was added. Higher concentrations reduced it from the 60 nM/h maximum but it was even higher than without PPIX.

Discussion

The high reactivity of HO• requires sufficient TA-probe concentrations to compete with other components. Although fluorescein based ROS-reporters are widely used, they’re rendered unsuitable by their photo-instability and poor HO• selectivity. The advantages of TA for probing HO• production have been discussed [22], its photo-stability and producing HO-TA in the presence of iron were essential for this work as were its inertness towards other ROS.
and not darkening photo-redox-reactive species (if more than a few percent forms, darkening can arise from HO-TA [24]). Fluorescence is convenient for detecting HO-TA, providing sub-nano- and pico-molar range detection limits with low background fluorescence, as applied to both freshwater and test media. The results revealed that HO$^\bullet$ formation hydroxylating TA-reporter depends on other reacting components, preferably those containing carbon, nitrogen and sulphur. When FeIIIEDTA exceeds TA, the radicals produced prefer to react with another FeIIIEDTA-molecule and EDTA is oxidatively degraded [27], explaining why excess TA was required to convert TA into HO-TA significantly.

Under the same irradiation, adding 1 µmol/L FeIIIEDTA to river water gave a four-fold increase in rate than in the synthetic reaction medium. Due to its 2 mg/L DOC and double [HCO$_3^-$], most HO-radicals are unavailable for reaction with TA, as shown by increasing TA up to a suboptimal 1.8 ratio of TA to DOC-carbon, substantially increasing HO-TA-formation. The concentration of photoactive FeII-complex concentrations in river water are below 1 µM (total Fe $<$ 1 µM) and the possibility of colored DOM producing 1O$_2$ is very low ($E_{280}=0.02$, 1 cm), also 'O$_2$ reacting with TA was reported to be 105 times slower than with HO$^\bullet$ [24]. TA- concentrations far below saturation suggest high rates arise from photo-catalysis accelerating river water components.

PPIX, a good photo-sensitizer [28] also strongly affected HO-TA-formation, whether its rate acceleration arises from improved HO$^\bullet$ formation or 'O$_2$ production was answered by introducing 0.5% methanol, it quenched HO-TA- formation completely (results omitted). The reaction with 'O$_2$ is unaffected by methanol, but efficiently traps HO$^\bullet$, PPIX increases HO$^\bullet$ formation. PPIX didn’t improve the formation rate in spent Talaquil medium as it did in river water, suggesting sufficient rate accelerating components are already present in the medium.

Decaying algae release chlorophylls containing a porphyrin moiety probably coordinating with FeIIIEDTA to form a ternary complex, improving its light harvesting efficiency and HO$^\bullet$ production. To quantify HO$^\bullet$-production, observed HO-TA-formation rates need be transferred into HO$^\bullet$-rates. A detailed mechanistic study of HO-TA-formation from TA and HO$^\bullet$ in water [23] showed side-reactions keep maximal yield at 35%. In oxygenated water, side reactions further reduce yield, they’re usually 15-20% [21]. Side reaction products haven’t been determined in this work but it’s reasonable to assume total HO-TA-yield matches that reported in the literature, so HO-TA rates reported here need multiplying by about 5 or 6 to give corresponding HO$^\bullet$-formation rates. Then HO$^\bullet$-production rates for river water can be calculated as 45 to 54 nmol/h, ~ 100 nmol/h for algal medium and ~ 8 nmol/h for the artificial reaction medium. Spent algal growth medium gave higher rates due to their high algal density and resulting higher concentration of rate accelerating compounds. A 200-400 times higher concentration of reactive carbon as organic buffer countered double HO$^\bullet$ growth medium production, reducing levels far below those of natural waters. Allowing for natural DOC (0.1-1 mM C) quenching HO$^\bullet$ and producing 'O$_2$ and despite lower photo-reactive iron concentration, natural conditions impose a much higher burden of HO$^\bullet$ and other ROS than 10 mM organic salt buffered laboratory test conditions.

Conclusions

Experiments in this study were not optimized for HO$^\bullet$ formation rate but for realistic relative rates under low light conditions for a conservative assessment. TA and Br-TA were used as probes to react with HO$^\bullet$, they represent the susceptibility of aromatic and halogenated aromatic moieties containing toxicant to it. Their TA reaction rates can be anticipated to be representative of environmental toxicants. Using TA to trap HO$^\bullet$ was straightforward, allowing measurement of HO$^\bullet$ production by light driven iron redox- cycling in algal test media and natural freshwater. TA-trapping efficiency in oxygenated water appears constant, enabling reliable calculation of HO$^\bullet$-rates. HO$^\bullet$-production depends on the solutes present. Apart from reactive atoms in molecules, such factors as catalysis need consideration. A combination of redox-active species with particular biological ligands is probable, increasing HO$^\bullet$-production.

Inorganic and organic carbon determine HO$^\bullet$-availability. Usually, algal growth and test media concentrate organic carbon (as buffer salts), rendering more HO$^\bullet$ available in river water than in growth media. This hasn’t been considered and should be allowed for to transfer data reliably between test systems and natural conditions. Recommendations for better agreement are presented. Increasing pH close to its natural equilibrium value associated with natural carbonate, yields pH 7.8-8.2, typical of both fresh- and sea-water, reducing the necessary dissolved organic salts. Decreasing algae density in test experiments would reconcile the two conditions. Decreasing rate accelerating exudate concentrations would better represent real aquatic systems but algal density in test systems will usually be greater which can compensated by lower (1-2 mM) organic salt levels than commonly used (10 nM). Refractive chelated redox-reactive metals are dangerous for microbial communities, a factor which has been vastly underestimated.

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References


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