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Decreased biodegradability of algal DOC due to interactive effects of UV radiation and humic matter

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ABSTRACT: The effect of dissolved humic matter (DHM) and UV radiation of intensities similar to solar radiation on the bacterial degradability of algae-derived dissolved organic carbon (DOC) was examined. A ^{14}C-labeled algal extract from a phytoplankton culture was dissolved in organic-free artificial lake water with or without the addition of humic substances. The water was exposed to UV radiation or kept in darkness for 8 h. Subsequently, an inoculum of lake water bacteria was added, and microbial mineralization to $^{14}CO_2$ was monitored during 8 d in darkness. DHM and exposure to UV radiation had interactive effects on the susceptibility of algal DOC to microbial mineralization. The DOC became less available to the microbes after UV radiation in the presence of DHM. However, it was not affected by DHM in darkness, and there was also no impact of radiation in the absence of DHM. The interactive effects of DHM and radiation did not occur when DHM was applied as an optical filter outside of the experimental vessel. Thus, the effect was independent of the absorbing properties of the colored humic substances and was probably the result of direct physico-chemical reactions between DHM and DOC freshly derived from algae. Our findings suggest a pathway for the production of recalcitrant DOC in pelagic waters.

KEY WORDS: DOC · Bioavailability · Recalcitrance · Diagenesis · UV radiation · Humic matter

INTRODUCTION

Most of the organic matter in aquatic systems is in the dissolved state. Hence, dissolved organic carbon (DOC) is one of the major pools of organic carbon in the biosphere (Hedges 1987). Only a minor fraction of the DOC is readily available for utilization by heterotrophic bacteria, while the remainder is recalcitrant towards microbial degradation (Søndergaard & Middelboe 1995). In lakes and coastal seawater, the recalcitrant DOC is largely comprised of recalcitrant dissolved humic matter (DHM) originating from the lignocellulose of terrestrial plant support tissues. However, even in oceanic environments, where the terrestrial origin of DOC is negligible (Nissenbaum & Kaplan 1972, Meyer-Schulte & Hedges 1986), most of the DOC is generally resistant towards microbial degradation (Søndergaard & Middelboe 1995). The mechanisms that regulate the recalcitrance of DOC are poorly understood.

Because DHM strongly absorbs photons, especially of UV radiation (Frimmel 1994), it takes part in a variety of photochemical reactions. This may be of special importance in surface waters of high DOC concentration, e.g. in humic lakes. Photolysis contributes to the degradation of DHM (Strome & Miller 1978, Kieber et al. 1990, Lindell et al. 1995, Wetzel et al. 1995) and may in this way enhance carbon cycling. In addition, photochemical reactions may also promote interactions with labile DOC. The formation of radicals (Mill et al. 1980, Mopper & Zhou 1990) can lead to reactions between humic substances and other DOC components. Recently, Keil & Kirchman (1994) showed that labile protein can be transformed into more recalcitrant forms in sea water. Sunlight exposure and presence of seawater DOC promoted this 'aging' process. Possibly, similar reactions occur between labile DOC and freshwater DHM in lakes.

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We tested the hypothesis that UV radiation promotes abiotic interactions between DHM and recently produced DOC, which makes the DOC less labile. We used a ^{14}C-labeled algal extract to examine the impact of UV radiation and the presence of humic substances on fresh DOC. Bacterial mineralization of the labeled DOC was used as a measure of biodegradability.

MATERIAL AND METHODS

Isolation of dissolved humic matter. We isolated DHM from water collected in November 1993 in a highly humic pond (108 mg DOC l^{-1}) in southern Sweden. The water was filtered (Whatman GF/F glassfiber filters) and stored in a 5 l polyethylene container in darkness at room temperature until February 1995. Thus, it had been extensively aged to minimize the content of labile organic compounds. Prior to extraction of DHM, flocculates were removed by GF/F filtration.

DHM was adsorbed onto XAD-8 resin (Amberlite) in a column with approximately 35 ml bed volume (Thurman & Malcolm 1981). Before initial use, the XAD-8 resin was rinsed carefully to remove impurities according to Thurman & Malcolm (1981). Before each reuse, the resin was cleaned with 0.1 N HCl and 0.1 N NaOH. After acidification of the sample to pH 1.5 with HCl, about 1.8 l of water was dispensed onto each of 2 columns at a rate of about 6 bed volumes h^{-1}. The DHM adsorbed onto the resin and was eluted with about 60 ml 0.1 N NaOH at the same flow rate. The humic extract was adjusted to pH 7 with 0.1 N HCl and stored chilled until further use.

To remove inorganic ions, we desalted the DHM extract by passage through a cation/anion exchange resin (AG 11A8, BioRad). The DHM extract was pumped through the column (bed volume ca 28 ml) at a rate of 1.5 bed volumes h^{-1}. Conductivity of the effluent was recorded to check the efficiency of the desalting procedure. In the experiments the DHM extract was applied at a concentration of 7 mg DOC l^{-1}, corresponding to an absorptivity of 0.04 cm^{-1} at 430 nm. This is representative of moderately humic boreal lakes.

Preparation of algal DO^{14}C. An algal enrichment culture from a eutrophic pond, dominated by *Chlamydomonas* sp. and other chlorophytes, was grown on a rotary shaker under fluorescent tubes (cool white) in filtered (Whatman GF/F) pond water amended with 2.4 mg N and 0.24 mg P l^{-1}, using solutions of $Ca(NO_3)_2$ and KH_2PO_4. To obtain a uniform labeling of algal cells, cultures in exponential growth were incubated with sodium [^{14}C]bicarbonate (Amersham CFA.3; specific activity 58 mCi $mmol^{-1}$) at a radioactive concentration of 1.45×10^6 dpm ml^{-1} for 3 d. This corresponded to several algal generations.

After incubation, the cultures were sonicated to break the algal cells. Subsequently, the cultures were acidified with HCl to pH 2 and purged with N_2 for at least 60 min to remove remaining inorganic radiocarbon. Samples for $^{14}CO_2$ estimation, taken after 30, 60, and 90 min in a previous test run, showed that all inorganic carbon was removed after 60 min. The algal extract was readjusted with 0.1 N NaOH to pH 7, sonicated again, filtered (0.2 µm Durapore, Millipore Corp.) to obtain a dissolved algal extract, and stored at −20°C until further use. It had a specific activity of 8.9×10^6 dpm mg^{-1} DOC. In the subsequent experiments, it was added at a concentration of 0.2 mg algal DOC l^{-1} (estimated with a Shimadzu TOC 5000 total organic carbon analyzer).

Hydrogen peroxide concentration. The concentration of hydrogen peroxide was derived from the fluorogenic reaction with N-acetyl-3,7-dihydroxyphenoxazine (Molecular Probes), 'APOXA', in the presence of peroxidase. A stock solution of APOXA was prepared by dissolving 1.0 mg of the reagent in 1 ml of DMSO (dimethyl sulphoxide). A fresh working solution of the reagent was prepared just prior to analysis by mixing 25 µl of the APOXA solution and 1 ml of Milli-Q water into 2 ml of horse radish peroxidase (Sigma Type VI, 50 units ml^{-1} in a 0.25 M Tris buffer, pH 7.2). For analysis, 1 ml samples were amended with 30 µl of the working solution, and fluorescence was measured after a few minutes on a Shimadzu RF-1501 spectrofluorometer (excitation 570 nm, emission 585 nm). Concentrations of H_2O_2 were derived from fluorescence, using internal standards. The H_2O_2 stock solution was calibrated from absorbance at 240 nm (molar absorptivity 38.1 mol^{-1} cm^{-1}; Miller & Kester 1988). Blanks were prepared as described by Miller & Kester (1988), using catalase to degrade H_2O_2.

Bacterial mineralization. To investigate the effect of abiotic transformations caused by radiation and humic matter, the algal DO^{14}C was exposed in organic-free artificial lake water (Lehman 1980), with or without the addition of DHM, incubated in the light or in darkness. For the light treatments, 33 ml quartz test tubes were used, while 32 ml borosilicate tubes, darkened with aluminum foil, were used for the dark treatments. The tubes contained 30 ml of water, thus having a small headspace. They were sealed by silicone stoppers penetrated by 2 Teflon tubes, one of which reached into the headspace and the other into the water. The Teflon tubes were sealed by silicon stoppers. Algal DO^{14}C and DHM extract were filter sterilized (0.2 µm, Acrodisc, Gelman). Artifical lake water, glassware, silicone stoppers, and tubing were autoclaved. Syringes and other plastic equipment were sterilized by microwaves (Sandborn et al. 1982). The tubes were placed horizontally in plastic boxes with white bottoms and covered

by a 1.5 cm thick water layer (distilled water). Samples to investigate separately the effect of the absorbing properties of humic substances were likewise covered by a solution of humic substances in distilled water (7 mg DOC l^{-1}). The tubes were irradiated for 8 h at 20°C under a set of Q-Panel UVA-340 lamps. Radiation intensity was measured with a radiometer (International Light Inc., IL 1400 A). PAR (Photosynthetically Available Radiation, 400 to 700 nm) was 6.1 W m^{-2}, UV-A (320 to 400 nm) 20 W m^{-2}, and UV-B (300 to 320 nm) 0.3 W m^{-2}. The PAR intensity was much lower than normal sunlight, while the intensities of UV radiation were comparable to those detected on a sunny day in Lund with the same radiometer (54°N; typically 300 W m^{-2} of PAR, 35 to 40 W m^{-2} of UV-A, and 1.0 to 1.5 W m^{-2} of UV-B). Hence, the photochemical effects are probably not stronger than similar effects in surface waters.

After irradiation, a 5 ml sample was taken from each replicate with a syringe via the Teflon tubes for $^{14}CO_2$ determination. After purging the CO_2 from these samples, their bacterial abundance was checked by epifluorescence microscopy (Porter & Feig 1980). In all samples, bacterial abundance was very low, similar to the abundance in controls consisting of freshly filter-sterilized Milli-Q water, indicating that no growth or contamination occurred before or during the 8 h period of exposure.

One replicate of each treatment was poisoned with formaldehyde (2% v/v, final), and each tube received 1.5 ml of bacterial inoculum. The bacterial inoculum consisted of water from a pond in Lund that had been filtered through Whatman GF/F filters to remove bacterivores, and was amended with NH_4Cl and KH_2PO_4 to reach final concentrations in the experimental tubes of N and P of 2.4 and 0.24 mg l^{-1}, respectively. This is within the range of N and P concentrations typically found in eutrophic lakes. The tubes were then incubated in darkness at room temperature (ca 20°C), and 2 ml samples for $^{14}CO_2$ determination were taken with syringes after 2, 4, 6, and 8 d, or for experiments where no time-course of mineralization is reported, only after 8 d. The $^{14}CO_2$ samples were acidified to approximately pH 1 with 2 N HCl and collected into a CO_2 absorbing solution (Carbosorb, Packard) for analysis of ^{14}C by liquid scintillation counting (Tranvik 1993).

All comparisons of treatments (effects of DHM and radiation) on $^{14}CO_2$ production were made by 1- or 2-way ANOVAs, with repeated measures when applicable. Probabilities of within-subject effects (those involving the repeated measure) were corrected for inherent correlation of repeated measures by Greenhouse-Geisser adjustment factors. Between-subject differences were identified post hoc by Tukey test. All statistics were calculated with SuperAnova (Abacus Concepts, USA) software.

RESULTS

At most about 0.2% of the initially added DOC was mineralized during 8 h of radiation (Fig 1). The production of inorganic carbon was 4 times higher in irradiated treatments than in darkened controls. No effect of humic substances could be detected ($p > 0.05$), while the effect of radiation was significant ($p \leq 0.001$, 2-way ANOVA). Thus, abiotic mineralization of algae-derived DOC was similar in humic and in clear water.

The amount of $DO^{14}C$ that was bacterially mineralized during the experiments was about 2 orders of magnitude higher than the initial abiotic mineralization (Fig. 2). In general, roughly 10% of the added algal DOC was converted to $^{14}CO_2$. The production of inorganic carbon suggested a batch culture pattern, with an exponential phase from the start until Day 6, followed by a stationary phase with essentially no further mineralization taking place. Analysis by 2-way ANOVA showed significant effects of radiation ($p = 0.0001$) and humic substances ($p = 0.0018$), as well as an interaction between the 2 factors ($p = 0.0068$). Subsequently, we performed a separate analysis of the effect of radiation in the samples with or without DHM and the effect of DHM in samples with or without UV treatment. There was no significant effect of DHM in samples that were not exposed to UV radiation ($p = 0.683$, 1-way ANOVA with repeated measures), but this effect was highly significant in exposed samples ($p = 0.0008$). Similarly, the effect of radiation was not as obvious in the absence of humic substances ($p = 0.0807$) as it was when humic matter was present ($p = 0.0012$). Thus, in combination with UV radiation,

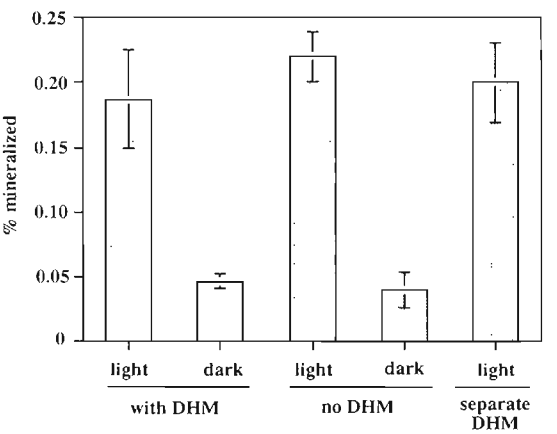

Fig. 1. Abiotic production of $^{14}CO_2$ during 8 h of UV exposure or darkness, as the percentage of added $DO^{14}C$ mineralized. Bars indicate mean values, error bars are standard deviation (n = 4 or 5). Separate DHM (dissolved humic matter) refers to a treatment where DHM was not present in the solution with algal DOC (dissolved organic carbon), but was applied as a separate optical filter

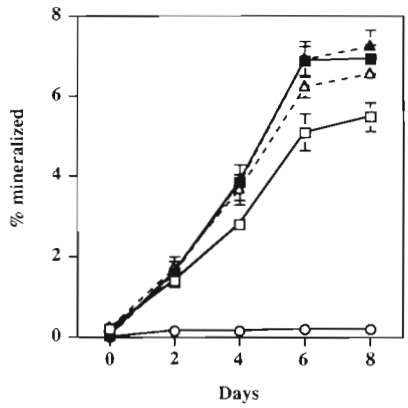

Fig. 2. Microbial $^{14}CO_2$ production from algal DO^{14}C (percentage of added DO^{14}C mineralized), with DHM after UV (□) or dark treatment (■); and without DHM after UV (△) or dark treatment (▲). Error bars indicate standard deviation (n = 5 or 4). Formaldehyde-poisoned samples (○) from all treatments were averaged (n = 4, 1 replicate of each treatment)

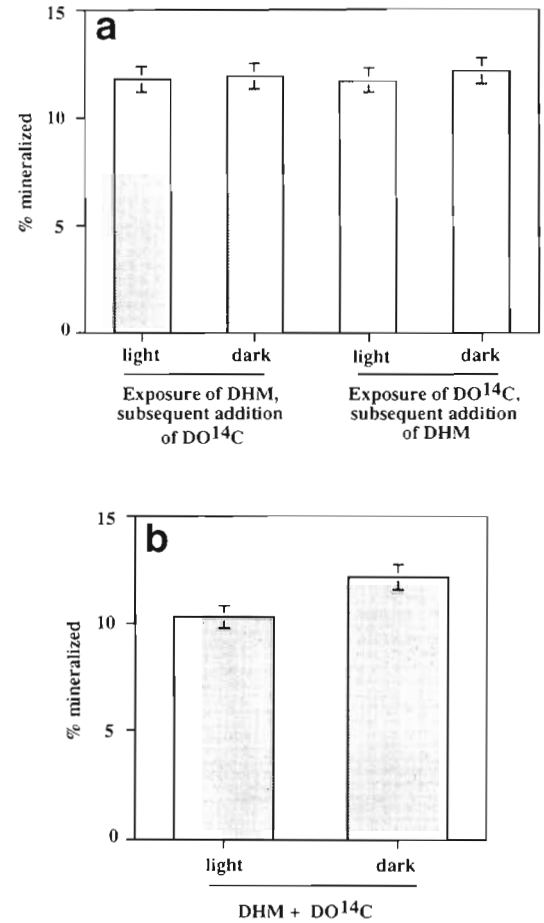

Fig. 3. (a) Microbial $^{14}CO_2$ production (percentage of added DO^{14}C mineralized) from algal DO^{14}C in water with DHM that was previously incubated in the dark or exposed to UV, and in water with algal DO^{14}C that was incubated with UV or in the dark and subsequently amended with DHM. (b) Microbial $^{14}CO_2$ production from algal DO^{14}C that was previously incubated together with DHM in the presence of UV or in the dark. Values are corrected for $^{14}CO_2$ production in formaldehyde-poisoned controls. Error bars indicate standard deviation (n = 5)

humic substances caused significantly lower mineralization of labile DOC.

In a subsequent experiment, we used dark controls and exposed samples of DHM that received algal extract after the exposure period. In addition, we employed dark and irradiated samples of algal DOC that were amended with DHM after the irradiation. After these treatments, the samples were subject to microbial mineralization, and respired CO_2 was measured after 8 d. There were no differences among the 4 different treatments in microbial mineralization (Fig. 3, $p = 0.74$). A subsequent mineralization experiment with an algal DOC-DHM mixture that was either pre-exposed to UV radiation or kept in darkness again indicated decreased mineralization of algal DOC upon UV exposure (Fig. 3, $p = 0.0008$). This further supports the proposition that the inhibitory effect of DHM on the mineralization of the algal DOC was directly dependent on radiation, as well as on immediate co-occurrence with the algal DOC. In the various experiments, the inhibition amounted to 15 to 20%.

To test whether the effect of DHM was due to a chemical interaction of DHM and algal DOC, or if it was due to the absorbing properties of DHM, we compared the mineralization of DOC that was previously exposed to UV together with DHM, DOC that had been exposed but shielded by a separate UV-absorbing filter consisting of DHM, and DOC that had been irradiated in total absence of DHM (Fig. 4). Mineralization was not different in samples without DHM or with DHM as a separate filter ($p > 0.05$), but it was significantly higher in these 2 treatments than in water containing both DHM and algal DOC ($p < 0.05$, 1-way ANOVA followed by Tukey post hoc test). Thus, decreased mineralization occurred only when the algal DOC was in direct contact with the DHM.

Photochemical reactions with DHM result in the production of hydrogen peroxide and other reactive species. A possible reason for the negative effect of DHM upon the degradation of algal DOC is that radicals survive in the water during the microbial mineralization experiments and in this way inhibit bacterial activity. To evaluate this possibility, we exposed DHM to UV and subsequently measured the concentration of hydrogen peroxide, which is the most slowly decaying of the photochemically produced reduced-oxygen species. Almost 0.5 µM of H_2O_2 was present in the samples

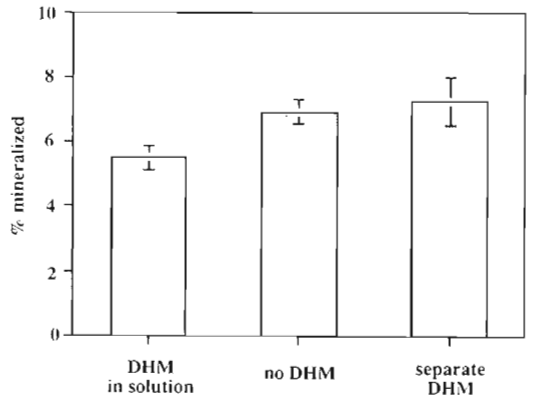

Fig. 4. Microbial $^{14}CO_2$ production (percentage of added $DO^{14}C$ mineralized) from algal $DO^{14}C$ exposed to UV with DHM present in the solution, without DHM, or with DHM as a separate, optical filter. Values are corrected for $^{14}CO_2$ production in formaldehyde-poisoned controls. Error bars indicate standard deviation (n = 5)

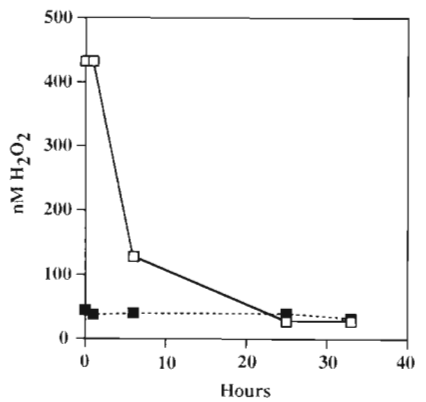

Fig. 5. Hydrogen peroxide concentration in a DHM solution in the dark 0 to 33 h after exposure to UV radiation (□). (■) Controls that were not irradiated

immediately after exposure, but the concentration decreased to a level similar to that in dark controls (ca 30 nM) within 24 h (Fig. 5). The first-order decay rate of H_2O_2 in the dark was 0.09 h^{-1} (calculated as the slope of linear regression of $\ln[H_2O_2]$ vs time, $r^2 = 0.92$) and the half-life of H_2O_2 was 5.5 h.

DISCUSSION

DOC comprises roughly 90% of the total organic carbon in surface waters (Thurman 1985), most of which is recalcitrant. Recalcitrant DOC is formed along several pathways, abiotic as well as biotic (Brophy & Carlson 1989, Tranvik 1993, Keil & Kirchman 1994, Lara & Thomas 1995), and probably combinations of both. Harvey et al. (1983) proposed that marine DHM is formed in water exposed to solar radiation by free radical oxidative cross-linking of fatty acids. Naganuma et al. (1996) noted that the growth of *Escherichia coli* in a peptone medium was reduced by up to 60% when the peptone was exposed to UV-B before inoculation. There was no loss of organic C or N from the peptone during exposure, indicating that the radiation resulted in photoalteration of the peptone. Hence, DOC can be transformed by radiation into more recalcitrant forms, and as suggested by our results this may involve interactions between diagenetically altered DOC (DHM) and freshly produced DOC.

Keil & Kirchman (1994) showed that proteins dissolved in seawater became increasingly recalcitrant towards microbial degradation during incubations in sterile seawater that lasted 0 to 40 d. The rate of this 'aging process' was reduced at decreased concentrations of DOM, suggesting that the aging involved binding to bulk DOM (cf. Carlson et al. 1985). Aging occurred in dark samples, but the rate of aging was enhanced by exposure to sunlight. Our experiments indicate that the presence of freshwater humic matter, in combination with exposure to sunlight in the upper layer of the water column, plays a role in such transformations. Unlike the experiments by Keil & Kirchman, which involved seawater DOM and a specified protein, our experiments with freshwater DHM and a complex mixture of dissolved organic compounds of phytoplankton origin revealed an aging effect of the bulk organic carbon on the fresh DOC only in the presence of radiation.

Contradictory results were presented by Thomas & Lara (1995), who found no UV degradation of aged DOC of algal origin. On the other hand, we found both a UV-enhanced direct abiotic mineralization (Fig. 1), and in the presence of DHM also an effect of UV radiation on the microbial degradability of the algal DOC (Fig. 2). We studied freshly produced algal DOC, while the DOC employed by Thomas & Lara (1995) had been aged in the dark in the presence of microbes for 8 mo. A possible explanation for the different results may be that, during the extensive incubation, heterotrophic bacteria consumed the components of the algal DOC that are susceptible to photolytically induced changes. Thus, certain DOC constituents would be degradable by photolysis as well as by bacteria. Thomas & Lara (1995) measured remaining DOC rather than produced CO_2. In our experiments, the minor change in DOC due to abiotic photolysis (less than 1% of the DOC) would not be detectable as a change in remaining $DO^{14}C$, but was readily seen from the different $^{14}CO_2$ production in dark and irradiated treatments (Fig. 1).

Humic substances efficiently absorb short-wavelength radiation. They have a pronounced impact on the radiation climate of lakes (Scully & Lean 1994,

Morris et al. 1995) and protect aquatic organisms from damage due to UV radiation (e.g. Nielsen & Ekelund 1993). The effect of DHM on microbial mineralization of algal DOC in our study was independent of these optical characteristics, because DHM applied as a filter outside of the experimental tubes had no effect (Fig. 4). In contrast, the abiotic mineralization of DOC was similar regardless of whether DHM was present in solution or applied as a filter (Fig. 1).

Radiation of recalcitrant DOC results in low molecular weight compounds as well as free radicals (Mopper & Zhou 1990) which can react in oxygenated water to form hydrogen peroxide (Frimmel 1994). Hydrogen peroxide is formed in most natural waters when they are exposed to sunlight (e.g. Scully et al. 1996). In our experiments, there was a substantial photoproduction of hydrogen peroxide and a rapid decay (half-life 5.5 h) of the peroxide in the dark. Hydrogen peroxide served as an indicator for the presence of reactive species, while there were probably also more reactive, but much more short-lived, radicals (e.g. singlet oxygen, hydroxyl radical, superoxide radical; Cooper et al. 1989) in the water during and immediately after the exposure to UV radiation. These radicals were probably consumed during reactions with DHM (Frimmel 1994) that may also have included reactions with algal DOC. The radical production can result in the formation of side chains and ring products (Mill et al. 1980) which may bind labile DOC to structures of humic substances. Such a mechanism is in accordance with our result that DHM makes algal DOC less labile upon exposure to UV radiation, but not in the dark.

Within 1 d after exposure, photochemically produced hydrogen peroxide was not detectable (Fig. 5), while the microbes were allowed to metabolize the DOC for a period of 8 d. Hence, it is not likely that peroxide or any of the more short-lived reactive species formed during UV radiation interfered directly with the microbial mineralization of algal DOC. This suggests that the lower mineralization of DOC upon UV exposure in presence of DHM was an indirect effect due to photochemical modifications of the DOC.

In many freshwater systems, the fraction of the recalcitrant DOM that is of phytoplankton origin is probably minor. However, the transformation of recently produced autochthonous DOC into less labile components may be a significant pathway within the fraction of the cycling of organic carbon in these lakes that originates in the indigenous primary production. Fry et al. (1996) found that about one-third of the DOC produced during an experimental coastal diatom bloom persisted for more than 2 yr, with no enhancement of the degradation occurring when organic or inorganic nutrients were added. In a whole lake experiment, Schindler et al. (1992) added ^{14}C-labeled inorganic carbon. Initially, there was a rapid increase in radioactive DOC, due to excretion of ^{14}C fixed during primary production. Most of this DOC was degraded during the following winter and spring, but a substantial fraction remained in the water for at least 1 more year without showing any decreasing trend, except for the loss that could be attributed to hydrologic flushing of the lake.

Consequently, autochthonous DOC can relatively rapidly enter the pool of recalcitrant DOC. Our experiments suggest that sunlight and DHM in combination contribute to the formation of recalcitrant DOC. The observed 15 to 20% inhibition of the mineralization of DOC may have a crucial effect on the transfer of some of the organic carbon from a labile fraction that turns over rapidly to the pool of recalcitrant DOC with long turnover times. Hence, in addition to the extensively documented increased biodegradability of bulk DOC upon UV exposure (Geller 1986, Kieber et al. 1989, Lindell et al. 1995, Wetzel et al. 1995), the UV radiation may also convert initially labile DOC into recalcitrant forms.

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