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# Physiological constraints on bromoform ($CHBr_3$) production by *Ulva lactuca* (Chlorophyta)

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*Abstract*

Physiological factors affecting bromoform ($CHBr_3$) production by *Ulva lactuca* were investigated using metabolic inhibitors and presumed substrates of bromoperoxidase (BrPO). The metabolic inhibitors were used at a verified physiologically active concentration. Bromoform production was nearly tripled in the light (376 ± 92 pg cm$^{-2}$ h$^{-1}$) compared to the dark (114 ± 70 pg cm$^{-2}$ h$^{-1}$), was inhibited in the light in the presence of the photosynthetic inhibitor DCMU, and was inhibited in the dark in the presence of mitochondrial respiratory inhibitor rotenone. Removal of $H_2O_2$ from seawater (treatment with catalase) decreased $CHBr_3$ production in the light and dark. Addition of $H_2O_2$ to incubations at either 1.0 mM or 100 $\mu$M significantly decreased $CHBr_3$ production in the light and inhibited photosynthesis. In the dark, $CHBr_3$ production was decreased and respiration inhibited in the presence of 1 mM $H_2O_2$; $CHBr_3$ production was enhanced and respiration was not affected in the presence of 100 $\mu$M $H_2O_2$. Removal of dissolved organic matter (DOM) from seawater decreased $CHBr_3$ production, as did the addition of alternative BrPO substrates. These results suggest the presence of an extracellular and intracellular BrPO that protects the alga from both internally produced and externally present $H_2O_2$. The results show that $H_2O_2$ produced as a result of photosynthetic and respiratory electron transport, presumably by superoxide dismutase, is available to BrPO for bromination, and that the bromination of an unidentified metabolite (presumably $\beta$-keto acids) and a component of DOM leads to the production of volatile polybromomethanes.

Bromoform ($CHBr_3$) is an important source of tropospheric organobromine that can penetrate into the stratosphere in chemically significant amounts, in spite of its relatively short atmospheric lifetime (Sturges et al. 2000). Over the past decade, marine algal production of bromoform and other polyhalomethanes have been repeatedly demonstrated and it has been estimated that marine algae produce approximately 70% of global bromoform (Carpenter and Liss 2000).

Bromoperoxidase (BrPO) has been identified as the brominating enzyme. Its activity requires $H_2O_2$, bromide ion, and a suitable organic substrate. Theiler et al. (1978) proposed a mechanism for polybromomethane biosynthesis that involved the bromination by BrPO of $\beta$-keto acids (e.g., 3-oxooctanoic acid) and cyclic $\beta$-diketones that are found in marine algae. Bromination produces an intermediate brominated heptanone that yields $CH_2Br_2$, $CHBr_3$, and 1-pentyl bromide on hydrolysis. Wever et al. (1991) proposed that $CHBr_3$ is produced in situ from components of DOM (dissolved organic matter) that are brominated by a reaction with hypobromous acid (HOBr), which itself is a product of BrPO activity.

In vivo bromination of phenol red by *Ascophyllum nodusum* was shown to be enhanced in the presence of exogenous $H_2O_2$ (Wever et al. 1991). Similarly, bromoform ($CHBr_3$) production was enhanced with the addition of $H_2O_2$ to incubations of various red and brown seaweeds, and decreased with its removal (Collen et al. 1994; Pedersen et al. 1996). Potential sources of $H_2O_2$ for algal BrPO activity include the Mehler reaction of photosynthesis (PS), mitochondrial respiration (*R*), enzymatic catalysis of certain $H_2O_2$ yielding reactions (e.g., glycollate oxidase of photorespiration), and illuminated seawater. The purpose of our study was to determine the relative contributions of PS and *R* on $CHBr_3$ production and to determine if in vivo $CHBr_3$ production was influenced by the addition or removal of substrates (e.g., $H_2O_2$, organic substrates, DOM) using the green macroalga, *Ulva lactuca.* Such information will lead to a refinement of global production estimates.

## Materials and methods

*Determination of $CHBr_3$ Production by* U. lactuca—The green seaweed *U. lactuca,* collected at Alamitos Bay Marina from April 1996 through September 2000, was used as the experimental organism. *Ulva* has several characteristics that make it the marine macroalga of choice for studying the physiology of $CHBr_3$ production. *Ulva* is a prolific producer of $CHBr_3$ (Gschwend et al. 1985; Manley et al. 1992; Nightingale et al. 1995). It lacks the peroxisomal enzyme, glycollate oxidase, and contains little or no catalase (Tolbert 1980; Pedersen et al. 1996). Therefore, *Ulva* does not produce photorespiratory $H_2O_2$ and relies mainly on peroxidases (presumably BrPO) to dissipate deleterious levels of $H_2O_2$. Being a thin, distromatic species should facilitate the penetration of $H_2O_2$ (Pedersen et al. 1996) and various metabolic effectors throughout the tissue.

Rectangular tissue samples of known area (72 cm$^2$) were cut from the collected algae. The tissue was placed in a serum bottle (160 ml) with 140 ml of filtered (0.45 $\mu$m pore diameter), off-shore (3.2 km from Long Beach Harbor, CA)

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surface seawater and sealed with a rubber stopper. The serum bottles ($n = 5$) were incubated with shaking for 2 h in the light (530 $\mu$mole photons $m^{-2}$ $s^{-1}$, cool white and grow-lux combination light bank) and 4 h in the dark, in a temperature-controlled incubator (16°C). Seawater blanks were incubated as controls. The incubations were terminated with the quick removal of tissue and the addition of 15 ml *n*-pentane (pesticide grade; Fisher Scientific). The bottles were shaken vigorously for 1 min and allowed to settle for phase separation (5 min). A GC-ECD (Perkin-Elmer 3920, with a Valco Inst. 140 BN ECD) was used to measure $CHBr_3$ in 25–100 $\mu$l of the pentane extract (Manley et al. 1992). The extract was injected into a stainless steel column (20% SP2100 on 80/100 Supelcoport) and carried in argon : methane (95% : 5%; 25 ml/min flow rate) isothermally at 110°C. Peaks were identified by comparison to known standards prepared in pentane and quantified by an integration of peak area (HP 3390A). Chromatographic results were normalized to pg $cm^{-2}$ $h^{-1}$. Eighty percent of the seawater $CHBr_3$ partitioned into the pentane in the first extraction. An additional 16% of the original seawater $CHBr_3$ partitioned into the pentane in the second extraction. Production values were calculated from single extractions assuming 80% recovery.

To ensure that photosynthetically produced $H_2O_2$ was not a factor in the short-term dark incubations, tissues were incubated in continuous darkness for 14 d. Respiration (*see section below*) and $CHBr_3$ production were measured daily. Based on this experiment, tissue samples were preincubated in the dark for 4 d before dark incubation under experimental conditions to ensure that any $CHBr_3$ production seen in the dark was not a result of residual $H_2O_2$ from PS.

*Effects of metabolic effectors on PS, R,* and $CHBr_3$ *production*—Prior to determining whether various photosynthetic and respiratory effectors influenced $CHBr_3$ production by *Ulva,* the effective concentration of a given effector on PS (532 $\mu$mole photons $m^{-2}$ $s^{-1}$) or *R* was determined by measuring DO (dissolved oxygen) production or consumption, using a YSI 5331 oxygen probe and a YSI Model 58 DO meter (Arnold and Manley 1985). *U. lactuca* was incubated in DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea; Diuron®] dissolved in seawater (final concentration 50 $\mu$M) for 2 h in the light. *Ulva* was incubated separately in DNP (final concentration 200 $\mu$M) and rotenone (final concentration 55 $\mu$M) for 4 h in the dark. DCMU and DNP were delivered to the seawater dissolved in ethanol ($[EtOH]_{final}$ = 0.3ppt [v/v]). Rotenone was delivered to the seawater dissolved in acetone ($[acetone]_{final}$ = 0.3ppt [v/v]). *Ulva* was then incubated as previously described: $CHBr_3$ production in the presence of these metabolic effectors was compared to $CHBr_3$ production in the absence of the effector. Tissues used in the DNP and rotenone experiments were preincubated in the dark for 4 d. Controls containing tissue and solvents only (ethanol or acetone) were also incubated. Rotenone, a respiratory inhibitor, was added to catalase (500 U $ml^{-1}$) treated seawater to ensure no $H_2O_2$ was present for BrPO activity (*see below*).

*Effects of extracellular $H_2O_2$ on PS, R,* and $CHBr_3$ *production*—Bromoform production by *Ulva* in the light (2 h) and the dark (4 h) in the presence of added extracellular $H_2O_2$ (1 and 0.1 mM) was compared to control tissue incubations (no added $H_2O_2$). *Ulva* was separately incubated for 2 h in the dark in seawater at $H_2O_2$ concentrations between 50 $\mu$M and 1 mM after which the tissue was rinsed in seawater and the rate of PS and *R* determined and compared to a control (tissue not treated). The effect of each $H_2O_2$ concentration ($n = 5$) was determined along with a paired control ($n = 5$).

Hydrogen peroxide, present in seawater and thus potentially available to BrPO, was removed from the seawater with treatment of catalase for 16 h prior to use (3500 units $mg^{-1}$ protein, Sigma Chemical; final concentration 500 U $ml^{-1}$). Bromoform production by *Ulva* was determined in the light and dark in seawater treated with catalase and compared to $CHBr_3$ production in untreated seawater ($n = 7$). Treated and untreated seawater was refiltered (0.45 $\mu$m pore diameter) prior to use and the catalase remained in the seawater during the incubations.

*Effects of BrPO substrates on $CHBr_3$ production*—Presumed BrPO substrates were added to the seawater to determine whether they enhanced or inhibited $CHBr_3$ production. Bromoform production in the light and the dark by *Ulva* exposed to oxaloacetic acid (OAA; final concentration 1 mM), $\beta$-ketoadipic acid ($\beta$-KAA; final concentration 0.5 mM), and phenol red (phenol-sulfonphthalein; final concentration 1 mM) was compared to $CHBr_3$ production in the absence of these possible substrates. To remove extracellular organic compounds available for bromination by BrPO, filtered seawater was treated with activated charcoal (20–40 mesh, Matheson, Coleman, and Bell). Charcoal was added to seawater and filtered (Whatman 1 paper filters). This process was repeated twice more with a final filtration through a membrane filter (0.45 $\mu$m pore diameter). Bromoform production by *Ulva* in charcoal treated seawater was compared to $CHBr_3$ production in untreated seawater.

*In situ bromoperoxidase activity*—The modified protocol of Wever et al. (1991) was used to measure BrPO activity. One circular disc of *Ulva* tissue (surface area = 56.5 $cm^2$) was placed in a capped test tube containing 25 ml of 25 $\mu$M phenol red seawater and exposed to 1,444 $\mu$mole photons $m^{-2}$ $s^{-1}$ of light. Test tubes were continuously rotated in the incubator (16°C) for 2 h. Tissue discs were separately incubated in DNP (final concentration 200 $\mu$M), rotenone (final concentration 55 $\mu$M), OAA (final concentration 1 mM), and varying $H_2O_2$ concentrations (0, 0.5, 1.0, and 1.5 mM) in seawater containing phenol red. DNP was delivered to seawater in ethanol, and rotenone was delivered to the seawater in acetone. Therefore, these solvents were separately incubated with *Ulva* as controls. Aliquots (500 $\mu$l) from each test tube were taken at the start of the experiment and at half hour intervals to 2 h. Morpholinoethane-sulfonic acid (250 $\mu$l) was added to the aliquot to make the final concentration 0.1 M and to bring the samples to pH 6.5. Absorbance was measured at 433 nm to determine the decrease in phenol red as it was brominated by BrPO to tetrabromophenol blue using the extinction coefficient of 19.7 $mM^{-1}$ $cm^{-1}$ (Wever et al. 1991). Absorbance at 592 nm ($\epsilon$ = 67.4 $mM^{-1}$ $cm^{-1}$;

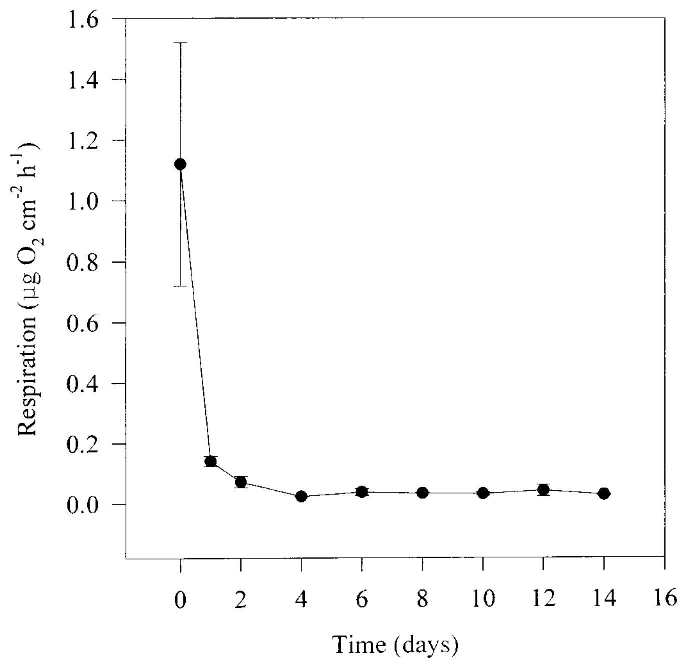


Fig. 1. Effect of continuous darkness on *Ulva* respiration. Brackets = ±s.d. ($n = 3$).

Wever et al. 1991) was also measured to determine the formation of tetrabromophenol blue; however, measurements at this wavelength were less sensitive because of the requirement of the substrate to remain in the active site for four bromination steps. The two data sets were in agreement.

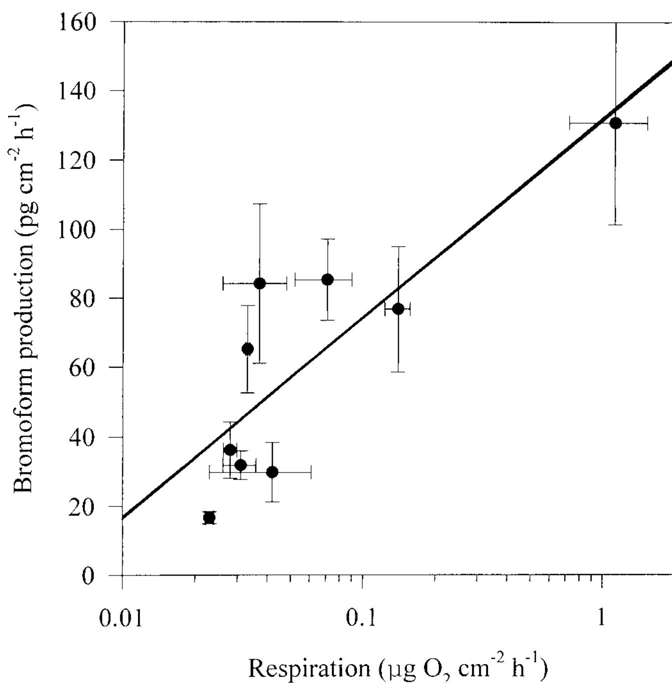


Fig. 2. Bromoform production versus log of respiration over 14 d of continuous darkness; same experiment as in Fig. 1. Brackets = ± s.d. ($n = 3$). Line is from regression analysis, $r^2 = 0.70$.

Table 1. External effectors influence on photosynthesis by *U. lactuca* ($n = 3$).

| Effector | Relative photosynthesis* | Significant from control ($p < 0.05$) |
|---|---|---|
| Control† | 100% | — |
| DCMU (50 $\mu$M)‡ | −14% | y |
| EtOH (0.3 ppt, v/v) | 84% | n |

* Percent $O_2$ production in the light (532 $\mu$mole photons $m^{-2}$ $s^{-1}$).
† Tissue with seawater only (mean = 18.7 $\mu$g $O_2$ $cm^{-2}$ $h^{-1}$).
‡ $O_2$ consumed.

## Results

*Bromoform Production by* U. lactuca—The mean of all the $CHBr_3$ production rates by *Ulva* over the entire several year long study was 281 ± 407 pg $cm^{-2}$ $h^{-1}$ in the light ($n = 66$), and 64 ± 102 pg $cm^{-2}$ $h^{-1}$ in the dark ($n = 65$; with no dark preincubation). These means are statistically different ($p = 0.0001$). No temporal variation was observed. Direct comparison experiments found $CHBr_3$ production rates in the light consistently and significantly higher than $CHBr_3$ production rates in the dark. Minimal background $CHBr_3$ was observed in seawater controls. Tissue incubated in the dark for 14 d showed a leveling off of *R* after 4 d (Fig. 1). Bromoform production also showed a decrease in production over the 14-d dark incubation period and was linearly correlated to the log of *R* ($r^2 = 0.70$; Fig. 2).

*Effects of metabolic effectors on PS, R,* and $CHBr_3$ *production—Ulva* PS ($O_2$ production) was significantly inhibited by DCMU while its solvent, EtOH, had no significant effect (Table 1). Respiration ($O_2$ consumption) was significantly inhibited by rotenone and stimulated by DNP (Table 2). While DCMU significantly decreased $CHBr_3$ production in the light, the presence of DNP and rotenone significantly decreased $CHBr_3$ production in the dark (Table 3). The controls containing ethanol or acetone had no effect on $CHBr_3$ production.

*Effects of extracellular* $H_2O_2$ *on PS, R,* and $CHBr_3$ *production*—Addition of $H_2O_2$ at either 1.0 mM or 100 $\mu$M to incubations significantly decreased $CHBr_3$ production in the light (Table 4). In the dark, $CHBr_3$ production was significantly decreased in the presence of 1 mM $H_2O_2$ but enhanced

Table 2. External effectors influence on respiration by *U. lactuca* ($n = 3$).

| Effector | Relative respiration* | Significant from control ($p < 0.05$) |
|---|---|---|
| Control† | 100% | — |
| DNP (200 $\mu$M) | 189% | y |
| Rotenone (55 $\mu$M) | 36% | y |
| Acetone (0.3 ppt, v/v) | 90% | n |

* Percent $O_2$ consumption.
† Tissue with seawater only (mean = 1.2 $\mu$g $O_2$ $cm^{-2}$ $h^{-1}$).

Table 3. External effectors influence on $CHBr_3$ production by *U. lactuca* ($n = 5$). The effect of each compound was determined in a separate paired experiment with a control (no effector present). The relative production was determined by comparison with the paired control and then adjusted by comparison with the light control (376 pg $CHBr_3$ $cm^{-2}$ $hr^{-1}$; 532 $\mu$mole photons $m^{-2}$ $s^{-1}$) below. EtOH (0.3‰ v/v) and acetone (0.3‰ v/v) had no effect. All effectors were significant from paired control ($p < 0.05$).

| Effector | Relative $CHBr_3$ production |
|---|---|
| Light (control) | 100% |
| DCMU (50 $\mu$M) | 47% |
| Dark* | 41% |
| Dark† | 9.2% |
| DNP (200 $\mu$M)† | 2.6% |
| Rotenone (55 $\mu$M)† | 3.5% |

* No dark preincubation.
† After 4 d dark preincubation.

in the presence of 100 $\mu$M $H_2O_2$ (Table 4). The removal of $H_2O_2$ from seawater with catalase prior to incubation of *Ulva* lowered $CHBr_3$ production in the light and in the dark (Table 4).

Inhibition of PS was detected at $H_2O_2$ concentration $\geq$49 $\mu$M, with significant inhibition occurring at a concentration $\geq$196 $\mu$M (Fig. 3). At 290 $\mu$M $H_2O_2$, PS was completely inhibited and respiration ($O_2$ consumption) was observed. Respiration was significantly inhibited at $H_2O_2$ concentration >390 $\mu$M and was not completely inhibited at 980 $\mu$M (Fig. 3).

*Effects of BrPO substrates on $CHBr_3$ production*—Addition of potential BrPO substrates, OAA and $\beta$-ketoadipic acid, was anticipated to cause an enhancement in $CHBr_3$ production. Instead they caused a decrease in $CHBr_3$ production in the light and in the dark as did the presence of phenol red (Table 5). The removal of DOM from the seawater with activated charcoal prior to the incubation of *Ulva* decreased $CHBr_3$ production >80% in the light (Table 5).

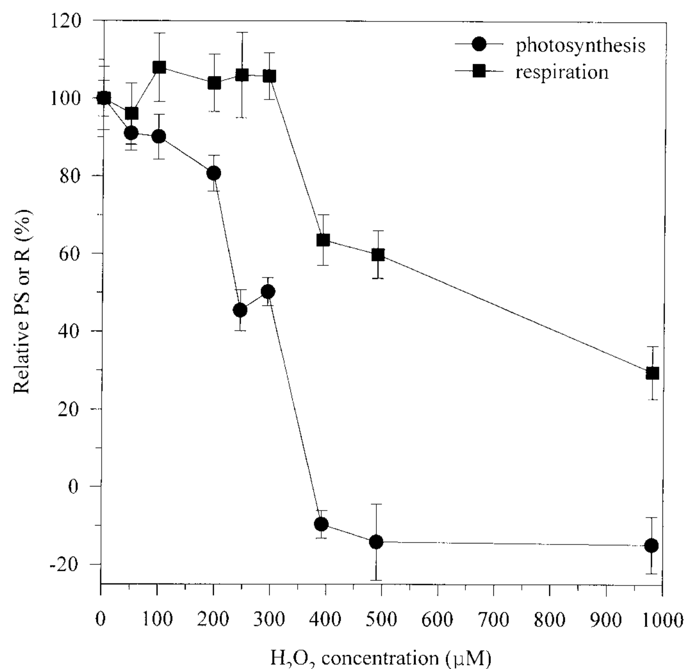


Fig. 3. The effect of exposure to $H_2O_2$ on *Ulva* photosynthesis and respiration. Brackets = $\pm$s.d ($n = 4$). Each point is based on a paired experiment (treatment and control). The treatment is expressed as a percent of the paired control. Significant differences in photosynthesis between the treated and control tissue occurred at $[H_2O_2] \geq 196$ $\mu$M, and at $[H_2O_2] > 390$ $\mu$M for respiration.

*In situ bromoperoxidase activity*—The BrPO in situ assay determined that DNP and OAA were inhibitors of BrPO activity while rotenone was not (Table 6). Hydrogen peroxide at 0.5 mM stimulated BrPO activity, while the higher concentrations showed no significant effect (Table 7). Ethanol and acetone (0.03‰ v/v), the carrier solvents for DNP and rotenone, respectively, showed no significant effects.

Table 4. The effect of $H_2O_2$ on $CHBr_3$ production by *U. lactuca* ($n = 5$). The effect of each treatment was determined in a separate paired experiment with a light or dark control. The relative production was determined by comparison with the paired control and then adjusted by comparison with the appropriate light or dark control below. All treatments were significant from paired control ($p < 0.05$).

| Condition/$[H_2O_2]$* | Relative $CHBr_3$ production |
|---|---|
| Light/control | 100% |
| Light/0 $\mu$M† | 44% |
| Light/100 $\mu$M | 52% |
| Light/1 mM | 19% |
| Dark‡/control | 100% |
| Dark‡/0 $\mu$M | 23% |
| Dark‡/100 $\mu$M | 200% |
| Dark‡/1 mM | 33% |

* Concentration based on addition.
† Seawater treated with catalase to remove $H_2O_2$ ($n = 7$).
‡ No dark preincubation.

Table 5. The effect of potential BrPO substrates and activated charcoal treated seawater on $CHBr_3$ production by *U. lactuca* ($n = 5$). The effect of each treatment was determined in a separate paired experiment with a light or dark control. The relative production was determined by comparison with the paired control and then adjusted by comparison with the appropriate light or dark control below. [OAA] = 1 mM; [$\beta$-KAA] = 0.5 mM; [phenol red] = 1 mM. All treatments were significant from paired control ($p < 0.05$) unless noted.

| Treatment | Relative $CHBr_3$ production |
|---|---|
| Light/control | 100% |
| Light/OAA | 16% |
| Light/$\beta$-KAA | 3.4% |
| Light/phenol red | 30% |
| Light/act. charcoal | 19% |
| Dark* | 100% |
| Dark*/OAA | 13% ($p = 0.15$) |
| Dark*/phenol red | 2.8% |

* No dark preincubation.

Table 6. The effect of potential substrates on the in situ bromination of phenol red by *U. lactuca* in the light ($n = 3$). [Phenol red] = 25 $\mu$M; [DNP] = 200 $\mu$M; [OAA] = 1 mM; [rotenone] = 55 $\mu$M.

| Treatment | Relative bromination rate | Significant ($p < 0.05$) |
|---|---|---|
| Control | 100% | — |
| +DNP | 33% | y |
| +OAA | 56% | y |
| Rotenone | 98% | n |

Table 7. The effect of added $H_2O_2$ on the in situ bromination of phenol red by *U. lactuca* in the light ($n = 3$). [Phenol red] = 25 $\mu$M.

| [$H_2O_2$] | Relative bromination rate | Significant ($p < 0.05$) |
|---|---|---|
| 0 (control) | 11% | — |
| 0.5 mM | 100% | y |
| 1.0 mM | 18% | n |
| 1.5 mM | 11% | n |

## Discussion

Production of $CHBr_3$ by *Ulva* is intrinsically variable, as the mean and s.d. of all light and dark runs carried out over the period demonstrate. The observed variability was most likely due to a combination of physiological and environmental factors. Because all of the incubations were run under identical conditions, this variability most likely reflected differences in the physiology (e.g., photosynthetic, respiratory or nutritional state) of the tissue used. Some variability, however, may have been introduced when different batches of seawater were used for the incubations. Bromide ion, the major species of inorganic bromine in seawater (~800 $\mu$M; Brewer 1975), was presumed constant during this study. Paired experiments using *Ulva* consistently demonstrated significantly greater $CHBr_3$ production in the light than in the dark.

*Physiological parameters of $CHBr_3$ production*—The initial step in the formation of polybromomethanes is the bromination of a suitable substrate (i.e., $\beta$-keto acids; Theiler et al. 1978). (Monohalomethanes are products of a methyltransferase reaction that does not require $H_2O_2$; Wuosmaa and Hager 1990.) Hydrogen peroxide is available from several cellular sources. Hydrogen peroxide is produced by the dismutation of the superoxide radical ($O_2^-$) by superoxide dismutase (SOD; Halliwell 1984). The production of $O_2^-$ that results from the transfer of an electron from ferredoxin of PS I to $O_2$ is termed the Mehler reaction. DCMU inhibits electron flow on the acceptor side of PSII by binding to the secondary acceptor quinone (Falkowski and Raven 1997). The observation that $CHBr_3$ production by *Ulva* increases in the light and is inhibited by DCMU is consistent with the Mehler reaction supplying $H_2O_2$ to BrPO. The DCMU concentration that inhibited $CHBr_3$ production (Table 3) also inhibited PS (Table 1). Other experiments using DCMU have shown conflicting results. Goodwin et al. (1997) found $CHBr_3$ production by the brown seaweed *Macrocystis pyrifera* inhibited in the light in the presence of DCMU (50 $\mu$M), while Sundstrom et al. (1996) found no effect (400 $\mu$M) with the red seaweed *Eucheuma denticulatum.* Cota and Sturges (1997) found mixed results using 1 $\mu$M DCMU, no effect with the brown seaweed *Agarum cribosum,* and decreased $CHBr_3$ production with an assemblage of ice microalgae. Goodwin et al. (1997) confirmed DCMU inhibition of photosynthesis (PS) at the concentration used. Sundstrom et al. (1996) and Cota and Sturges (1997) did not report photosynthetic inhibition by DCMU at the concentration used.

Superoxide radicals are also produced as a result of electron transfer from the mitochondrial electron transport chain to $O_2$ in plant tissues and $H_2O_2$ is produced via mitochondrial SOD (Cadenas 1989). *Ulva R* was initially ($t = 0$) high because of a high residual $O_2$ present in the tissue as a result of PS (Fig. 1). This value is typically obtained when respiration is measured without dark preincubation. Because the tissue was continually aerated in the dark for the 14 d the [$O_2$] attained equilibrium rapidly at a lower concentration, and after day 4 little change in *R* was observed (Fig. 1). *Ulva* was still able to produce $CHBr_3$ after day 4 and there was a correlation between $CHBr_3$ production and the log of *R* (Fig. 2). Rotenone inhibits mitochondrial electron transport acting between the flavoprotein and nonheme proteins of complex I (Moreland 1980). In the presence of rotenone, *R* by *Ulva* was inhibited 36% (Table 2) and $CHBr_3$ production was deceased 38% (Table 3). These experiments demonstrate that mitochondrially produced $H_2O_2$ can be used by BrPO to produce $CHBr_3$.

A consequence of uncoupling oxidative phosphorylation from mitochondrial electron transport is an increase in electron flow and $O_2$ reduction. The increased *R* rate of *Ulva* in the presence of DNP, therefore, demonstrated that DNP was indeed acting as an uncoupler at the concentration used. With increased electron flow an increase in $O_2^-$, $H_2O_2$, and $CHBr_3$ production was anticipated. Surprisingly, $CHBr_3$ production dramatically dropped. This can be attributed, however, to DNP itself being brominated by BrPO and acting as a competitive inhibitor (*see in situ formation of $CHBr_3$ section below*).

Another probable source of $O_2^-$ is the cytochrome P450 system and the enzymes xanthane oxidase and galactose oxidase in which $O_2^-$ is a result of catalysis; all have been identified in higher plants (Elstner 1987). The $O_2^-$ produced from these sources is also removed by SOD. The resulting $H_2O_2$ should be available to BrPO. Hydrogen peroxide can be enzymatically produced by the glycollate oxidase, urate oxidase, and amino acid oxidases. Glycollate oxidase is replaced by glycollate dehydrogenase in the Ulvophyceae and is not a factor in our experiments (Gross 1993). Urate oxidase, involved in purine metabolism, has been reported in Chlorophytes and Ulvophytes (Gross 1993). Although present in higher plant tissue, amino acid oxidases have not been reported to be present in algae. The contribution of these other sources of $H_2O_2$, except glycollate oxidase, to $CHBr_3$ production is unknown. However, the lowered amounts of

$CHBr_3$ produced in the dark experiments in the presence of rotenone may, in part, be due to these other $H_2O_2$ generating systems.

*Bromoperoxidase and other $H_2O_2$ using enzymes*—Hydrogen peroxide severely inhibits carbon fixation by acting on certain Calvin cycle enzymes; it also slowly inactivates SOD (Halliwell 1982). The inactivation of SOD results in the reaction of $O_2^-$ with $H_2O_2$ to form the highly reactive hydroxyl radical, which causes the peroxidation of membranes (Halliwell 1982). As a means of coping with internally generated $H_2O_2$, a suite of enzymes have evolved to catalyze its removal.

As first noted by Pedersen et al. (1996), BrPO present in marine algae may help alleviate ''oxidative'' stress imparted by the presence of $H_2O_2$. Other enzymes that degrade $H_2O_2$ include catalase and other peroxidases. Catalase is found in the peroxisome of all higher plants and most green algae (Gross 1993) but not in the chloroplast (Halliwell 1984). *U. lactuca* has been reported to have less than 10% the catalase activity of those algae with glycollate oxidase (Tolbert 1980), whereas no catalase activity has been detected in *Ulva rigida* (Pedersen et al. 1996). Catalase is very efficient in removing $H_2O_2$. It has one of the highest molecular activities known for enzymes ($H_2O_2$ molecules consumed$\cdot$molecule catalase$\cdot s^{-1}$). However, it is less effective at breaking down $H_2O_2$ at low concentrations, because of its higher $K_m$ for $H_2O_2$ (1.1 M, pH 7; Borman 1969), than peroxidases (Halliwell 1984). The $K_m$ (pH 7) for BrPO is 28 $\mu$M $H_2O_2$ and 90 $\mu$M $H_2O_2$ (*Fucus* and *Macrocystis,* respectively; Soedjak and Butler 1991).

Ascorbate peroxidase (AP) is found primarily in the chloroplast fractions of higher plants (Halliwell 1984) and algae (Takeda et al. 1997). It has been detected in *U. rigida* (Pedersen et al. 1996). This enzyme catalyzes the oxidation of ascorbate, found in higher plant chloroplasts at high concentrations (~50mM), to dehydroascorbate as part of the Halliwell-Asada pathway (Halliwell 1984). Bromoperoxidase has been localized in chloroplast fractions of the brominating red algae *Odonthalia floccosa* (Manley and Chapman 1979) and of *U. lactuca* (Manley unpubl. data). If BrPO coexists in the chloroplast of *Ulva* with AP, whether or not it is the main peroxide utilizer will depend upon their relative concentrations and $K_m$ values.

BrPO has also been located on the outer cellular surface of marine seaweeds (Vitler 1983). The in situ bromination of phenol red by seaweeds has also been interpreted as the result of an external BrPO (Wever et al. 1991; Pedersen et al. 1996; Sundstrom et al. 1996). Phenol red was brominated in situ by *Ulva* (Table 7). No phenol red was detected by spectroscopy within the tissue after several washings in filtered seawater (data not shown). Phenol red would not be expected to pass through the cellular membrane because of its large size (MW 354), its ionization in seawater and its three aromatic rings. Thus it appears that a BrPO exists outside of the cell membrane in *Ulva.*

*Environmental parameters of $CHBr_3$ production*—Those environmental factors that influence photosynthetic and respiratory electron transport should affect the production of $CHBr_3$ and other polyhalomethanes. Therefore, light is a major environmental factor influencing $CHBr_3$ production.

Hydrogen peroxide is present in seawater at concentrations between 10 and 200 nM with the higher values found in illuminated surface waters as a result of biotic and abiotic processes (Moffet and Zafiriou 1993). Hydrogen peroxide can leak out of seaweeds with thin thalli (Pedersen et al. 1996) and single-celled phytoplankton (Palenik et al. 1987). It is also formed from the photochemical degradation of DOM (Cooper et al. 1988). Seawater $H_2O_2$ is available to BrPO. $H_2O_2$ added to seawater at 500 $\mu$M enhanced BrPO activity over samples in which no $H_2O_2$ was added (Table 7). BrPO activity at 500 $\mu$M $H_2O_2$ was also greater than the activity at concentrations $\geq$1 mM.

Although $CHBr_3$ production was determined directly in the presence of $H_2O_2$, PS and *R* could not be. The tissue first had to be treated with $H_2O_2$ and rinsed of interstitial $H_2O_2$ prior to determining PS and *R* by the $O_2$ method. When PS and *R* measurements were attempted in the presence of $H_2O_2$, a rise in $O_2$ was measured because of the enzymatic breakdown of $H_2O_2$ present. External $H_2O_2$ was shown to both activate and inhibit $CHBr_3$ production depending on the concentration and the presence or absence of light. At 1 mM $H_2O_2$, $CHBr_3$ production was inhibited in both the light and the dark; PS and *R* were also inhibited. At 100 $\mu$M $H_2O_2$, $CHBr_3$ production was still inhibited in the light but stimulated in the dark. At 1 mM $H_2O_2$, the in situ activity of the external BrPO (bromination of phenol red) was not enhanced over the control but was much less than the activity at 0.5 mM. The decline of $CHBr_3$ production in the presence of $H_2O_2$ at 100 $\mu$M in the light and 1 mM in the light and dark corresponded to declines in PS and *R* under these conditions (Fig. 3). The inhibition of PS and *R* was probably due to membrane destruction by peroxidation (Halliwell 1982). The decline in $CHBr_3$ production at the elevated $H_2O_2$ levels may have been due to the loss of internal BrPO activity brought about by membrane disruption. Bromoform production was never completely inhibited, possibly because the external BrPO was still functioning.

The physiological effect of $H_2O_2$ was dependent upon the exposure duration and whether or not $H_2O_2$ was being internally produced. The critical external $H_2O_2$ concentrations for PS and *R* were different because of the higher rate of $H_2O_2$ production during PS than from *R*. The $H_2O_2$ concentration within the chloroplast in the light was probably much greater than the added external concentration of 50 $\mu$M, where PS inhibition was detected, and greater than the mitochondrial $H_2O_2$ concentration at the same external concentration. Therefore the inhibition of PS was seen at a lower external concentration than that for *R*. Interestingly *R* was not completely inhibited even at 1 mM.

Studies exposing red and brown seaweeds to 1–2 mM $H_2O_2$ showed enhanced $CHBr_3$ production in the light (Wever et al. 1991; Collen et al. 1994; Pedersen et al. 1996; Sundstrom et al. 1996). These seaweeds contain many more cell layers than the two-cell layer thick *Ulva* and also have thicker cell walls. It may be that $H_2O_2$ rapidly penetrates the thin thallus of *Ulva* disrupting metabolism, while its slower penetration into these other seaweeds allows it to be more thoroughly scavenged by the $H_2O_2$ using enzymes. Addition of

$H_2O_2$ to incubations of the brown seaweed *Macrocystis* at 190 nM enhanced $CHBr_3$ production in the dark, but it had no effect in the light (Goodwin et al. 1997).

Removal of $H_2O_2$ from seawater by catalase decreased $CHBr_3$ production by more than half. Being a large protein (MW 247,00) it does not pass through biological membranes (Halliwell 1984). The addition of catalase to seawater (100 units $ml^{-1}$) incubations of *Meristiella* had no effect on $CHBr_3$ production (Pedersen et al. 1996). This contrasts with our results. However, we used a higher catalase concentration (500 units $ml^{-1}$) and let the seawater sit overnight in the dark before use.

The production of $CHBr_3$ in seawater in which the DOM had been removed was lower than untreated seawater (Table 5). There appears to be a component of DOM that can serve as a substrate for BrPO (or released HOBr) to produce $CHBr_3$. Both the charcoal-treated and control seawater were filtered (0.45 $\mu$m), so the presence of microorganisms was not a factor. We anticipate that this effect will vary depending on the source of the seawater and the season, i.e., the unknown external substrates ($S_{ext}$) concentration should vary temporally and spatially. The dramatic effect demonstrated in this experiment may be an unusual occurrence because of an unusually high concentration of $S_{ext}$. The concentration of $S_{ext}$ in seawater and those factors involved in its production and degradation are, therefore, important environmental parameters influencing $CHBr_3$ production.

Wever et al. (1991) first proposed that $CHBr_3$ could be produced from components of DOM that have been brominated by a reaction with HOBr, an active intermediate of BrPO activity, that is released from the active site. This mechanism of haloperoxidase catalysis (i.e., release of HOBr) is not accepted by all biochemists working with isolated bromoperoxidase; studies do not support the enzyme release of HOBr (Soedjak and Butler 1990; Tschirret-Guth and Butler 1994). The release of HOBr from BrPO inside of the cell (i.e., chloroplast) would probably result in its rapid reaction with cellular constituents that could be deleterious. If BrPO exists on the outer cell surface, as the bromination of phenol red suggests (Tables 6 and 7), then the bromination of $S_{ext}$ and production of $CHBr_3$ need not invoke the release of HOBr.

*In situ formation of $CHBr_3$*—When phenol red (1 mM) was added to seawater the production of $CHBr_3$ by *Ulva* was inhibited (Table 5). This inhibition was probably due to phenol red outcompeting the natural substrate for $CHBr_3$ formation for the active site of BrPO. Phenols are excellent BrPO substrates for bromination. The observation that the phenol DNP inhibited $CHBr_3$ production when it was expected to enhance it (Table 3) and inhibited the bromination of phenol red (Table 7) was probably due to it binding to the active site of BrPO. Rotenone, which does not contain a phenol moiety, inhibited $CHBr_3$ production as expected by blocking electron transport. It did not inhibit the phenol red bromination and, therefore, did not compete with phenol red for the active site. DCMU also does not contain a phenol group and is not a substrate for BrPO (Manley, unpubl. data).

Because the addition of phenol red inhibited $CHBr_3$ production, it appears that the external BrPO produces a significant amount of $CHBr_3$, especially in the dark. The inhibition of $CHBr_3$ production in the presence of OAA (1 mM) and $\beta$-KAA (0.5 mM) can be similarly explained. It is not known whether these two compounds can enter into the cell and bind to the internal BrPO (i.e., competitive inhibition). OAA did inhibit the bromination of phenol red (Table 6). A similar experiment with $\beta$-KAA was not performed. The greater degree of inhibition with these compounds than with phenol red could be explained either by these compounds inhibiting both the internal and external BrPO or by BrPO having a greater affinity for these substrates than for phenol red. The inhibition of $CHBr_3$ production by these two compounds was unexpected because when OAA was added to a red algal BrPO extract, $CHBr_3$ production was enhanced (Itoh and Shinya 1994), and because $\beta$-KAA is structurally very similar to other $\beta$-keto acids known to be brominated by BrPO extracts to yield $CHBr_3$. We offer no explanation for these differences except that we were using intact *Ulva* tissue, whereas the other studies were using extracts from red and brown seaweeds.

We interpret our results and those of others in the following model of $CHBr_3$ and polyhalomethane production by marine macroalgae. Macroalgae contain both an internal (chloroplast) and external BrPO, that can brominate the $CHBr_3$ yielding substrate(s) using $H_2O_2$ produced metabolically and $H_2O_2$ found externally in seawater. As demonstrated in this study, $H_2O_2$ is deleterious to both PS and *R*, although the external concentration supplied was much higher than that usually found in illuminated seawater. The internal BrPO may replace AP as the major $H_2O_2$-removing enzyme within the cell. We do not favor the interpretation of HOBr being released from BrPO for bromination of the $CHBr_3$ yielding substrate. We surmise that bromination occurs within the active site. Although the internal $CHBr_3$ yielding substrates are probably $\beta$-keto acids, the nature of the external $CHBr_3$ yielding substrate, a component of DOM, is not known.

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