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# Photochemical effects on the interaction of enzymes and dissolved organic matter in natural waters

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*Abstract*

Extracellular enzymes such as phosphatase (Pase) and glucosidase (Gase) can be inactivated in natural waters through photochemical processes. In this study, we examined the mechanisms involved in enzyme inactivation. We first explored the possibility of direct photoinactivation. The quantum yield spectrum ($\Phi_\lambda$) for the direct photoinactivation of Pase increased exponentially with decreasing wavelength, with a much steeper slope relative to other $\Phi_\lambda$. This, combined with modeled half-life values for direct photoinactivation in excess of 5 d, indicates that direct photoinactivation of Pase by natural sunlight in lakes is negligible. Nonetheless, photoinactivation of enzymes occurred rapidly in light-exposed natural waters and suggested an indirect mechanism. The pH of natural waters greatly affected photoinactivation. In acid humic lake water exposed to ultraviolet radiation, the half-lives of both Pase and Gase were 4 h. The half-life of these enzymes under the same conditions were twofold higher for lake waters obtained from a limed humic lake (8 h). The higher rate of inactivation in acid water was likely caused by a pH-mediated increase in Fe photoreduction and enzyme binding. Solutions of Pase with Fe(II) and $H_2O_2$ resulted in rapid inactivation (half-life 7 min at 8.9 $\mu$mol $L^{-1}$ Fe). There was no significant inactivation of controls without $H_2O_2$, indicating that enzymes are inactivated through Fe(III)/enzyme binding, which is enabled through the oxidation of Fe(II) by $H_2O_2$ to Fe(III). Direct inactivation by reactive oxygen species (ROS) was ruled out by tests with ROS scavengers.

The photolysis of dissolved organic matter (DOM) by sunlight in natural waters can result in the release of DOM-bound inorganic nutrients that can increase microbial growth (Bushaw et al. 1998; Kieber et al. 1999). Bioavailable low–molecular weight compounds such as carboxylic acids and other small organic moities (Kieber et al. 1990; Wetzel et al. 1995; Bertilsson and Tranvik 1998, 2000) are also produced by photolysis of complex DOM. In addition to facilitation of microbial processing of DOM through photolysis of recalcitrant organic moieties, sunlight can also inhibit degradation. A fraction of this photolyzed DOM can be transformed into less available forms by photochemical transformation (Benner and Biddanda 1998; Tranvik and Kokalj 1998; Tranvik and Bertilsson 2001). Direct photochemical damage to the heterotrophic microorganisms can also slow degradation (Herndl et al. 1993). Some of this inhibition might occur through the inactivation of extracellular enzymes released by the microbial community for utilization and incorporation of DOM. These extracellular enzymes play an important role in nutrient and carbon cycling in natural waters (Wetzel 1992; Münster and De Haan 1998). Extracellular enzyme activity of phosphatase, $\beta$-glucosidase, and aminopeptidase in a humic-stained lake were found to be high and was attributed in part to low availability of utilizable DOM (Münster 1991). This suggests that, in aquatic systems with particularly high amounts of recalcitrant organic matter, such as humic lakes, heterotrophic bacteria could be especially dependent on extracellular enzymes for acquisition of energy and nutrients.

Recent studies have provided evidence that the photolysis of DOM by ultraviolet radiation (UV-R) might alter the activity of phosphatase enzymes, but the precise mechanisms behind this process is still unknown (Boavida and Wetzel 1998; Garde and Gustavson 1999; Espeland and Wetzel 2001). Loss of enzyme activity through UV-R–mediated processes could potentially constrain DOM metabolism by bacteria, as well as acquisition of organically bound phosphorus by both algae and bacteria through the use of phosphatase.

The loss of enzyme activity could be especially important in humic-stained lakes and rivers, which, according to the arguments above, could be systems where carbon metabolism is highly dependent on extracellular enzymes. In these waters, high concentrations of DOM and transition metal species result in elevated photochemical activity (Voelker et al. 1997; Gao and Zepp 1998). Gao and Zepp (1998), for example, demonstrated that the presence of high concentrations of Fe and a low pH enhance photobleaching of colored dissolved organic matter (CDOM) and production of $CO_2$, CO, and $NH_4^+$. Similar conclusions were drawn from a correlative study of photobleaching and $CO_2$ production in UV-R–exposed Swedish lake waters (Bertilsson and Tranvik 2000). Low pH and high iron concentration were found to be associated with enhanced photobleaching and photochemical $CO_2$ production. The high photobleaching rate in sys-

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tems with low pH and high Fe concentration likely is due to enhanced hydroxyl radical production. The hydroxyl radical is a powerful oxidant that can be optimally produced through DOM photolysis and the photo-Fenton reaction under acidic high-Fe concentrations (Zepp et al. 1992; Vaughn and Blough 1998; Goldstone et al. 2002).

Thus, we tested this hypothesis using microbial extracellular enzymes in acidic humic systems through photochemical processes. Processes leading to enzyme activity in aquatic ecosystems are extremely complex (Overbeck 1991), and the study of the role of photochemistry in these processes is at its very beginning. Therefore, rather than using field data to make inferences on the possible mechanisms involved in photochemical enzyme inactivation, we used a laboratory-based mechanistic approach with clearly defined controls. We first examined the possibility of enzyme inactivation through direct photolysis by UV-R and then compared the photochemical inactivation rate between waters from a naturally acidic humic lake and another that has been limed repeatedly over a 10-yr period. In addition, we performed a series of experiments to elucidate the mechanisms of enzyme inhibition. Our results demonstrate that extracellular enzymes in natural waters are inactivated through a complex process involving the photolysis of CDOM and oxidation–reduction reactions with iron and $H_2O_2$, and not by reactive oxygen species (ROS).

The humic-stained waters are common not only in Scandinavia but throughout the world. There is an increasing awareness of the complex and major role of humic matter as a regulator of aquatic ecosystems (Hessen and Tranvik 1998; Findlay and Sinsabaugh 2003). This study provided, for the first time, a clearly defined mechanism that described in detail the photochemical inactivation of extracellular enzymes in humic-stained lakes and provided further impetus to study the interaction between biogeochemical and photochemical processes.

## Materials and methods

*Direct photolysis of enzymes*—The quantum yield spectra ($\Phi_\lambda$) for the inactivation of phosphatase (Pase) was obtained by exposing phosphatase (from *E. coli* Sigma P5931) Pase solutions of 3 mg $L^{-1}$ in Milli Q water with a monochromatic light source at several wavelengths. The monochromatic irradiation system (Spectral Energy) consisted of a LH151N/2 housing with a 1,000 W Xe/Hg arc lamp, quartz focusing optics, and an LPS 256 SM power supply. Monochromatic wavebands with a half-bandwidth of 5 nm were obtained with a GM 252 Quarter meter monochromator with a GMA 252-20 grating and a GMA 2560, 90° entrance prism. Samples were placed in a 10-cm quartz cuvette within an RCA 250 sample chamber. Selected wavelengths were 280, 295, 313, and 333 nm. Light intensity was measured with an International Light 1700 radiometer having a UV-R–calibrated probe. The $\Phi_\lambda$ was calculated as the number of moles Pase inactivated per mole of photon absorbed at a specific wavelength and can be represented by Eq. 1.

$$\Phi_\lambda = \frac{\text{Pase*}}{E_{a\lambda}} \quad (1)$$

$\Phi_\lambda$ is the quantum yield for phosphatase photoinactivation, $E_{a\lambda}$ is the photon absorption rate (mol photons absorbed $ml^{-1}$ $s^{-1}$), and Pase* is the phosphatase inactivation rate (mol Pase inactivated $ml^{-1}$ $s^{-1}$). The $E_{a\lambda}$ was calculated using Eq. 2 (Miller 2000).

$$E_{o\lambda}(1 - 10^{-(A\lambda l)})S/V \quad (2)$$

$E_{o\lambda}$ is the irradiance (mol photons $cm^2$), $A$ is the spectrophotometric absorption ($cm^{-1}$), $l$ is the pathlength of the irradiation cuvette (cm), $S$ is the surface irradiation area ($cm^2$), and $V$ is the volume of the irradiation vessel (ml).

*Enzyme inactivation in lake waters*—Samples from the midepilimnion of Lake Skärshultssjön and Lindhultsgölen in southern Sweden were obtained in July 2001 and stored at 4°C (lakes described in detail in Bertilsson and Tranvik [2000]). The two lakes are located in the same area and have similar optical characteristics with $a_{350}$ $m^{-1}$ and [Fe] values of 66.1 and 54.9 $m^{-1}$ and 9.1 and 15.1 $\mu$mol $L^{-1}$ for Lake Lindhultsgölen and Skärshultssjön, respectively. The watershed of both lakes is dominated by coniferous forest and sphagnum bogs, and lakes are oligotrophic with typical summer chlorophyll *a* concentrations of $<5$ $\mu$g $L^{-1}$. Lake Lindhultsgölen differs in that it has been limed repeatedly over a 10-yr period. Before irradiation, water samples were filtered through a 0.2-$\mu$m-pore-size filter and allowed to equilibrate to room temperature. Enzyme solutions of Pase and Gase (from *E. coli* Sigma, P5931 and G451) were added to the lake water samples in 50-ml quartz cuvettes at concentrations of 0.3 and 0.9 mg $L^{-1}$, respectively, and irradiated in a solar simulator for 0.5–6.5 h. The activity levels of 15 and 30 $\mu$mol $L^{-1}$ 4-methylumbelliferone (MUF) $h^{-1}$ used in these experiments are high but similar to reported values in the natural environment (6 $\mu$mol $L^{-1}$ MUF $h^{-1}$, Boyer et al. 1997).

The solar simulator was a Q-Sun 1000 test chamber (Q-Panel Lab Products) with a full-spectrum 1,000-W Xenon arc lamp equipped with a temperature-regulated 280- by 400-mm water-filled aluminum block capable of holding 18 (50 mm depth; 35 mm diameter) 50-ml quartz cuvettes. Irradiance was monitored and controlled with a CR 20 calibration radiometer (Q-Panel Lab Products) that was set at 0.65 W $m^{-2}$ at 340 nm. The inactivation rate was calculated by measuring the activity over time, fitting the data to an exponential decay equation, and using the slope of the equation as the loss rate.

*Enzyme inactivation by ROS*—The role of indirect photochemical processes by ROS on the activity of Pase was also investigated through the use of ROS scavengers and enzymes. The ROS scavengers, furfuryl alcohol (200 $\mu$mol $L^{-1}$) and mannitol (100 $\mu$mol $L^{-1}$) that efficiently scavenge singlet oxygen ($^1O_2$) and hydroxyl radical ($^{\cdot}OH$) were added to natural water samples and irradiated for 2 h. Catalase, peroxidase, and superoxide dismutase (SOD) also were added at concentrations of 130 mg $L^{-1}$ (2,330 units $mg^{-1}$), 20 mg $L^{-1}$, (200 units $mg^{-1}$), and 1.3 mg $L^{-1}$ (5,000 units $mg^{-1}$) to eliminate hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^{\cdot-}$). In a separate experiment as an enzyme activity control, Pase solutions in Lake Skärshultssjön water were irradiated for 3

h with albumin at concentrations ranging from 2 to 400 mg $L^{-1}$. The irradiation experiment with ROS scavengers and enzymes was also repeated but with the more environmentally realistic concentrations of catalase and SOD of 1.0 mg $L^{-1}$ and 0.4 mg $L^{-1}$, respectively. The ROS scavengers do not absorb light above 290 nm and thus are not involved in any direct photochemical reactions (Scully et al. in press).

*Enzyme analysis*—Phosphatase analysis was modified from the method of Pettersson and Jansson (1978) and was measured with a Turner Designs model TD-700 fluorometer with a standard PMT, near-UV lamp (P/N 10-049), and long-wavelength UV filter kit (P/N 7000-967). The increase in fluorescence over time was measured for a 7.76-ml sample containing 40 $\mu$l of 2 mol $L^{-1}$ Tris pH 8.3 buffer (Sigma T5128) and 200 $\mu$l of 48 $\mu$mol $L^{-1}$ 4-methylumbelliferyl phosphate, free acid (Sigma M8883) in 0.05 mol $L^{-1}$ Tris buffer at pH 8.3. The activity of $\beta$-glucosidase was obtained using identical procedures and instrument configuration as Pase analysis, but with the free acid fluorophore 4-methylumbelliferyl glucose (4-MUG) at a concentration of 200 $\mu$mol $L^{-1}$. Enzyme activity was estimated as production ($\mu$mol $L^{-1}$ $h^{-1}$) of 4-methylumbelliferone (Sigma M1508), from a 10–1,000 nmol $L^{-1}$ calibration curve. Phosphatase activity measurements for quantum yield data were obtained using a Turner Designs model 10 fluorometer.

*$H_2O_2$ and Fe analysis*—Fe photoreduction and $H_2O_2$ production rates were also measured in separate experiments but under identical irradiation conditions for both Lake Skärshultssjön and Lindhultsgölen. Ferrous iron was measured according to the method outlined by Voelker and Sulzberger (1996). Briefly, 500 $\mu$l of 2 mol $L^{-1}$ $H_2SO_4$ was added to a 5-ml sample followed by 500 $\mu$l of 10 nmol $L^{-1}$ ferrozine and 1.0 ml of acetate buffer (13.2 ml $NH_4OH$ and 2.4 ml glacial acetic acid to 100 ml Milli Q water). The absorbance at 562 nm was then read with a Perkin Elmer Lambda 40 spectrophotometer. For total iron measurements, 500 $\mu$l of acid reducing agent (0.03 M $NH_2OH$ HCl in 4 mol $L^{-1}$ HCl) replaced the $H_2SO_4$. Calibration curves were obtained from a series of $FeCl_2$ concentrations.

$H_2O_2$ was determined by measuring the peroxidase catalyzed oxidation of *N*-acetyl-3,7-dihydroxyphenoxanine (Amplex Red from Molecular Probes) (Tranvik and Kokalj 1998). Eighty microliters of 0.25 mol $L^{-1}$ phosphate buffer and 40 $\mu$l of 1 g $L^{-1}$/4 g $L^{-1}$ Amplex Red/horseradish peroxidase working solution were added to a 680-$\mu$l sample and incubated at room temperature in the dark. After 5 min, 200 $\mu$l of the solution was injected into a 250-$\mu$l cuvette, and the fluorescence was measured at an excitation wavelength of 560 nm and emission wavelength of 585 nm. Working solutions of Amplex Red/horseradish peroxidase were prepared before analysis by adding 200 $\mu$l of 5 g $L^{-1}$ Amplex Red in dimethyl sulfoxide to 1 ml of 0.5 mol $L^{-1}$ phosphate buffer containing 4 g $L^{-1}$ horseradish peroxidase (Sigma P8250). $H_2O_2$ calibration curves were obtained by standard additions to lake water samples previously held in the dark for ~24 h. Standard solutions of $H_2O_2$ were calibrated by measuring absorbance at 240 nm. Fluorescence was measured with a Turner Designs model TD-700 fluorometer with a standard PMT, daylight white lamp (P/N 10-045), and excitation filter (AM-33021-02) and emission filter (P/N 10-052R). In order to obtain an estimate of the binding capacity of Fe(II) and Fe(III) to protein structures, solutions of albumin (400 mg $L^{-1}$) containing 8.9 $\mu$mol $L^{-1}$ of Fe(II) were passed through >50 kDa hollow fiber filters (MicroKros X15S-100-04N) with and without the addition of 10 $\mu$mol $L^{-1}$ $H_2O_2$.

## Results and discussion

*Direct photolysis of enzymes*—The quantum yield of Pase inactivation increased exponentially with decreasing wavelength (Fig. 1). The log slope (s) of the $\Phi_\lambda$ was particularly steep (0.095 $nm^{-1}$) relative to other photochemical quantum yield spectra, such as hydrogen peroxide (0.01 $nm^{-1}$; Yocis et al. 2000). This is not surprising since the Pase enzyme absorption peak is centered within a narrow wavelength range of 270–290 nm, as generally expected for a protein (Fig. 1). The relatively low quantum yield values and light absorption above 300 nm coupled with negligible radiation below 300 nm (Fig. 1) indicate that inactivation through direct photolysis is negligible in natural waters. Irradiations of Pase in Milli Q water with a full-spectrum solar simulator (0.65 W $m^2$ at 340 nm, Fig. 1) support this conclusion. There was no significant loss of Pase activity over dark controls for samples irradiated for 5 h. The activity of the irradiated Pase samples in Milli Q water was 0.93 ($n$ = 3, SD = 0.06) of dark controls. Furthermore, we modeled the half-life due to direct photolysis for Pase exposed to ambient levels of 313 and 333 nm radiation at a 5-cm depth in Lake Skärshultssjön with Eq. 3.

$$t^{1/2} = \frac{([\text{Pase}]/Ea_\lambda)0.5}{\Phi_\lambda} \quad (3)$$

$t^{1/2}$ is the half-life for phosphatase, and [Pase] is the molar phosphatase concentration. The resulting $t^{1/2}$ of 5.4 and 7.4 d were much higher than values of irradiated Pase samples in lake waters (*see below*). The response of glucosidase to direct photolysis was not tested. However, the absorption of enzymes are similar; therefore, the response or lack thereof to direct photoinactivation should also be similar to Pase.

These results are contrary to those of Boavida and Wetzel (1998), in which irradiations with natural sunlight caused significant Pase inactivation. Because light absorption is required for any photochemical reaction to occur, it is possible that their enzyme samples contained light-absorbing organic matter from algal exudates that behaved as photosensitizers and initiated secondary photochemical reactions. This was apparently the case for the studies on an algal phosphatase, which were based on a crude extract from algal cultures and included other cellular components. More surprisingly, the authors also demonstrated enzyme photolysis in a pure extract of *E. coli* phosphatase. Interestingly, in a more recent study by Espeland and Wetzel (2001), phosphatase and glucosidase activity was significantly reduced directly through UV-R irradiations, but only in samples exposed to artificial UV-R. There was no significant reduction in activity for enzyme solutions exposed to natural sunlight. Garde and Gus-

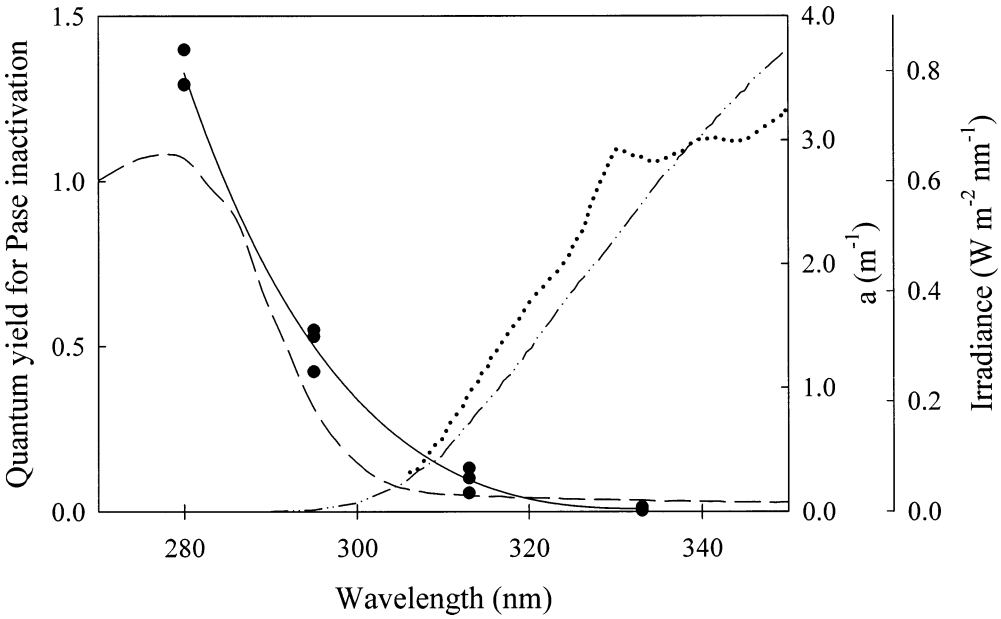


Fig. 1. The quantum yield action spectra for the photoinactivation of phosphatase from 280 to 380 nm (solid line). Absorption coefficient spectrum of the phosphatase from *E. coli* at a concentration of 3 mg $L^{-1}$ (dashed line). Irradiance of Q-Sun 1000 solar simulator (dash dotted line) and typical midsummer solar irradiance (dotted line). Irradiance data was provided by Q-Panel Lab Products.

tavson (1999) found UV-B radiation to be particularly effective in inactivating enzymes. With the use of natural sunlight and enhanced artificial UV-B, the authors demonstrated that UV-R below 320 nm is especially effective in inactivating enzymes. Similarly, the artificial irradiation system of Espeland and Wetzel (2001), consisting of fluorescent and incandescent lights, might have been rich in UV-B radiation relative to other wavebands and not consistent with the radiation spectrum of sunlight.

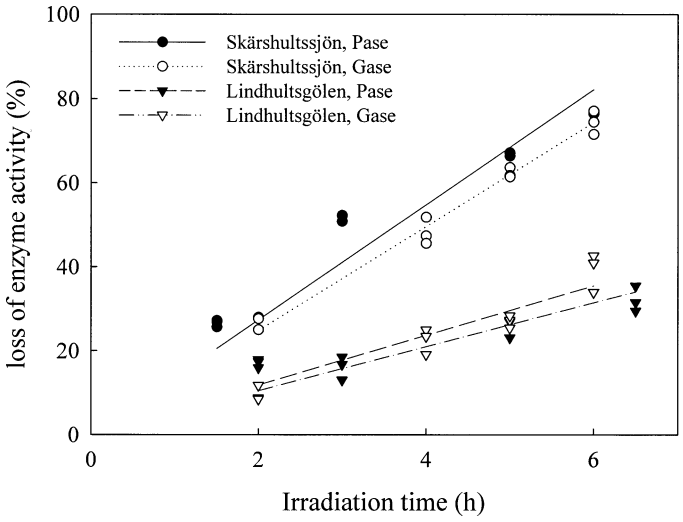


Fig. 2. Phosphatase and $\beta$-glucosidase activity of enzyme solutions in Skärshultssjön and Lindhultsgölen lake waters irradiated with a solar simulator over time.

*Interactions of iron, DOM, and enzymes*—The pH of natural waters greatly affected photoinactivation. Both enzyme activities in an acid humic lake water (pH 6.0) exposed to identical irradiance conditions as in the experiments with Pase in Milli Q water were nearly completely eliminated after 8 h (11% inactivation per hour). The enzyme inactivation rates were twofold lower for lake waters obtained from a limed humic lake (pH 6.9) (5% inactivation per hour) (Fig. 2). Production rates of both $H_2O_2$ and photoreduction of Fe in the two lake waters were also influenced by the pH. The acid humic lake water was characterized by low net $H_2O_2$ production and high Fe reduction, whereas the limed lake water produced $H_2O_2$ at a relatively high rate but the reduction rate of Fe was low (Fig. 3). Although at first glance the difference in pH would seem minor, pH is on a log scale, and small changes in pH can have dramatic effects on metal speciation in natural waters.

*Enzyme inactivation by ROS*—The higher rate of enzyme inactivation in the acid humic lake could be the result of the oxidizing capacity of the hydroxyl radical that can be produced through the photo-Fenton reaction at an increased rate under low-pH conditions. There was an 82% decrease in photoinactivation when samples were irradiated for 2 h with mannitol (100 $\mu$mol $L^{-1}$), furfuryl alcohol (200 $\mu$mol $L^{-1}$), and high concentrations of peroxidase (20 mg $L^{-1}$, 200 U $mg^{-1}$), catalase (130 mg $L^{-1}$), and SOD (1.3 mg $L^{-1}$). However, the addition of albumin at equal concentrations provided similar protective effects. The inactivation of Pase in spiked Lake Skärshultssjön water irradiated for 3 h decreased with the addition of albumin (alb) and was nearly completely eliminated at a concentration of 400 mg $L^{-1}$ (Fig.

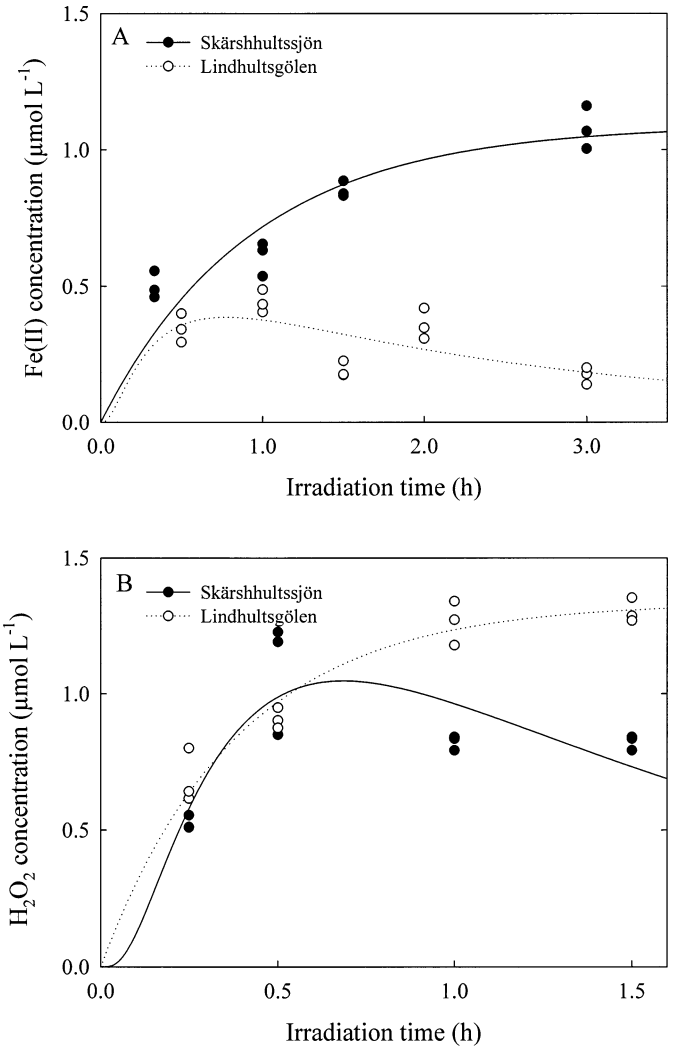


Fig. 3. (A) photoreduction of Fe (B) and production of $H_2O_2$ in irradiated water samples from Lakes Skärshultssjön and Lindhultsgölen.

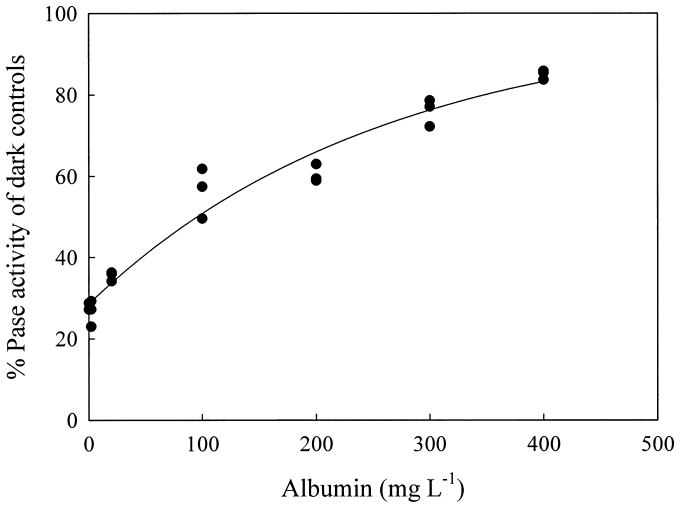


Fig. 4. The phosphatase activity of Pase-spiked Lake Skärshultssjön water irradiated for 3 h with additions of albumin.

4): %Pase = 28.60 + 69.57(1 − exp(−0.0039 × [alb])); $p < 0.0001$. Hence, addition of a nonenzymatic protein had a similar effect. The addition of the furfuryl alcohol (200 $\mu$mol $L^{-1}$) and mannitol (100 $\mu$mol $L^{-1}$) and enzymes catalase and SOD at reduced concentrations of 1.0 and 0.4 mg $L^{-1}$, respectively, did not decrease the inactivation by light of Pase in Lake Skärshultssjön water samples. Accordingly, the results were not indicative of a direct ROS effect because (1) the ROS scavengers were ineffective in decreasing the inactivation of the enzymes and (2) the effect of enzymes that eliminate ROS (peroxidase, catalase, and SOD) was similar to the general effect of adding protein (albumin) to the sample. Furthermore, even if the addition of protein as enzyme or albumin can block the direct effects of ROS, such concentrations do not exist in natural waters. Hence, high concentrations of another protein (albumin) can block the inactivation of enzymes, but apparently in natural waters, this process is not directly mediated by ROS.

*Fe(III) binding inactivation mechanism*—From the studies described above, conducted with pure solutions of enzymes in Milli Q water and ROS scavengers, enzyme inactivation through direct photolysis and reactive oxygen species activity were eliminated as possible mechanisms. We propose instead that extracellular enzymes are inactivated photochemically through a process of binding to Fe. Under this previously untested mechanism, Fe from DOM–Fe(III) complexes is released through photolysis as free Fe(II) (Eq. 4). The photoreduced iron is then oxidized by photochemically produced $H_2O_2$ (*see* Cooper et al. 1994 for reaction mechanism) to Fe(III) and rapidly bound to the enzyme (Eq. 5, enzyme* = active enzyme).

$$\text{DOM–Fe(III)} + \text{enzyme*} + h\nu \rightarrow [\text{DOM}]_{\text{oxd}} + \text{Fe(II)} + \text{enzyme*} \quad (4)$$

$$H_2O_2 + \text{Fe(II)} + \text{enzyme*} \rightarrow \text{enzyme–Fe(III)} + {}^{\cdot}OH + OH^- \quad (5)$$

To further test this hypothesis, we measured the enzyme inactivation rate of Pase solutions containing $FeCl_2$ (0.36–17.9 $\mu$mol $L^{-1}$) with and without $H_2O_2$ (10 $\mu$mol $L^{-1}$). There was a positive relationship between Fe concentration and inactivation rate. Solutions of Pase with Fe(II) and $H_2O_2$ resulted in a maximum inactivation rate of ~0.1 $min^{-1}$ ($t^{1/2}$ = 7 min) at 8.9 $\mu$mol $L^{-1}$ Fe (Fig. 5): Pase* = 0.113(1 − exp(−0.219 × [Fe])); $p < 0.0001$. Enzyme activity for samples without $H_2O_2$ did not decrease below controls (Fig. 5) (Pase* = 0.0056 − 0.0002 × [Fe]; $p = 0.33$) and suggests that Fe(II)–enzyme binding did not occur.

Further support for a Fe(III) binding inactivation mechanism was provided through ultrafiltration of solutions of albumin, which served as a general model for the interaction of proteins (including enzymes) with iron. The ultrafiltration of albumin solutions with Fenton's reagent (8.9 $\mu$mol $L^{-1}$ Fe(II) and 10 $\mu$mol $L^{-1}$ $H_2O_2$) with a >50 kDa hollow fiber filter revealed that Fe(III) was rapidly bound to the protein structure. There was an 81% decrease in total Fe concentra-

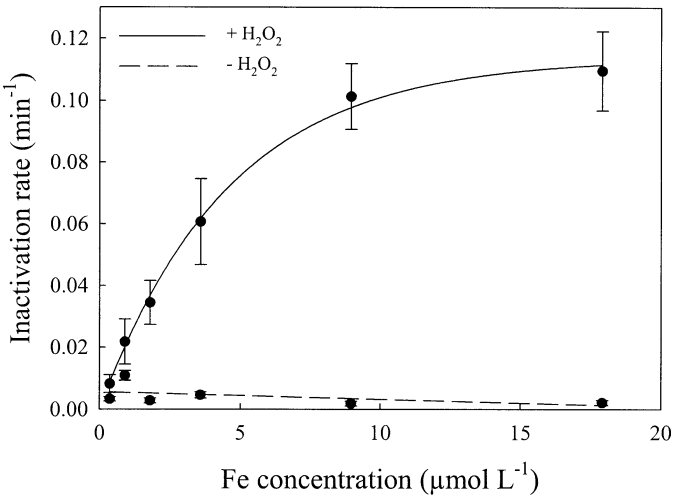


Fig. 5. The inactivation rate of Pase incubated at room temperature with and without 10 $\mu$mol $L^{-1}$ $H_2O_2$ and additions of $FeCl_2$.

tion in the permeate of the $H_2O_2$/Fe(II)/albumin treatment compared to a 38% reduction in the Fe(II)/albumin treatment. Hence, when $H_2O_2$ was present and Fe oxidized, the iron was largely bound to the protein size fraction (>50 kDa), most likely as complexed Fe(III). When $H_2O_2$ was absent, most of the iron remained in the smaller size fraction, probably as free Fe(II). When exposed to sunlight, reduction of Fe will result in both proteins and other DOM being in the free form. When Fe is reoxidized by $H_2O_2$, it will form complexes with organic compounds in the water, including enzymes and other proteins, as well as other DOM components.

*Overall importance of Fe–enzyme complex formation*—The extent of inactivation of enzyme by Fe(III) binding will depend on the concentration of Fe in the water, as well as on the concentration and Fe(III) affinity of other organic compounds in the water. Accordingly, and as shown above (Fig. 4), the presence of other proteins in the water will diminish the extent of inactivation of enzymes. Similarly, humic matter and other DOM compounds, which also form complexes with Fe(III), will provide Fe(III) binding sites that compete with those on the enzymes. Furthermore, acidity of the water will affect enzyme binding patterns, as we demonstrated by comparing an acid and a limed lake. Altogether, this suggests that enzyme–Fe(III) complexes are subject to altered binding patterns as they are exposed to altered water chemistry. When they are transported downstream in surface waters, they are intermittently exposed to sunlight, resulting in reduction of Fe and release of the enzyme, followed by recomplexation when the Fe is oxidized again. If conditions change (e.g., with more alkaline water and altered concentration of humic matter downstream), some of the enzymes that were inactivated upstream through complexation with Fe(III) could remain free after photoreduction of Fe, because of less efficient recomplexation with Fe(III), and again contribute to the enzymatic processing of organic matter in the water.

Concurrent with the photochemically driven changes in the activity of phosphatase and other enzymes, independent photochemical reactions could result in increased availability of phosphate. Accordingly, Francko and Heath (1982) demonstrated that phosphate can be photochemically liberated from DOM–Fe complexes. This release of phosphate might counteract our proposed photochemical inhibition of phosphatases, illustrating the complexity of interactions of solar radiation with aquatic biogeochemical reactions.

Transport of enzymes as complexes was previously suggested by Wetzel (1992). Boavida and Wetzel (1998) suggested a scenario roughly similar to that we propose here but proposed that enzymes bind directly to DOM and are released from DOM photochemically. Recent studies by Espeland and Wetzel (2001) also provided some support for a DOM binding mechanism. The addition of macrophyte leachates and Suwannee River DOM extracts caused both inhibition and stabilization of enzymes depending on the species of macrophyte, the enzymes, and the type of DOM extract (humic or fulvic acid). The authors speculated that the stabilization or inhibition of enzymes depends on the relative location of both the enzyme activity and DOM binding site. They suggested that under a DOM–enzyme complex stabilization scenario, the hydrolytic site of the enzyme remains exposed and active, with the added benefit of protection from denaturing processes. Conversely, they speculated that inhibition of enzyme activity is caused by the blocking of the active hydrolytic site. The possible influence of iron redox reactions was not addressed in either of these experiments. It has been recognized for a long time in plant physiology and soil biology that polyphenolic compounds and humic substances might interact with enzymes and that humic substance–metal–protein interactions are important in these interactions. Accordingly, ''microbial extracellular enzyme—humic substance interactions seem to be more a fact than an exception or vision in most humic waters, but we know rather little about their mechanisms'' (Münster and De Haan 1998). In this paper, we demonstrate a photochemical mechanism for these interactions of DOM and enzymes that also involves metal redox reactions.

We suggest that the influence of DOM on the photochemical effects of enzyme activity is indirect, as a competitor with the enzymes for Fe(III) binding. The inactivation of enzymes through photochemically induced Fe–enzyme binding could be a key process in limiting the supply of bioavailable nutrients and carbon to the microbial community in solar-exposed humic-stained systems, especially at low pH. This negative pressure on the presence of microbial enzymes in natural waters could be a counterbalance to the often positive influence of photochemical processes on microbial activity (Moran and Zepp 1997; Tranvik and Bertilsson 2001). Hence, photochemical and enzymatic processing of DOM might be negatively correlated in time and space, being constrained to shallow, solar-exposed layers and daylight hours and to deeper waters and dark periods, respectively.

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