Daily bursts of biogenic cyanogen bromide (BrCN) control biofilm formation around a marine benthic diatom

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The spatial organization of biofilms is strongly regulated by chemical cues released by settling organisms. However, the exact nature of these interactions and the repertoire of chemical cues and signals that micro-organisms produce and exude in response to the presence of competitors remain largely unexplored. Biofilms dominated by microalgae often show remarkable, yet unexplained fine-scale patchy variation in species composition. Because this occurs even in absence of abiotic heterogeneity, antagonistic interactions might play a key role. Here we show that a marine benthic diatom produces chemical cues that cause chloroplast bleaching, a reduced photosynthetic efficiency, growth inhibition and massive cell death in naturally co-occurring competing microalgae. Using headspace solid phase microextraction (HS-SPME)-GC-MS, we demonstrate that this diatom exudes a diverse mixture of volatile iodinated and brominated metabolites including the natural product cyanogen bromide (BrCN), which exhibits pronounced allelopathic activity. Toxin production is light-dependent with a short BrCN burst after sunrise. BrCN acts as a short-term signal, leading to daily "cleaning" events around the algae. We show that allelopathic effects are H_2O_2 dependent and link BrCN production to haloperoxidase activity. This strategy is a highly effective means of biofilm control and may provide an explanation for the poorly understood role of volatile halocarbons from marine algae, which contribute significantly to the atmospheric halocarbon budget.

allelopathy | chemical ecology | marine ecology | signal molecule

Biofilm formation in marine habitats is a rapid and ubiquitous process and most submerged surfaces, natural or man-made, are covered with complex microbial communities. Intense efforts are made to control biofilm formation on industrial surfaces such as ship hulls because this biofouling can result in severe economic loss (1). Among the early settlers, microalgae play a key role in the biofilm development and diatoms, especially, are able to settle on even the most fouling resistant surfaces (2). In this context, it is interesting to observe that certain microalgae can obviously control their microenvironment because the patchy variation in species composition observed around these algae (3–5) cannot be explained by abiotic heterogeneity or bioturbation by grazers (4). This spatial organization of species is characterized by complementary distribution patterns and negative correlation of species densities (4). Allelopathic interactions have been suggested as a possible explanation for such observed patchiness (4). Because biofilms are composed of densely packed cells embedded within a matrix of exuded polymeric compounds, secondary metabolites produced by any cell can efficiently target its neighbors rather than diffusing into the surrounding water column (6).

Studies that focused on interspecific interactions between biofilm-forming diatoms revealed that synergistic (7) and antagonistic (8) interactions are common and can have a strong influence on biofilm performance (7). The underlying chemistry of these interactions is unknown, but several modes of action of allelochemicals on susceptible target cells have been demonstrated, including the inhibition of photosynthesis (9, 10), membrane damage (11), inhibition of enzymes (12), reduced motility (13) and oxidative damage (10, 11, 14). In this study we selected the common biofilm forming diatom *Nitzschia cf pellucida* due to the high allelopathic activity observed in preliminary bioassays. Several *Nitzschia* species are known for their production of volatile halocarbons (15–17) and a first screening revealed that the selected alga is also a rich source of such compounds. The formation of low molecular weight halogenated metabolites is widely distributed in macro- and microalgae which contribute significantly to the atmospheric halocarbon budget (18–20). Local maxima of volatile halogenated metabolites are often observed in coastal regions but the function of these metabolites is poorly understood. Here we directly link the halocarbon chemistry of microalgae to an allelopathic activity by establishing that the natural product cyanogen bromide (BrCN) is highly inhibitory against competitors. This metabolite is released during a short period after the onset of light in quantities sufficient to kill or inhibit the growth of competing microalgae.

Results

Allelopathic Effects of Nitzschia *cf pellucida*. Experiments with cocultures of biofilm forming diatom species revealed that the diatom *Nitzschia cf pellucida* (Fig. 1*A*) exerted strong allelopathic effects. We observed that the naturally co-occurring diatoms *Navicula arenaria* (Fig. 1*D*), *Cylindrotheca closterium* and *Entomoneis paludosa* (Fig. 1*B*) were inhibited and killed after 24-h exposure to relatively low cell densities (7,000–10,000 cells mL^{-1}) of *N. cf pellucida*. Another diatom, *Stauronella* sp. (Fig. 1*C*) was more resistant but was killed within 24 h when exposed to circa 80,000 cells mL^{-1} of *N. cf pellucida*. The mechanism of inhibition appeared to be the same for all species investigated: exposure to *N. cf pellucida* cells resulted in loss of pigmentation, shriveling of chloroplasts and finally cell death (Fig. 1).

Application of nutrient-enriched spent *N. cf pellucida* culture medium on cells of different diatom species induced a collapse of photosynthetic efficiency (Fig. S1). Microscopic investigations showed a massive cell death within 10 h for *E. paludosa* (Fig. 2) and *C. closterium*. The diatom *Stauronella* sp. was again more resistant and was able to maintain its photosynthetic efficiency, but its growth was suppressed for 2 d (measured as the initial

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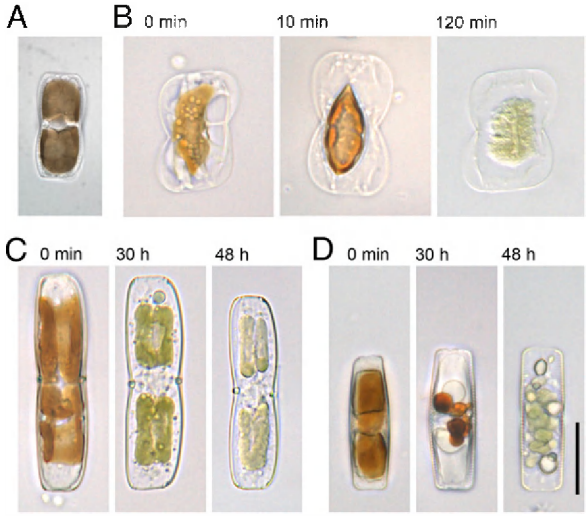

Fig. 1. Antagonistic effects of *N. cf pellucida* on a naturally co-occurring diatom species. (*A*) *N. cf pellucida*. (*B*) *E. paludosa*. Healthy cell (*Left*), cell after 10-min exposure to *N. cf pellucida* cells (80,000 cells mL^{-1}; *Center*), and dead cell after 120-min exposure (*Right*) are shown. (*C*) *Stauronella* sp. Healthy cell (*Left*), cell after 30-h exposure to *N. cf pellucida* cells (80,000 cells mL^{-1}; *Center*), and cell after 48-h exposure (*Right*) are shown. (*D*) *Navicula arenaria*. Healthy cell (*Left*), cell after 30-h exposure to *N. cf pellucida* cells (80,000 cells mL^{-1}; *Center*), and cell after 48-h exposure (*Right*) are shown. (Scale bar: 20 μm.)

fluorescence, F_0, a proxy for algal biomass) after which cell growth at rates similar to the control was restored.

An additional response was detected in *E. paludosa*: *N. cf pellucida*-spent medium induced a loss of motility and cells displayed a strong condensation of the protoplast within 10 min after exposure (Fig. 1*B*). As this species is highly sensitive toward *N. cf pellucida*, we selected it as a model for further bioassay experiments.

The allelochemical potential of *N. cf pellucida* varied dramatically with time of day. Using a bioassay with *E. paludosa* and cell free spent medium from *N. cf pellucida* cultures sampled in time intervals, we showed that allelopathic activity is highest between 2 and 4 h after daybreak when application of spent medium resulted in nearly complete eradication of *E. paludosa* cells (Fig. 2). Six hours after the onset of light, the activity diminished and spent medium had nearly no effect. The proportion of healthy cells increased to nearly 100% toward the end of the night. This striking pattern suggests that labile, reactive or volatile metabolites are responsible for the allelopathic activity. Further studies therefore focused on the characterization of metabolites present 3 h after the onset of light when toxicity was maximal.

Extraction and Structure Elucidation of *N. cf pellucida* Allelochemicals. We performed GC-MS analyses of ethylacetate extracts (EAe) of cell-free spent culture medium and of volatile organic compounds collected by headspace solid phase microextraction (HS-SPME) and revealed the occurrence of 18 different brominated and iodinated volatiles in *N. cf pellucida* cultures. These compounds were a mixture of methylhalogens (CH_3Br, CH_3I), dihalomethanes (CH_2Br_2, CH_2I_2, CH_2ClI*, CH_2BrI*) as well as trihalomethanes ($CHBr_3$, CHI_3, $CHBr_2I$*, $CHBr_2Cl$*, $CHClI_2$*) (EAe and SPME), 1-iodopropane (EAe), di and trihalogenated acetaldehydes (dibromoacetaldehyde*, bromochloroacetaldehyde*, chlorodibromoacetaldehyde*, EAe), and 1,2-dichloroethane (EAe). We identified these compounds based on their retention time and mass spectra using GC-MS and, if not indicated otherwise, compared with commercially available or synthetic (21) standards (compounds marked with an asterisk were identified only by mass spectrometry and retention time). In addition to these metabolites that are known from marine algae (18), we could also detect the volatile and highly toxic cyanogen bromide, BrCN using SPME. A commercially available synthetic standard of this natural product provided material for the confirmation of the structure, quantification and a dose–response assessment in bioassays with *E. paludosa*.

To check if these halocarbons caused the observed allelopathic interactions, we assessed the toxicity of different concentrations of the nine most abundant halogenated compounds and sodium cyanide (NaCN) on the diatom *E. paludosa* (Fig. 3). In these bioassays, cyanogen bromide clearly turned out to be the most toxic halogenated metabolite produced by the algae. The minimal lethal concentration was 2 μM (causing 96% of the *E. paludosa* cells to die within 3 h). BrCN is also by far more potent compared with NaCN, which is active only in concentrations above 40 μM. BrCN concentration in the *Nitzschia* cultures (220,000 cells mL^{-1}) determined by SPME GC-MS with $CDCl_3$ as an internal standard strongly varied over time and was highest 2–4 h after daybreak (up to 7.46 ± 1.77 μM BrCN) (Fig. 2). To ensure that BrCN is also causing the observed allelopathic effects at low *N. cf pellucida* cell densities in the cocultures described above, we measured local concentrations of BrCN in cultures of lower cell densities. Therefore, we sampled small aliquots (2.5 mL) of culture medium just above the diatoms growing at the bottom of the culture vial. Fig. S2 shows that cultures with cells densities even below 20,000 cells mL^{-1} can locally produce cell inhibitory concentrations of BrCN. The concentration of BrCN can thus fully explain the lethal effect of the spent *N. cf pellucida* medium.

No other tested halogenated metabolites were active in the concentration range reached in cultures. Cells of *N. cf pellucida* are more resistant to BrCN and could cope with concentrations

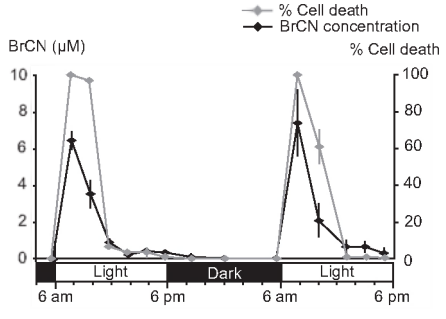

Fig. 2. Time-dependent production of BrCN in *N. cf pellucida* cultures (180,000 cells mL^{-1}) throughout the day. Black line shows BrCN concentrations (μM). Gray line shows the effect of *N. cf pellucida* cell-free spent medium harvested at different time points on *E. paludosa* cells. We measured the effects 10 h after exposure to *N. cf pellucida* filtrate (means ± SD, $n = 3$).

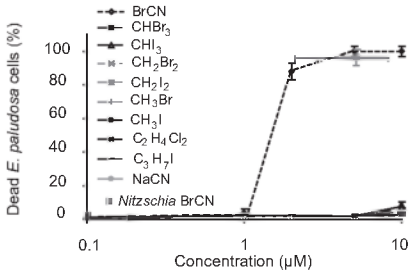

Fig. 3. Dose–response curve for *E. paludosa* for nine halogenated compounds detected in *N. cf pellucida* cultures. The gray square represents the average BrCN concentration in *N. cf pellucida* cultures (3 h after onset of light) and the average response of *E. paludosa* to the *N. cf pellucida* filtrates (means ± SD, $n = 4$).

of up to 16 μM with minor or no growth reduction, and only displayed a reduced growth and photosynthetic efficiency at 32 μM BrCN (Fig. S3).

Light-Dependent BrCN Production and Stability. We show that BrCN production is light dependent: Changing the light regime from a standard 12 h:12 h light/dark rhythm to an extended darkness of 18 h prevented BrCN production (Fig. S4). BrCN production levels could be restored to 76.68 ± 17.38% if cells were exposed to light after 15 h of darkness (instead of 12 h). After a prolonged darkness of 18-h illumination could not trigger BrCN production. Because a pronounced reduction of the BrCN concentration was observed 6 h after the onset of illumination we verified if an abiotic degradation of this metabolite is causing this process. If BrCN was incubated in medium under conditions identical to the culturing we did only observe minor degradation over time. Even after 6 h, more than 80% of the initial BrCN concentration was recovered, excluding abiotic degradation (Fig. S5).

Biotic Interactions and Bacteriacidal Activity. Competing co-occurring species did not affect the BrCN production (Fig. S6). If cells of *N. arenaria* or *E. paludosa* at cell densities of 70,000–80,000 cells mL^{-1} were added to *N. cf pellucida* cultures of 180,000 cells mL^{-1}, no differences in BrCN production were observed [*N. arenaria*: Student's *t* test ($P = 0.9157$), *E. paludosa:* Student's *t* test ($P = 0.4076$)].

In contrast to the pronounced activity against algae, no inhibitory activity is found against bacteria. Agar diffusion assays of up to 64 μM BrCN concentrations and *N. cf pellucida*-spent medium filtrate against six bacterial strains isolated from estuarine intertidal mudflats showed no inhibition zones after up to 8 d of incubation.

BrCN Biosynthesis. To elucidate the source of halogens for the BrCN production we deprived *N. cf pellucida* cultures of bromide and iodide. Omitting these halogens from the culture medium almost completely eliminated the production of BrCN. When we altered the I^-:Br^- ratio in the culture medium from ~1:2,000 in natural seawater to 1:16, production of brominated hydrocarbons ceased and was replaced by the formation of iodinated compounds. Likewise, BrCN production was reduced at the expense of ICN formation, a second natural product. Allelopathic assays revealed that the Br^- and I^- deprived cultures lacked allelochemical activity (Fig. S7). In contrast, spent medium of *N. cf pellucida* grown at high I:Br ratios was even more toxic than spent medium derived from natural seawater and caused massive cell death of *E. paludosa* cells within 45 min after application (Fig. S7).

Because BrCN and cyanogen iodide (ICN) are not known as natural products, we aimed to verify their biogenic origin by addressing their biosynthesis. Therefore, we incubated *N. cf pellucida* cells in culture medium enriched with ^{13}C labeled bicarbonate for 5 d. Analysis of the isotope distribution of the cyanides provided proof for a biosynthetic origin. BrCN and ICN exhibited a ^{12}C: ^{13}C ratio of 1:1.13 and 1:1.18 in BrCN and ICN, respectively, whereas BrCN from cells in natural seawater exhibited the natural ratio of 1: 0.012 (Fig. 4). An axenic *N. cf pellucida* culture enriched with ^{13}C-labeled bicarbonate exhibited a ^{12}C:^{13}C ratio of 1:0.93. GC/MS analysis revealed that isotope enrichment after ^{13}C bicarbonate treatment was also observed for other metabolites.

Biosynthetic considerations of BrCN or ICN suggested the presence of an oxidized halogen species. Known enzymes that could be involved are H_2O_2 consuming haloperoxidases (22). To test whether BrCN production is linked with cellular H_2O_2 production, we assessed the effect of the H_2O_2-decomposing enzyme catalase incubated with *N. cf pellucida* cultures. BrCN production was significantly reduced in the presence of catalase (70.7 ± 13.4% reduction, $P = 0.0002$).

In a second assay for haloperoxidase activity, we added phenol red (phenolsulfonphthalein) at 36 μM to *N. cf pellucida* cultures and spectrophotometrically checked for conversion into brominated phenol blue (3′,3′′,5′,5′′-tetrabromophenolsulfonphthalein). Halogenation of phenol red occurred shortly after daybreak and phenol red concentration decreased to 26.38 ± 2.26 μM (mean ± SD, $n = 5$). Bromophenol blue (8.60 ± 1.86 μM, $n = 5$) was formed within 3 h after daybreak.

Discussion

In this study, we show that the benthic diatom *N. cf pellucida* produces allelochemicals that cause chloroplast bleaching, a reduced photosynthetic efficiency, growth inhibition and massive cell death in naturally co-occurring competing microalgae. The allelopathic compounds are effective even at low cell densities: 8,000 *N. cf pellucida* cells mL^{-1} were sufficient to suppress competitors. Local concentrations of BrCN in dilute cultures with cell counts below 20,000 cells mL^{-1} were sufficient to cause the effects in bioassays. Given the rather slow diffusion processes in water over a biofilm, the local concentrations around *N. cf pellucida* will most likely be even higher. This result demonstrates that the concentration required to trigger activity in bioassays is found in vicinity of the producing cells.

Our work adds to a very limited number of studies demonstrating the molecular basis for chemically mediated interactions between biofilm forming microalgae. Using a combination of chemical analyses and bioassays, we identified the highly reactive metabolite BrCN as the causative agent of the observed activity. This compound has not been previously detected as a natural product. BrCN is highly toxic and has been applied as fumigant and pesticide and was even briefly used as a chemical weapon during World War I (23). BrCN is currently used to fragment proteins by hydrolyzing peptide bonds at the C terminus of methionine residues (24). In addition to this effective metabolite, *N. cf pellucida* also produces a diverse mixture of iodo- and bromocarbons with comparatively lower allelopathic properties. The halomethanes, halogenated acetaldehydes, and iodopropane detected have been reported previously from micro- and macroalgae (22, 25, 26). Cyanogen bromide is hydrolyzed by water to release hydrogen cyanide (HCN), but we can exclude that the allelopathic effects were caused by cyanide alone because hydrolysis is comparably slow and because NaCN caused only minor effects when applied to our bioassay species *E. paludosa*.

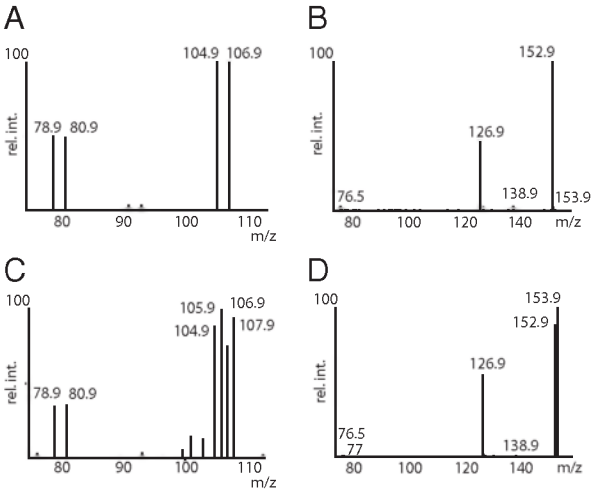

Fig. 4. GC-EI-MS spectra of BrCN (*A* and *C*) and ICN (*B* and *D*). *A* and *B* were harvested from *N. cf pellucida* cultures grown in natural seawater based culture medium. *C* and *D* were from cultures grown in $NaH^{13}CO_3$ enriched medium.

Concentrations of 40 μM NaCN (20 times higher than the active BrCN concentration) caused no short-term (8 h) effect on *E. paludosa* cells.

We observed that BrCN targets competing algae with different efficiency. Whereas *E. paludosa* and *C. closterium* are dramatically affected by this metabolite, leading to reduced photosynthetic activity and growth rate (Fig. S1), *Stauronella* sp. is more resistant. As the producer *N. cf pellucida* itself, this alga shows resistance against the metabolite using hitherto unidentified mechanisms. BrCN production can be seen as a mechanism that is not requiring an inducing partner because its release is independent of the presence of competing diatoms. In contrast to the pronounced effects on certain diatoms, BrCN is virtually inactive against bacteria. Even if incubated over elevated concentrations of 64 μM this metabolite did not affect the growth and morphology of six bacterial strains from intertidal mudflats. BrCN can thus be considered as a metabolite targeted specifically against competing algae.

Because BrCN is not a common natural product, we verified whether it might result from abiotic transformations in the medium or whether it is a true natural product biosynthesized by the alga. ^{13}C-labeled bicarbonate was incorporated into BrCN and ICN in high yields, confirming unambiguously a biogenic origin. Given the degree of labeling of all extracted products after administration of labeled bicarbonate it can be concluded that the CO_2 from bicarbonate is fueling the photosynthesis and the ongoing metabolism leads to a labeling of all metabolites in the algae. The bicarbonate is thus most likely not directly transformed to CN^- but rather via complex metabolic processes. The cyanide source for BrCN is still unclear. Several plants are known to produce cyanides through cyanogenic glucosides (27), but these compounds have never been detected in algae. The green algae *Chlorella* is known to produce cyanides from aromatic amino acids via an amino acid oxidase enzyme system (28), but such mechanism has not been described for diatoms. CN^- production is known from a broad range of organisms, including bacteria, fungi, insects, algae, and plants, as a means to avoid predation (27, 29), but apparently *N. cf pellucida* has found a means to release even more active metabolites by a simple modification.

Application of the H_2O_2-decomposing enzyme catalase significantly reduced the BrCN production in the *N. cf pellucida* cultures, which suggests that haloperoxidase (HPO) activity (22) is involved in the BrCN synthesis. Furthermore, we observed a preference for iodide over bromide incorporation, which corresponds with the halide selectivity for haloperoxidases (30). Also, the absence of ClCN in Br- and I-depleted cultures matches with the halide preference of algal HPO. Lastly, the conversion of phenol red into brominated phenol blue in the *Nitzschia* cultures points to the involvement of haloperoxidase enzymes. These enzymes catalyze the oxidation of halide ions to hypohalous acid by H_2O_2. Hypohalous acid (or a similar oxidized intermediate) can then react with organic substrates that are susceptible to electrophilic halogenation (22). We cannot, however, conclude whether biogenic CN^- (or equivalent) reacts with "Br^+" from the haloperoxidase reaction or if the transformation of halomethanes or other precursors to BrCN is involved in the biosynthetic pathway.

Haloperoxidase enzymes are distributed in marine organisms including Rhodophyta, Phaeophyta, Chlorophyta (18), and Bacillariophyta (16). An important function of HPO is to scavenge harmful H_2O_2 produced during photosynthesis, photorespiration, and other metabolic processes (31). It has also been suggested that HPO of marine organisms are involved in defense mechanisms such as the mediation and prevention of bacterial biofilm formation, but evidence for the involved metabolites was not given till now (32, 33). Here we provide a link between HPO activity and allelopathic potential supporting an ecological role for this enzyme in diatoms.

It is interesting to note that we only detected BrCN during the morning hours in the culture and only if we quickly extracted this reactive metabolite. Given the methodological difficulties in detecting highly reactive metabolites, it would be worth verifying the potential of other microalgae to produce this reactive metabolite, which would most likely not have been picked up in other determinations of halogenated volatiles. The mechanism introduced here could thus have a broader occurrence.

The production of BrCN only within few hours after daybreak coincides with the time span of H_2O_2 production in algal cells due to the Mehler reaction (34). It is clearly light dependent: Prolongation of the dark period also leads to a delayed formation of BrCN. If the dark period is prolonged for 3 h, BrCN production can still be observed. If, however the dark period is prolonged for 6 or more hours, light is not sufficient to elicit BrCN release. Therefore, we conclude that BrCN production is light dependent, but is only possible during a certain timeframe within the day when other physiological prerequirements are in place. Three alternative but not mutually exclusive mechanisms may underlie this phenomenon. First, the observed patterns could be explained by the fact that diatoms exposed to prolonged darkness show an increase in the photoprotective xanthophyll pigment diatoxanthin and higher non-photochemical quenching (NPQ) (35). NPQ dissipates excess absorbed light energy and thereby diminishes the energy arriving at the photosystems and thus less potential for H_2O_2 formation during photosynthesis (34, 36). Second, several algae are known for circadian activities of antioxidative enzymes or low-molecular-weight antioxidants (37–39), often with an increase of antioxidative activities at subjective noon, when oxidative stress is most severe (38). A third potential mechanism is the occurrence of a circadian pattern in the formation of the unknown cyanide source for BrCN production.

A short-term release of a toxic metabolite to suppress growth of competitors might be a highly efficient allelopathic strategy. Like a "molecular toothbrush" BrCN could eliminate the surrounding flora daily after sunrise, leading to increased access to nutrients and light present in the environment and even elevated concentrations resulting from nutrients leaking from killed cells. The clean and nutrient-rich area could then be used by *N. cf pellucida* for effective proliferation in the absence of toxins. This diatom species is more resistant to BrCN compared with its competitors (Fig. S3) but at elevated concentrations it is still sensitive to the toxin. Thus, the short-term toxin burst is an effective means of reducing the risk of autotoxicity and represents a strategy for allelopathic interactions.

In this study, we illustrate that a highly active simple metabolite from diatoms has the potential to promote daily cleaning events around a biofilm-forming diatom. Our results provide a mechanism by which diatoms can generate microscale chemical territoria in which competitors are deterred or killed. Obviously, such strategy contributes to complex microlandscapes maintained by interacting species and may boost the small-scale patchy growth habits of biofilm-forming species. Our results also suggest a potential link between the globally significant emissions of volatile halocarbons released by marine algae and allelopathic activity.

Methods

Bialgal Culture Experiments. The origin of the algal strains and the culture conditions are described in *SI Methods*. Growth interactions between *Nitzschia cf pellucida* and 3 benthic diatom species (*Navicula arenaria Cylindrotheca closterium*, and *Entomoneis paludosa*) were examined by using bialgal cultures as described in *SI Methods*.

Effects of Spent Medium. *N. cf pellucida* spent medium was prepared by filtering exponentially growing cultures (200,000–250,000 cells mL^{-1}) on GF/F filters and subsequently on 0.2 μm membrane filters. This filtered spent medium was enriched with f/2 nutrients and then applied to cells of *C. closterium*, *Stauronella* sp. and *E. paludosa*. In parallel, cells of these species were cultured in f/2 enriched seawater as a control. The effect of *N. cf pellucida* spent medium was monitored by measuring the biomass and

photosynthetic efficiency using Pulse-amplitude-modulated (PAM) fluorescence (see *SI Methods* for details).

Liquid–Liquid Extraction of Allelopathic Compounds and GC-MS Analysis. A total of 150 mL of *N. cf pellucida* spent medium was filtered and extracted three times with 50 mL ethylacetate. The extract was dried with anhydrous sodium sulfate and was concentrated at reduced pressure. GC-EI-MS measurements of the concentrated extracts were performed with a Waters GCT premier (Waters) time of flight mass spectrometer (MS) coupled to an Agilent 6890N gas chromatograph (GC) equipped with a DB-5ms column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness and 10 m Duraguard precolumn, Agilent). The carrier gas was Helium 5.0 with a constant gas flow of 1.0 mL min^{-1}. The source temperature was at 300 °C with an electron energy of 70 eV. The column was held at 40 °C for 2 min, heated up from 40 °C to 150 °C with 5 °C min^{-1}, from 150 °C to 280 °C with a rate of 20 °C min^{-1} and held for 4.5 min. The samples were injected in splitless mode.

Volatile Organic Compounds. Solid phase microextraction (SPME, Carboxen/Polydimethylsiloxane, Supelco) was used to identify the volatile compounds emitted by *N. cf pellucida* cultures. The SPME fiber was exposed for 30 min to the headspace of 82 mL of magnetically stirred filtrate (GF/F filters) of *N. cf pellucida*. For the quantification of BrCN concentrations the SPME fiber was exposed for 5 min to the headspace of 2.5 mL magnetically stirred filtrate (0.2 μm filtered) of *N. cf pellucida*. We used $CDCl_3$ (Eurisotop) (at 0.124 μM) as an internal standard to enable quantification. The extracted compounds were analyzed using a Perkin-Elmer Autosystem XL GC coupled to a Perkin-Elmer TurboMass MS. An Agilent DB-5-MS column was used for separation. The GC was operating isothermally at 70 °C and the MS was recorded in single ion mode. The SPME extraction was calibrated by measuring a dilution series of commercially available BrCN (Sigma Aldrich) with $CDCl_3$ as an internal standard. BrCN concentrations were determined by calculating the GC peak area using standard program peak detection. BrCN peak area was normalized to the $CDCl_3$ peak area and used the calibration curve to calculate the BrCN concentrations in the *N. cf pellucida* cultures.

Bioassays. Bioassays were used to detect the presence of allelochemicals in *N. cf pellucida* spent medium and used *E. paludosa* as the susceptible strain. Cells of exponentially growing *E. paludosa* were inoculated in *N. cf pellucida* spent medium at a final density of 2,000 cells mL^{-1}. After 2 h of exposure to spent medium, we checked for the occurrence of resting cell formation and cell death using an inverted microscope (counting min 300 cells per replicate).

To ensure that our observations of cell death using normal light microscopy is valid, we assessed cell death using the membrane-impermeable DNA-specific stain Sytox (*SI Methods* and Fig. S8).

To check the occurrence of allelochemicals in the ethylacetate extracts, the extracts were concentrated using reduced pressure and finally dried the extract under a nitrogen enriched atmosphere. The residue was dissolved in acetone and added to an *E. paludosa* culture with 2,000 cells mL^{-1} at a final concentration of 1% acetone. This acetone concentration did not affect cell integrity itself within the timeframe of the bioassay.

The toxicity of nine halogenated compounds on the diatom *E. paludosa* was tested at concentrations of 0.1, 1, 2, 5, and 10 μM (Fig. 3). The halogenated compounds were first dissolved in acetone and added to an *E. paludosa* culture with 2.10^3 cells mL^{-1} at a final concentration of 1% acetone. We microscopically checked for dead cells, resting cells, and healthy cells 3 h after application. The same approach was used to check the toxicity of NaCN on *E. paludosa* cells at 2, 10, 20, and 40 μM NaCN.

The toxicity of BrCN on 6 bacterial strains isolated from estuarine intertidal mudflats was tested using agar diffusion assays (ADA, see *SI Methods* for details).

Synthesis of Cyanogen Iodide. ICN synthesis was performed as described (21). 0.25 mmol NaCN (Sigma Aldrich) was dissolved in 0.5 mL of water and cooled it to 0 °C. Iodine (0.25 mmol; Fluka) was gradually added, waiting until the last portion has reacted. The watery solution was extracted three times with diethyl ether, the ether extract was then dried with sodium sulfate, and the ether was removed by a stream of argon. ICN was received as colorless crystals.

Catalase Experiment. The effect of the H_2O_2-decomposing enzyme catalase (600 units bovine liver catalase mL^{-1} dissolved in water, Sigma Aldrich) on the toxicity of *N. cf pellucida* cultures was assessed by adding catalase to the *N. cf pellucida* cultures one hour before the onset of light and the presence of allelochemicals was tested 3 h after the onset of light using the *E. paludosa* bioassay. A control treatment in which we stirred *N. cf pellucida* cultures (analogous to the catalase treatment) one hour before the onset of light was included. Treatments were replicated four times.

Phenol Red Assay. The bromination of phenol red (phenolsulfonphthalein) into brominated phenol blue (3′,3′′,5′,5′′-tetrabromophenolsulfonphthalein) was used as an indicator for haloperoxidase activity (15). Phenol red (30 μM final concentration) was added to *N. cf pellucida* cultures (200,000–250,000 cells mL^{-1}) 3 h after daybreak. Two hours later, phenol red and brominated phenol blue were measured spectrophotometrically at 433 nm and 592 nm, respectively (15). Before the measurements, cells were removed by filtering on a 0.2-μm filter and we adjusted the pH to 6.5 with acetic acid.

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