Ubiquity and quantitative significance of detoxification catabolism of chlorophyll associated with protistan herbivory

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Chlorophylls are essential components of the photosynthetic apparatus that sustain all of the life forms that ultimately depend on solar energy. However, a drawback of the extraordinary photosensitizing efficiency of certain chlorophyll species is their ability to generate harmful singlet oxygen. Recent studies have clarified the catabolic processes involved in the detoxification of chlorophylls in land plants, but little is understood about these strategies in aquatic ecosystems. Here, we report that a variety of heterotrophic protists accumulate the chlorophyll a catabolite 132,173-cyclopheophorbide a enol (cPPB-aE) after their ingestion of algae. This chlorophyll derivative is nonfluorescent in solution, and its inability to generate singlet oxygen in vitro qualifies it as a detoxified catabolite of chlorophyll a. Using a modified analytical method, we show that cPPB-aE is ubiquitous in aquatic environments, and it is often the major chlorophyll a derivative. Our findings suggest that cPPB-aE metabolism is one of the most important, widely distributed processes in aquatic ecosystems. Therefore, the herbivorous protists that convert chlorophyll a to cPPB-aE are suggested to play more significant roles in the modern oceanic carbon flux than was previously recognized, critically linking microscopic primary producers to the macroscopic food web and carbon sequestration in the ocean.

Chlorophylls are crucial to sustaining most life forms on Earth, the majority of which ultimately depend on solar energy. Photoexcitation of chlorophylls initiates various photosynthetic reactions that convert the energy of photons into chemical potentials, which in turn, drive the full range of metabolic reactions throughout the global ecosystem. Chlorophylls play a central role in the photosynthetic apparatus by absorbing light and transferring the excitation energy to the reaction centers of photosystems before photosynthetic electron transport. However, without measures to contain the excited energy, chlorophylls can harm organisms because of their high photosensitizing potential. Photoexcited chlorophylls generate singlet oxygen, a highly reactive oxygen species that can cause severe cellular damage (1). Therefore, the phototoxicity of chlorophylls has been a continuous concern for the Earth’s ecosystem since the global rise in atmospheric oxygen about 2.3 billion years ago (2).

Given its potential risks, chlorophyll metabolism is thought to be carefully controlled in the cells of phototrophic organisms. Recent works have revealed that the biodegradation of chlorophyll a (Chl-a) (Fig. 1) in land plants (embryophytes) is regulated as carefully as its biosynthesis (3). The chlorin moiety found in Chl-a is a highly π-conjugated tetrapyrrolic macrocycle that acts as a potentially phototoxic chromophore or fluorophore. It is converted stepwise into an unconjugated and hence, colorless and nonfluorescent linear tetrapyrrole (SI Text, section 1.1). However, many algae and cyanobacteria apparently lack the programmed detoxifying catabolism of chlorophyll observed in embryophytes (SI Text, section 1.2). The inability of unicellular protists to detoxify chlorophyll can be rationalized by their lack of the need to remobilize nutrients before death, unlike multicellular land plants. Regarding heterotrophs, the digestive systems of most terrestrial herbivores are dark, whereas the digestive systems of most aquatic herbivores, such as multicellular and unicellular zooplankton, are small and translucent. This finding renders the microscopic aquatic herbivores susceptible to damage by the singlet oxygen generated when ingested chlorophylls are exposed to light (SI Text, section 1.3).

Thus, strategies to protect against the accumulation of phototoxic chlorophyll derivatives should be critical for the survival of the microscopic aquatic herbivores feeding under illumination.

We conducted feeding experiments on several microorganisms to screen for potentially detoxified chlorophyll catabolites. Here, we report that 132,173-cyclopheophorbide a enol (cPPB-aE) (Fig. 1) is the dominant product derived from Chl-a that accumulates in cells of various aquatic heterotrophic protists (i.e., unicellular eukaryotes) that feed on algae and that cPPB-aE is a virtually nonfluorescent and nonphotosensitizing chlorophyll derivative incapable of generating singlet oxygen. These results strongly suggest that herbivorous protists generate cPPB-aE as a detoxified catabolite of Chl-a. We also show that cPPB-aE is ubiquitous in all of the aquatic environments that we tested, frequently as the most abundant Chl-a derivative in the surface sediments, and that it is actively generated in the water near illuminated surfaces.


The authors declare no conflict of interest.

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See Commentary on page 17311.

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Ubiquity and quantitative significance of detoxification catabolism of chlorophyll associated with protistan herbivory

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and (132 of total amounts of chlorins derived from Chl-a after they were fed on fresh unialgal cells are as high as 80 mol% only major chlorophyll derivative associated with herbivory. We served in darkness for months to years (6), suggesting that it is dif attributed to metabolic processes in the heterotrophic protists.

Results and Discussion

Feeding Experiments. We showed that three genetically distant heterotrophic protists—a stramenopile, a cercozoan (Filosa), and a heliozoan (Centrohelida)—accumulated cPPB-aE as the only major chlorophyll derivative associated with herbivory. We estimated that levels of cPPB-aE in extracts from these protists after they were fed on fresh unialgal cells are as high as 80 mol% of total amounts of chlorins derived from Chl-a (Fig. 2). However, a heterotrophic discristioidean did not accumulate any cPPB-aE. No trace of cPPB-aE was detected in control experiments, where the unialgal cultures fed to the protists were incubated under conditions identical to those conditions used for the feeding experiments (Table S1). Organic synthesis of cPPB-aE requires a strong base to form the C–C bond between 132 and 172 carbons (4, 5), suggesting that it is difficult to generate cPPB-aE in vitro under normal conditions. In fact, the generation of cPPB-aE has not been reported in so-called dark incubation experiments involving pure unialgal cultures sealed and preserved in darkness for months to years (6–8). Therefore, cPPB-aE detected in the present experiments must be conclusively attributed to metabolic processes in the heterotrophic protists.

Minor products of Chl-a degradation are pyropheophytin a (pPhe-a) (Fig. 1) as well as various polar chlorins, including (132R)- and (132S)-hydroxychlorophyllones a [(R/S)-hCPLs-a] (Fig. 1). Dark incubation experiments of unialgal cultures revealed substantial accumulation of pPhe-a only after the incubations had proceeded for several months (6–8). Given that pPhe-a is absent from our control experiments involving unialgal cultures, its accumulation can also be attributed to the heterotrophic process. However, (R/S)-hCPLs-a may be a product of biotic processing (9, 10) and/or abiotic oxidation of cPPB-aE (11). All samples of heterotrophic protists also contain variable amounts of intact Chl-a, which may have been derived from undigested algal materials in phagosomes.

Our experiments, thus, suggest qualitatively that the herbivorous protists studied actively modify Chl-a into cPPB-aE during their course of phagotropic digestion (Fig. 3). Given that heterotrophic and phototrophic cells grew together in the cultures used in our experiments, quantitative estimations of the rate of cPPB-aE production from Chl-a as well as the stability of cPPB-aE in vivo were beyond the scope of the present work. Nonetheless, it seems reasonable to conclude that cPPB-aE is a major Chl-a catabolite produced by the herbivorous protists that we studied. Therefore, cPPB-aE could potentially emerge as a biochemical marker of protistan herbivory in aquatic environments.

Photochemical Significance. We determined various properties of cPPB-aE by analyzing an authentic sample that was semisynthesized from Chl-a (Materials and Methods). The most striking property of cPPB-aE is that it is essentially nonfluorescent (4), despite its highly π-conjugated structure (Fig. 4 A and B, SI Text, section 1.5, Fig. S1, and Table S2). This finding is consistent with our control experiments involving unialgal cultures, its accumulation can also be attributed to the heterotrophic process. However, (R/S)-

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rapid generation of singlet oxygen was approximately three times more rapid for Chl-a than Phe-a. The observed difference between Chl-a and Phe-a should partially reflect differences in the absorption intensities of Q, bands (Table S3). In conclusion, cPPB-αE is likely to be a colored but nonphotosensitizing Chl-a catabolite, which is comparable with the colorless nonfluorescent chlorophyll catabolites (NCCs) of higher plants (SI Text, section 1.1) insofar as its generation during the detoxification of chlorophylls protects against the accumulation of singlet oxygen in vivo.

Previous studies have frequently regarded cPPB-αE as an antioxidant that accumulates in marine macrofauna (11, 13–16). However, other workers have failed to highlight the possibility that the primary evolutionary advantage of cPPB-αE production by single-celled organisms is the detoxification of the phototoxic chlorophyll derivatives that are inevitably ingested on feeding chlorophyll-containing diets to protists in an illuminated and aerated environment. The reported antioxidant function of cPPB-αE accumulated in the intestines of metazoans would, thus, be a secondary adaptation if the derivative, indeed, functions as an antioxidant. We note that its antioxidant activity, which should protect against the oxidative damage exerted by reactive oxygen species, has not been shown experimentally. Furthermore, no direct evidence has yet been presented that metazoans produce cPPB-αE. Geochemical evidence that is consistent with the ability of microscopic plankton to produce cPPB-αE is discussed below.

Ubiquity and Geochemical Significance of cPPB-αE in Aquatic Environments. To determine the distribution of cPPB-αE metabolism among various aquatic settings, we used a geochemical approach that involved detecting cPPB-αE directly from particulate organic matter (POM) in water columns as well as surface sedimentary samples just beneath the columns. We analyzed extracts of glass microfiber filters (GF/F grade; Whatman) that presumably collected any particulate matters larger than 0.7 μm in diameter from the water samples examined, and therefore, they should have included any eukaryotic cells and most prokaryotic cells present as well as fecal materials and any organic aggregates.

The results showed that cPPB-αE is ubiquitous and abundant in all of the aquatic environments tested, confirming the ecological and biogeochemical significance of cPPB-αE metabolism (Fig. 5).

In summary, we detected cPPB-αE in all POM samples from diverse environments, although its abundance relative to the other Chl-α derivatives ranged from 5 mol% (samples collected from Ise Bay) to 42 mol% (samples collected from a dammed creek). These values correspond to ranges in the cPPB-αE/Chl-α ratios from 0.07 to 1.53. However, the relative concentrations of cPPB-αE in the surface sediments were generally very high (51–81%), representing virtually the most abundant Chl-α derivatives. In fact, the presence of cPPB-αE in sediments has been occasionally reported in previous works (11, 17–19), although it has been probably completely missed in other reports because of analytical artifacts (20).

The abundance of cPPB-αE relative to Chl-α in water columns implies that a considerable proportion of photosynthetic primary production should initially be processed by herbivorous protists that produce cPPB-αE. Given that Chl-α is generally regarded as standing biomass of phototrophic plankton, we similarly expect that the cPPB-αE content in POM should represent the mass of herbivorous protists in water. Depth profile patterns of cPPB-αE and the other chlorophyll derivatives (Fig. 6) are perhaps best explained by (i) in situ feeding activities of herbivorous protists in the upper parts of water columns and/or (ii) selective preservation into deeper water and sediments, which would explain the elevated relative concentration of cPPB-αE in the surface sediments.

Evaluation of those data in terms of either the rate of protistan herbivory or the rate of cPPB-αE production require additional investigation, including investigation of the rate of cPPB-αE pro-

microscopic observation that the autofluorescence of chlorophylls in the plastids of ingested algae rapidly disappears during their phagocytosis by herbivorous protists (Fig. 3 and Fig. S2). This observation suggests very rapid and nonradiative quenching of the photoexcited singlet state of cPPB-αE, which is ascribed to internal conversion(s) to the ground singlet state (12) and/or intersystem crossing to the excited triplet state. Considering the biochemical significance of producing cPPB-αE by some protists, the latter possibility seems unreasonable owing to the highly toxic singlet oxygen that is readily produced from the triplet state. The increase in the SOSG fluorescence caused by single-celled organisms is the detoxification of cPPB-αE metabolism (Fig. 5).

Fig. 3. A typical differential interference image (A) and a corresponding fluorescent image (B; excitation light = 400–440 nm) of cercozoans in different stages of phagocytosis. The cercozoan cells are indicated by the dashed lines on the fluorescent image. EP, a cell in an earlier stage of phagocytosis containing two chains of Skeletonema sp. in different stages of digestion; LP, a cell in a later stage of phagocytosis containing a chain of Skeletonema sp. in a later stage of digestion displaying no autofluorescence; NP, a cell exhibiting no phagosome formation. (Scale bars: 50 μm.) In EP cell, the longer chain of diatoms on the left is partially digested, and therefore, the plastids of several diatom cells still display the autofluorescence of chlorophyll (arrows), whereas the shorter chain on the right is in a later stage of digestion and contains remnant plastids (dark brown grains) displaying no autofluorescence. Additional information is given in Fig. S2.
duction by various herbivorous protists, its lifetime in their cells and the environment (e.g., in the excreta), and the sinking rate of POM in each environment. In particular, a better understanding of the chemical properties of cPPB-aE in vivo is required to explain its apparently high preservation potential in environmental samples, despite its instability in organic solutions. In fact, we do not exclude the possibility that protistan catabolism responsible for producing cPPB-aE would occur even in darkness (e.g., in deep water columns and sediments), despite our hypothesis that the process evolved primarily to avoid the photoxicity of chlorophyll derivatives.

**Evolutionary Significance of Protistan Herbivory.** We postulate that the ingestion of Chl-a by small, transparent, or translucent microbes in illuminated and oxygenated environments places an evolutionary constraint on microbial herbivory by necessitating appropriate biochemical strategies to contain levels of singlet oxygen-generating molecules after feeding on microalgae, cyanobacteria, or phototrophic bacteria. The evolution of aquatic microbial herbivory should have been accompanied by the establishment of some enzymatic pathways that catabolize chlorophylls into nonfluorescent substances. Unlike the NCCs of higher plants, cPPB-aE is a colored catabolite, despite its nonphotosensitizing properties. Given that conversion of Chl-a to cPPB-aE apparently requires fewer enzymatic steps than the generation of NCCs, it has emerged as a simple and efficient strategy for rapid detoxification of chlorophyll among aquatic protists.

Our evidences regarding the synthesis of cPPB-aE by protistan herbivores are comparable with the results of similar feeding experiments in the work by Goericke et al. (17) using heterotrophic protists. The work by Goericke et al. (17) also identified cPPB-aE as a major Chl-a derivative in the fecal material of three protists, the ciliate *Strombidinopsis acuminatum* and the heterotrophic dinoflagellates *Amphidinium* sp. and *Noctiluca scintillans*. Fig. 7 illustrates that cPPB-aE producers, thus, distribute widely among the protistan lineage, at least in two supergroups. These are the Stramenopile-Alveolate-Rhizaria (SAR) clade and the Cryptophyte-Centrohelid-Telonemid-Haptophyte (CCTH) clade (21, 22). In contrast, one species belonging to the opisthokonta clade lacks the ability to synthesize cPPB-aE. Significantly, over 70% of heterotrophic protists in the marine environment are known to belong...
CCTH protists are colorless and lack plastids, and thus, they are verse picophytoplankton species (24). The rest of the SAR and freshwater Lake Biwa (23), which is consistent with our geochemical data.

The SAR and CCTH clades include various phototrophic and mixotrophic protists other than the obligate heterotrophs analyzed here. Many of these phototrophic and mixotrophic protists possess plastids that had been derived from ancestral red algae through secondary symbiosis (Fig. 7). They include major marine algae, such as diatoms and dinoflagellates, as well as diverse picophytoplankton species (24). The rest of the SAR and CCTH protists are colorless and lack plastids, and thus, they are presumably heterotrophic. Nonetheless, it is still not certain whether these protists lost plastids during the course of evolution (i.e., secondary evolution of heterotrophy) or had never possessed plastids (i.e., multiple origins of secondary phototrophy). Our evidence implies that the generation of cPPB-aE, a key metabolic step in the heterotrophic lifestyle, is likely to have evolved at the root of the two clades, if not at an earlier evolutionary stage. The ability to synthesize cPPB-aE must, thus, be retained in heterotrophic protists of distant lineages, regardless of the reason for the absence of plastids.

Fig. 6. Depth profiles of absolute and relative abundances of chlorophyll derivatives in water columns. (A–C) Absolute abundances. (D–F) Relative abundances. POM in the pelagic Pacific Ocean (Kumano-Nada; A and D), POM and surface sediments from Ise Bay (B and E), and POM and surface sediments from freshwater Lake Biwa (C and F) are shown.

Fig. 7. Unrooted tree of eukaryotes comprising six distinctive supergroups. Yellow stars beside taxonomic groups denote that they contain protists that produce cPPB-aE, which was reported in this study or a previous report (17). Examined protists belonging to discicristoidea did not produce cPPB-aE (red x). The taxonomic groups with green circles contain phototrophs with true chloroplasts. Note that the stramenopiles include not only the cPPB-aE–producing heterotrophs but also phototrophic diatoms used as diets that did not produce any trace of cPPB-aE (Fig. 2). This finding suggests that cPPB-aE metabolism evolved primarily for heterotrophy. Modified from Walker et al. (22).
Ecological Significance of Protistan Herbivory. We postulate that herbivory, particularly of the picophytoplankton, by cPPB-aE-producing protists is one of the most important processes in the modern aquatic food web, but it has been poorly understood until now (Fig. 8). Importance of protistan herbivory as a part of the microbial loop in aquatic environments has been recognized for nearly three decades (25). During this time, the quantitative significance of picophytoplankton (ϕ ≤ 3 μm) has also been revealed (23, 24). Picophytoplankton includes coccoidal cyanobacteria and eukaryotic picophytoplankton. The former belong to the two major genera Prochlorococcus and Synechococcus, which account for up to 50% of pelagic marine oxygenic photosynthesis (26–28). Eukaryotic picophytoplankton are believed to account for 20–50% of aquatic photosynthesis (29–32). The use of the PCR to analyze environmental samples has also revealed the amazingly wide diversity and activity of eukaryotic picophytoplankton species (33, 34).

However, we are still largely ignorant of abundance as well as ecological and biogeochemical roles of colorless protists in the oceans, in large part because the majority of these protists has never been studied because of an inability to maintain them in culture. Indeed, only recent advances showed the tremendous diversity of protists in aquatic environments (32, 35, 36), thanks largely to molecular biological approaches, such as PCR surveys of the diversity of 18S rRNA sequences in environmental samples. Therefore, the ecological functions of aquatic colorless protists remain largely unexplored (37, 38).

Our evidence suggests that picophytoplankton-based protistan herbivory is a quantitatively important process in aquatic environments. In analyses of POM samples from the water column of Lake Biwa, cPPB-aE was substantially enriched in the fine POM fractions, which contained POM with diameters of 5–0.7 μm (Fig. S4), which indicates that cPPB-aE was either accumulated in picozooplanktonic protists (ϕ ≤ 3 μm) and/or concentrated in very fine particulate excreta most likely derived from variously sized zooplanktonic protists. Therefore, picophytoplankton are probably consumed by these protistan herbivores (Fig. 8). In fact, the small size of picophytoplankton precludes their consumption by metazoan zooplankton such as copepods, suggesting that they are instead likely to be preyed on by protists (23, 39).
Conclusions

The singlet oxygen generated when oxygen is photosensitized by chlorophylls is probably a major concern among the organisms living on the oxygenated and illuminated surface of the Earth. However, all organisms have evolved to somehow protect themselves against the potential phototoxicity of the chlorophylls in their respective environments. However, the significance of these detoxification processes is only readily apparent in such striking manifestations as autumnal tints. In the present work, we have shown a cryptic process of chlorophyll detoxification that is widely distributed among the SAR and CCTH protists, where colored but fluorescent cPPB-E is the catabolite of Chl-a. The ubiquitous occurrence of cPPB-E in all aquatic environments indicates that cPPB-E metabolism is one of the major chlorophyll detoxification mechanisms in the modern global ecosystem.

If we consider cPPB-E a biomarker of protistan activity, our results suggest the quantitative importance of herbivory by the SAR and CCTH protists in aquatic environments. Picophytoplanktons are particularly likely to be an important prey. Although the microbial loop tends to be pictured as a material flow starting from bacteria and archaea that are fed on POM or dissolved organic matter (i.e., protistan bacterivory), nano- and microplanktonic protists should also play primary roles in the picophytoplankton-based microbial loop (37, 40, 41) that, hence, makes substantial contributions to the flow of carbon and energy in aquatic environments (42). Additional studies of cPPB-E, including its suitability as a proxy for protistan herbivory, may provide approaches for quantifying the contribution of picophytoplankton to flux in the aquatic geochemical cycle.

A better understanding of cPPB-E metabolism within and outside the SAR and CCTH clades of protists may provide valuable insights into the evolutionary origin of the modern aquatic ecosystem, founded largely on protistan herbivory. Feeding on photosynthetic organisms in situ in the presence of molecular oxygen and light, would have enabled much more efficient bio-geochemical recycling and bioenergetic redistribution within environments near the water surface. Interestingly, chemical structures of fossil biomarkers that could be derived from cPPB-E have been reported from various aquatic sedimentary rocks as old as the Early Jurassic (ca. 183 Ma) (Fig. S5) (10, 43–50). Interestingly, fossil-based evidence suggests that this timing correlates roughly with the emergence of secondary algae containing red algae-derived plastids. These plastids include phototrophic protists within the SAR and CCTH clades, including dinoflagellates, coccolithophores, and diatoms (51). An understanding of aquatic microbial herbivory gleaned from combining biochemical, molecular biological, and geochemical evidence would provide critical insights into the evolutionary dynamics of the aquatic protistan lineages as well as the global geochemical cycles.

Materials and Methods

Preparation of Authentic Samples. Schemes for preparation of authentic samples of Chl-a, Phe-a, pPhe-a, pPPB-a, cholesteryl pPPB-a, cPPB-E, and (R/S)-hPLs are summarized in Fig. S6. Additional details are described in SI Text, section 2.1.

Development of HPLC Methods. We developed improved analytical methods that enable quantitative identification of unstable cPPB-E from microbiological and environmental samples with high sensitivity. Analytical difficulties arose as a consequence of the instability of cPPB-E during handling during extraction and analysis involving HPLC (11, 13, 17). Given that cPPB-E is rather unstable in most organic solvents in the presence of molecular oxygen, it was rapidly degraded, especially when present at low concentrations. Our results, thus, depend largely on the development of improved analytical methods that required the availability of the semisynthesized authentic standard. In fact, the analysis required (i) careful removal of molecular oxygen from the solvents for extraction and the mobile phases of HPLC, (ii) use of an end-capped reverse-phase HPLC column as well as its deactivation by preconditioning using mobile phase with 0.5% (wt/vol) trifluoroacetic acid, and (iii) use of stabilizing agents. In particular, the addition of imidazole in the mobile phase dramatically improved the quantitative analysis of cPPB-E using HPLC. In addition, cPPB-E was found to be particularly stabilized in anisole solution. Therefore, the use of the standard solution in anisole permitted accurate calibration on the HPLC analysis (SI Text, section 2.2). Consequently, the current methods significantly improved the quantitative analysis of cPPB-E (Fig. S7, Fig. S8, and SI Text, section 2.3). Detection limit of cPPB-E was ~30 fmol per injection on the current HPLC method.

Analytical HPLC (Fig. S9) was performed using a Shimadzu Prominance liquid chromatograph system, which comprised a CBM-20A communications bus module, a DGU-20A, degasser, two LC-20AD pumps constituting a binary pumping system, an SIL-20AAC auto sampler, a CTO-20AC column oven, and an SPD-M20Avp diode array detector. The system was coupled to a personal computer configured to run Shimadzu LC Solution software. All solvents used for the analytical HPLC were of HPLC-grade quality, and they were purchased from Nacalai Tesque. The standard solutions of various Chl-a derivatives used in the following analytical HPLCs were prepared using anisole (SI Text, section 2.2).

Analytical HPLC involved the use of a reverse-phase monomeric column ZORBAX Eclipse Plus C18 (4.6 x 30 mm, 1.8-μm silica particle size; Rapid Resolution HT). The solvent gradient program used is summarized in Table S5, including conditions used to precondition the column. Because only a binary automated programming technique with two pumps is available in our system, solvents B and C are introduced to the same second pump using a switching valve in the line. Therefore, switching of the solvents and purging of the pump system by the solvent were performed promptly within 1 min after preconditioning of the column. All three solvents were degassed in vacuo with ultrasonication and sealed under argon. The solvent reservoir bottles were specially customized for the convenience of degassing or sealing with argon as well as preventing the solvent from contacting the air. The flow rate of the mobile phase was 1.00 mL min-1. The column oven was set to 25 °C. The auto sampler tray was set to 15 °C. Given that standard solutions and samples were prepared in anisole, which is not a component of the eluent, the injection volume was generally 1.00 μL or smaller.

Feeding Experiments. Four combinations of heterotrophic protists with algae, a combination of a crustacean zooplankton with an alga, and a combination of a heterotrophic protist with plant materials were examined. Experiments were designed to provide insights into the feeding behavior of various heterotrophic feeding experiments as well as six control experiments without heterotrophs/crustacean zooplankton/bacteria. All protistan strains as well as the copepod Daphnia magna were maintained by feeding unialgal clones (Table S1). After certain incubation periods, these strains were collected on sterile glass microfiber filters (GF/F grade; 47 mm r; Whatman), which were then extracted and analyzed according to the analytical procedures used for natural samples described below. Information including specific names of organisms examined, their phylogenetic position, strain identities, and experimental conditions are summarized in Table S1.

Optical Spectrometry and Singlet Oxygen Detection Experiments. Electronic absorption spectra were measured using a Hitachi U-3500 spectrophotometer. Fluorescence spectra were measured using a Hitachi F-4500 spectrophotometer, and fluorescence quantum yields were obtained using a photoluminescence yield measurement system model C9920-02 comprising an excitation xenon light source, a monochromator, an integral sphere, and a multichannel CCD spectrometer (Hamamatsu Photonics). OD was about 1.0/10 mm at the Soret absorption maximum in both anisole and tetrahydrofuran (THF) for electronic absorption measurements. THF used for spectroscopy was distilled from a regent purchased from Nacalai Tesque. Anisole (RegentPlus grade) and tert-butyl methyl ether (ACS regent grade) were purchased from Sigma-Aldrich. All of the other solvents used herein were spectrometry-grade regents purchased from Nacalai Tesque, and they were used without additional purification. In optical spectroscopy, absorption and emission properties of chlorophyll derivatives in THF and anisole are listed in Table S2. In experiments involving cPPB-E dissolved in THF, we, therefore, prepared the solution under argon in freshly distilled THF and measured all optical properties immediately. In singlet oxygen detection experiments, generation of singlet oxygen was detected using SOSG (Invitrogen), a commercially available fluorescent probe (52). A chlorophyll derivative (10 μM) and SOSG (1 μM) were dissolved in anisole and methanol (1:1, vol/vol), placed in a quartz cell, irradiated with red light that was provided by a 250 W metal halide lamp (LS-250–7500; Sumita Optical Glass), and passed through a colored glass filter that failed to transmit light with a wavelength shorter than ~630 nm (AGC Techno Glass). Therefore, the irradiated light selectively excited chlorophyll derivatives but not SOSG. The aerated solutions were continuously stirred during irradiation.
experiments using a magnetic stirrer. The increase in fluorescence intensity (i.e., initial fluorescence ascribable to the purchased SOSG was subtracted) was then stored below −20 °C before analysis. Sediment samples were stored at 4 °C in the dark, with the extraction and sonication procedures repeated a total of three times. The combined extracts were then dried under a stream of argon in the dark. The residue was then dissolved in anisole. All of the above operations were performed in an argon atmosphere using a glove bag. Acetone and anisole were carefully weighed and purged with argon gas before use. The sample dissolved in anisole was then transferred for the vial for the autosampler of the HPLC system.

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Supporting Information

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SI Text

1. Notes. 1.1. Degradation pathway of chlorophylls in higher plants. In higher plants, the degradation pathway involves four to seven enzymatic steps, of which oxygenic opening of the macrocycle by pheophorbide a (PPB-a) oxygenase (PAO) plays a central role in the detoxification process. This role accounts for it being named the PAO pathway. In the PAO pathway, chlorophyll a (Chl-a) is eventually converted to nonfluorescent chlorophyll catabolites (NCCs), with the accumulation of only trace levels of phototoxic intermediate catabolites. This result protects the protease involved in recycling components of the photosynthetic apparatus from damage by singlet oxygen (1).

1.2. Abiotic and biotic degradations of chlorophylls in aquatic environments. In mesoscopic- to macroscopic-scaled observations/ experiments of aquatic systems, a variety of degradative chlorophyll derivatives have been reported. The majority of the derivatives retain the π-conjugated chlorin moiety, although its peripheral functional groups are modified and/or lost to different degrees. Algal senescence and death-related degradation of chlorophylls have been studied thoroughly by analyses of dark incubation experiments of unialgal cultures monitored for up to 10 y (2–4). The observed chlorophyll derivatives [e.g., pheophytin a (Phe-a), pyropheophytin a (pPhe-a), and pyropheophorbide a (pPPB-a)] still retain the π-conjugated tetrpyrrolic macrocycle that renders them phototoxic because of their abilities to efficiently generate singlet oxygen (Materials and Methods and Fig. 4C). In contrast, in growing unialgal culture experiments, the majority of chlorophylls was inferred to be converted into partially π-conjugated linear tetrpyrroles, the red chlorophyll catabolites, which were extracted from the culture media (5). Before the experiments, a green algae (Aurouochlorella protothecoides) was reported to excrete putative red chlorophyll catabolites, likely generated by the catalytic activity of a PAO-like enzyme (SI Text, section 1.1) on the opening of the macrocycle (6). A true colorless, non-fluorescent chlorophyll catabolite has not yet been reported from algae and cyanobacteria.

1.3. Toxicity of liberated chlorophylls in plant cells. Liberated chlorophyll derivatives in cells have been suggested to be highly toxic to land plants. For example, accumulation of phototoxic intermediates in lesion-mimic mutants causes cell death by damaging the proteins in the cells in which singlet oxygen is generated (7, 8). Even in WT plants, the accumulation of phototoxic PPB-a after the down-regulation of PAO expression generates local cell death as part of a strategy, known as the hypersensitive response, that resists pathogen infection and establishment (9).

1.4. Other possible mechanisms protective against phototoxic derivatives. We failed to identify any trace of 1517-cyclophorophorbide a enol (cPPB-aE) from either the extracts of any bacterial clones grown on Chl-a-containing culture media or the crustacean zooplankton (Daphnia pulex) that was fed an algal clone (Fig. 2 and Table S1). Our experiments, thus, did not agree with results from previous analyses of fecal pellets from crustacean zooplankton, where cPPB-aE had been identified in an euphausiid and a copepod (10). Therefore, our results imply the involvement of protists in the production of cPPB-aE within crustaceans (11) or their excrement, and they suggest that the cPPB-aE production in environments is only attributed to heterotrophic protists belonging to the Stramenopile-Alveolate-Rhizaria (SAR) and/or Cryptophyte-Centrohelid-Telomonid-Haptophyte (CCTH) clades.

All these organisms are translucent and therefore, are potentially subject to the phototoxicity of chlorophyll derivatives. Thus, our observations suggest the presence of other protective mechanisms in organisms that do not produce cPPB-aE, including discicristioideans. Various protective mechanisms against chlorophyll phototoxicity should be present in all translucent herbivores living in illuminated surface–water environments. Even the SAR–CCTH protists that we observed in the present study contain some amounts of phototoxic chlorophyll derivatives, such as PPh-ea, as well as nonphototoxic cPPB-aE, indicating the presence of an additional protective mechanism. Therefore, cPPB-aE metabolism is merely one of the protective mechanisms (but undoubtedly a major one) in aquatic microbial ecosystems.

1.5. On the fluorescence spectra of cPPB-aE. Only very weak fluorescence was observed for cPPB-aE dissolved in either tetrahydrofuran (THF) or anisole. An excitation spectrum of cPPB-aE dissolved in anisole (Fig. S1B) exhibited an intense maximum at 413 nm and two small peaks in the 500- to 600-nm range. This spectrum was very different from the absorption spectrum of cPPB-aE, which has split three Soret maxima at 360, 428, and 459 nm, with highly diminished Qy bands in the 500- to 600-nm range (Fig. 4B). In addition, the emission maximum of cPPB-aE in anisole at 676 nm (Fig. S1C) is 15 nm shorter than the Qy absorption maximum at 691 nm (Fig. 4B), suggesting that emission is derived from any compound structurally different from cPPB-aE. The excitation spectrum resembles the spectra of PPB-a–type pigments, which include strongly fluorescent hydroxychlorophyllones a (hCPLs-a), a potential oxidative product of cPPB-aE. The observations made using the THF solution were essentially the same.

Moreover, the quantum yield of the fluorescence emission from the pure cPPB-aE (its purity checked by NMR and TOF mass spectra) solutions varied between repeated experiments, generally increasing in time after preparation of the solution. The values varied between 0.2% and 0.7%, even among freshly prepared solutions, depending on the authentic samples from independent semisynthesis experiments (we prepared several batches of cPPB-aE throughout the course of this study). It, thus, suggests that any fluorescent compounds were produced before or during the measurements. All of the evidence supports our conclusion that the observed very weak fluorescence signals were attributable to trace amounts of fluorescent compounds in our authentic standards and that genuine cPPB-aE itself is, in fact, nonfluorescent. This conclusion is essentially the same as the conclusion in the work by Falk et al. (12), which also observed trace fluorescence in the synthesized samples. An absence of fluorescence has also been reported for other enol derivatives of chlorophyll, including Mg-chelated cPPB-aE (13) and the peripheral metal complexes of PPB-a (14).

2. Materials and Methods. 2.1. Preparation of authentic samples. 2.1.1. General. 1H-NMR spectra in CDC13 were recorded at 293 K on a JEOL ECA-600 (600 MHz) spectrometer; residual CHCl3 (δH = 7.26 ppm) was used as an internal reference. TOF mass spectra were obtained using direct laser desorption/ionization by a Shimadzu AXIMA-CFRplus spectrometer. Methyl pPPB-a (Fig. 1) was modified by naturally occurring Chl-a (Fig. 1) extracted from a commercially available cyanobacterium (Spirodinia powder) (15). Transesterification was performed according to reported procedures (16). Synthetic procedures were performed under argon or nitrogen atmosphere in the dark. Silica gel A (C-300, particle size = 45–75 μm; Wakogel) and silica gel B (Kieselgel 60, 63–200 μm; deactivated with 20% water; Merck) were used for flash column chromatography (FCC). Reverse-
phase HPLC (Cosmosil 5C18−AR-II, 10 × 250 mm) was performed on a Shimadzu liquid chromatograph system comprising an SCL-10Avp system controller, an LC-20AT pump, and an SPD-M10Avp photodiode array detector.

2.1.2. Preparation of Chl-a and Phe-a and preparation of pPhe-a. Dried cyanobacterial powder (Spirulina powder) was extracted by acetone, which was evaporated in vacuo (Fig. S6). The residue was purified by FCC (silica gel A, petroleum ether, and 2-propanol; 96:4, vol/vol) and HPLC (methanol and acetone; 90:10, vol/vol) to give pure Chl-a.

A hexane solution of Chl-a was treated with aqueous 1 M HCl solution and dried in vacuo. The residue was purified by HPLC (methanol and acetone; 75:25, vol/vol) to give pure Phe-a (Fig. 1).

A collidine solution of Phe-a was refluxed for 3 h under argon. After collidine was evaporated in vacuo, the residue was crystallized twice from dichloromethane and methanol to give pure pPhe-a (Fig. 1).

2.1.3. Synthesis of cholesteryl pPPB-a. Methyl pPPB-a (101 mg, 184 μmol), cholesterol (700 mg, 1.80 mmol), and bis(dibutylchlorotin oxide) [(Bu2SnCl)2] was added to the mixture (121 mg, 134 μmol; 73%) as a black solid: 1H NMR (CDCl3) δ = 9.44 (1H, s, 10-H), 9.33 (1H, s, 5-H), 8.55 (1H, s, 20-H), 7.98 (dd, 1H, J= 18, 11 Hz, 3-CH3), 6.26 (1H, dd, J= 18, 1 Hz, 32-CH trans to 3-H), 6.15 (1H, dd, J= 11, 1 Hz, 31-CH cis to 3-H), 5.28, 5.12 (each 1H, J = 19 Hz, 13C-CH2), 5.30–5.25 (1H, m, cholesterol 6-H), 4.55–4.46 (1H, m, cholesterol 3-H), 4.50 (1H, br-q, J = 7 Hz, 18-H), 4.31 (1H, dt, J= 9, 3 Hz, 17-H), 3.65 (3H, s, 12-CH3), 3.64 (2H, q, J = 8 Hz, 8-CH2), 3.40 (3H, s, 2-CH3), 3.20 (3H, s, 7-CH3), 2.74–2.63, 2.55–2.45, 2.37–2.28, 2.28–2.20 (each 1H, m, 17-CH2CH2), 2.20–2.10, 1.98–1.91, 1.91–1.85, 1.81–1.73, 1.71–1.63, 1.63–1.57, 1.56–0.93, 0.91–0.74 (2H+1H+1H+1H+1+18H+3CH3, m, cholesteryl CHx6, CHx11), 1.82 (3H, J = 7 Hz, 18-CH3), 1.68 (3H, t, J = 8 Hz, 8′-CH3), 0.89 (3H, d, J = 7 Hz, cholesterol 20-CH3), 0.870, 0.865 (each 3H, d, J = 7 Hz, cholesterol 25-CH3), 0.859 (3H, s, cholesterol 10-CH3), 0.62 (3H, s, cholesterol 13-CH3), 0.43–0.172 (each 1H, m, NHx2), MS (TOF) found m/z = 920.9 (calculated for C57H80N2O4: M+ 920.6).  

2.1.4. Synthesis of cPPB-aE. According to reported procedures (17), cPPB-aE (Fig. 1) was prepared by modifying, and the synthetic details are described below (Fig. S6). Note that all of the synthetic procedures must be done strictly under nitrogen atmosphere in the dark.

The distilled THF (30 mL) solution of synthetic methyl pPPB-a (276.2 mg, 0.503 mmol) was stirred for 30 min, to which commercially available sodium bis(trimethylsilyl)amide ([Me3Si]2NaN, 3.5 mL, 38% in THF, ca. 1.9 M; Tokyo Chem. Ind.) was added and further stirred for 30 min. After the color of the reaction mixture was changed from green to yellow, a nitrogen-purged mixture of an aqueous solution of sodium dihydrogen phosphate (NaH2PO4, 20 mL) and dichloromethane (400 mL) was added and stirred vigorously until the mixture became clear green. The separated organic phase was washed with water, dried over sodium sulfate, and evaporated under a reduced pressure. The residue was purified by FCC on silica gel B, and the desired eluate (sole dichloromethane as an eluent) was evaporated. After recrystallization from dichloromethane and hexane, analytically pure cPPB-aE was obtained as a green solid (20.0 mg, 0.039 mmol, 7.7%): 1H-NMR (600 MHz, CDCl3) δ = 13.49 (1H, s, 17-CH3), 9.01 (1H, s, 10-H), 8.99 (1H, s, 3-H), 8.08 (1H, s, 20-H), 7.86 (1H, dd, J= 18, 11 Hz, 3-CH2), 6.20 (1H, dd, J= 18, 1 Hz, 32-CH trans to 3-H), 3.62 (1H, dd, J=11, 1 Hz, 31-CH cis to 3-H), 4.08 (1H, dq, J= 8 Hz, 17-H), 3.74 (1H, dt, J = 8, 5 Hz, 17-H), 3.52 (2H, q, J = 8 Hz, 18-CH2), 3.43 (3H, s, 12-CH3), 3.25 (3H, s, 2-CH3), 3.10 (3H, s, 7-CH3), 3.13–3.07 (1H, m, 17-CH), 2.98 (1H, dt, J= 18, 3, 17-CH), 2.49–2.44 (2H, m, 17-CH2), 2.03 (3H, d, J= 7 Hz, 18-CH3), 1.62 (3H, t, J = 8 Hz, 31-CH), 0.68 (each 1H, s, NH; another NH could not be identified), MS (TOF) found m/z = 517.0 (calculated for C33H38N2O4: MH+ , 517.3).

2.1.5. Synthesis of (R)-hCPL-a and (S)-hCPL-a. (R)-hCPL-a and (S)-hCPL-a (Fig. 1) were prepared from the authentic cPPB-aE according to reported procedures (Fig. S6) (17). The products were further purified by HPLC (acetonitrile, water, 75:25, vol/vol, 0.5 vol % trifluoroacetic acid (TFA)) to give the titled compounds.

2.2. Standard solutions for HPLC analyses. We found that cPPB-aE is quite unstable in many organic solvents, even in darkness. We did not observe any substantial degradation of cPPB-aE when it was dissolved in deoxygenated anisole after being stored for more than 1 mo in the dark or after exposure to ambient light under normal laboratory conditions for up to 10 min. All of the authentic samples used in the present study were highly soluble in anisole and stable in the solution for an extended period.

Reliable standard solutions with accurate concentration values are a prerequisite for quantitative analytical HPLC using a diode array detector. We, thus, determined molar extinction coefficients of Chl-a derivatives in anisole using the authentic samples (Table S3). Using these values, molar concentrations of working standard solutions were determined spectrometrically.

Electronic absorption spectra were measured using a Hitachi U-3500 spectrophotometer. Anisole (RegentPlus grade), used for spectrometry as well as for the preparation of standard solutions for analytical HPLC, was purchased from Sigma-Aldrich.

2.3. Evaluation of the HPLC method. It is known that cPPB-aE is easily degraded into various compounds with pPPB-aE-like absorption spectra (18), which generally elute more quickly than cPPB-aE during reverse-phase HPLC. Consequently, the extent to which the peak of cPPB-aE is significantly diminished is highly variable, and the extent to which peaks are diminished was not perfectly reproducible, even when using an identical analytical method. This problem was successfully addressed by a combination of (i) preconditioning of the column using a mobile phase that comprises an aliquot of TFA (solvent C in Table S5) with (ii) the addition of imidazole to the mobile phase used during the analysis (solvents A and B in Table S5).

The column needed to be free of TFA during the analysis, because cPPB-aE seems to be protonated by TFA during HPLC, and the protonated species are eluted more rapidly than the solvent, causing it to behave as a distinct compound. Therefore, the entire HPLC line (including the column) was purged using a mobile phase with an identical composition to the phase used in the beginning of the gradient analysis. However, the efficacy of TFA conditioning gradually degrades as the non-TFA mobile phase is flushed. We have established that 6 min with the purge flow at 1.00 mL min−1 immediately before injection of a sample provides the best chromatographic profile of cPPB-aE (Table S5).

The concentration of imidazole in the mobile phase is critically related to the heights or areas of cPPB-aE peaks in chromatograms (Fig. S7A). As the concentration increases, the area values seem to increase, reaching a plateau. Considering the possibility of potential damage of the column caused by the use of a highly concentrated imidazole mobile phase, we decided to use 50 mM imidazole for the present analysis (Table S5). The calibration line in Fig. S7B shows that the high accuracy of quantitation on cPPB-aE in the present method is guaranteed down to 200 fmol per injection. We could still quantify 50 fmol cPPB-aE per injection with a fair degree of accuracy (Fig. S7C), and the detection limit was ~30 fmol per injection using our system. We also drew calibration lines for Chl-a, Phe-a, pPPB-a, and the cholesterol pPPB-a (Fig. S8).

Actual chromatograms of standard samples as well as an extract of natural samples are shown in Fig. S9.

Fig. S1. Fluorescence spectra of cPPB-aE in anisole. (A) 3D fluorescence spectra. (B) An excitation spectrum detected at 676 nm. (C) An emission spectrum excited at 413 nm. These spectra also suggest the absence of any fluorescent species related to absorption spectrum of cPPB-aE shown in Fig. 4B.
Fig. S2. A time series illustration of phagocytosis by a cercozoan protist engulfing the centric diatom, Skeletonema sp. (Left) Differential interference images. (Right) Fluorescent image showing chlorophyll auto-fluorescence from diatom plastids (excitation light = 400–440 nm; the cell of the cercozoan is traced by a dashed line). A cercozoan preying on a filament of Skeletonema sp. elongates its cell membrane from the right to left to form a phagosome (A and A'; 0 min); the red arrow in A indicates the front of the phagosome. The entire filament of the centric diatom Skeletonema sp. has been completely engulfed by the cercozoan cell (B and B'; 4 min). The chlorophyll auto-fluorescence in the phagosomes within the cells was significantly reduced at 13 min (C and C'); loss of auto-fluorescence occurred heterogeneously among the diatom plastid, indicated by the arrows pointing to a still highly fluorescent plastid. The auto-fluorescence of all of the plastids was completely lost at 28 min (D and D'), which is in marked contrast to the auto-fluorescence of the live diatom cells floating near the cercozoan. The cercozoan swam around actively using its flagella, even after it had engulfed the diatom (particularly in the first 30 min). By 65 min (E and E'), the plastids of the digested diatom had shrunk to dark brown, non-fluorescent grains that remained in the silicate cell walls until they were excreted from the cercozoan at 81 min (F and F'). (Scale bars: 50 μm.) Additional information is shown in Fig. 3.
Fig. S3. Schematic representation of the production of singlet oxygen by a photosensitizer. Most of organic compounds with π-conjugated systems, including chlorophylls and their derivatives, are in a singlet state in their ground energy states. When such compounds are photoexcited, their excited singlet states are generated, which can then relax to the ground singlet state either by fluorescence (the emission of a photon) or some internal conversion mechanisms (resulting in the release of heat). Alternatively, the excited singlet state may relax to a more stable excited triplet state through intersystem crossing. The quantum yield to produce the triplet state depends on the molecular species. However, molecular oxygen is in a triplet state in its ground state. Excited triplet energy of chlorophylls can be transferred to the ground state of molecular oxygen, resulting in the generation of a singlet molecular oxygen (singlet oxygen). Because direct excitation from the ground triplet to singlet oxygen is forbidden, singlet oxygen is usually produced only by triplet energy transfer from photoexcited (triplet) molecules. Chlorophylls and some of their derivatives relax to their excited triplet states with relatively high efficiency after their photoexcitation, hence readily generating singlet oxygen.

Fig. S4. Depth profiles of the absolute abundances of chlorophyll derivatives in extracts of the particulate organic matter (POM) from the euphotic layer of Lake Biwa on a spring day (March 15, 2012). Larger POM (>5 μm) was separated by passing the sample through 5-μm nylon mesh (PP-5n; Kyoshin Rikoh Inc.); the finer POM in the filtrate was then collected onto a glass microfiber filter (GF/F grade; Whatman) (pore size = 0.7 μm). Chl-a in the 0.7–5 μm fraction was thought to be derived from the small cryptophytes observed as the dominant phytoplankton on the day of sampling.
Fig. 55. Schematic representation of the possible transformation reaction path of cPPB-aE. Fossil porphyrins with structures related to those structures reported from geological samples were estimated to be generated after cPPB-aE. In early stages of diagenesis, cPPB-aE is thought to be degraded to such compounds as hCPL-a (refs. 1 and 2 and references therein) and meso-cPPB-aE (3, 4). Meso-hCPL-a has been reported from the Miocene Gessoso-Solfifera Formation (5) and as old as the Upper Cretaceous Demerara Rise boring core sample (6, 7). Their oxygen functional groups would then be reduced to generate free base chlorin as reported from the Pliocene Willershausen clay in the work by Keely et al. (8). Thus, chemical structures of bicycloalkanoporphyrins (BiCAPs) possessing fused five- and seven-membered rings reported from relatively matured sedimentary rocks are regarded to be homologous to those structures of cPPB-aE, hence strongly indicating that these fossil porphyrins should have been originated in cPPB-aE produced in herbivorous protists in the ancient oceans/lakes (2, 9, 10). BiCAPs have been reported from the Miocene Monterey Formation (11, 12), the Miocene Onnagawa Formation (9), the Upper Cretaceous El Lajjun shale (13), the Lower Cretaceous Bonarelli Shale (10), and the Lower Jurassic Toarcian Shale (11).

Fig. S6. Scheme for the preparation of authentic samples used for HPLC analysis.
Fig. S7. Quantitative detection of cPPB-aE using HPLC. (A) The HPLC peak area for 1 pmol cPPB-aE was dependent on the concentration of imidazole in the mobile phase. The cross-plots of data from experiments by mobile phase with 50 mM imidazole are illustrated after injection of a mass larger than 200 fmol (B) and a mass smaller than 200 fmol (C). The regression lines in these plots were used to calibrate the HPLC chromatogram peak area for quantitation of cPPB-aE in natural samples. Elution of cPPB-aE was detected at 686 nm.

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Fig. S8. Cross plots of peak areas against injected masses of various authentic samples. Plots are for Chl-a over 197 fmol (A) and below 197 fmol (B), Phe-a over 200 fmol (C) and below 200 fmol (D), pPhe-a over 200 fmol (E) and below 200 fmol (F), and cholesteryl pPPB-a over 196 fmol (G) and below 196 fmol (H). Elution of all compounds was detected at 664 nm.
Fig. S9. 3D HPLC chromatograms. (A) An authentic sample mixture. (B) Extract of the culture of the heterotrophic stramenoplile. (C) Extract of POM of Tokyo Bay at 3.5 m depth. (D) Extract of surface sediment of Tokyo Bay.
Table S1. Information on the feeding experiments

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<th>Scientific name</th>
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<th>Strain identification</th>
<th>Collection site</th>
<th>Isolator</th>
<th>Culture conditions</th>
<th>Media</th>
<th>Temperature (°C)</th>
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<td>E05</td>
<td>Mouth of Kano River, Shizuoka, Japan</td>
<td>T.S. IMK</td>
<td>20 14L:10D 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daphnia magna</td>
<td>Opisthokonta; Metazoa; Arthropoda</td>
<td>—</td>
<td>—</td>
<td>VT</td>
<td>20 12L:12D 7</td>
<td>NIES-281</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptomonas tetrapyreroidosa</td>
<td>CCH; Cryptomonad</td>
<td>NIES-281</td>
<td>Purchased from NIES</td>
<td>—</td>
<td>20 12L:12D 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>γ-proteobacteria</td>
<td>—</td>
<td>—</td>
<td>DS</td>
<td>30 12L:12D 14</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green juice powder (barley)</td>
<td>Archaeplastida; Streptophyta</td>
<td>—</td>
<td>—</td>
<td>DS</td>
<td>30 12L:12D 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Four combinations of heterotrophic protists with algae, a combination of a crustacean zooplankton with an alga, and a combination of a bacterium with plant materials were examined. Experimental conditions, thus, describe six feeding experiments as well as six control experiments without heterotrophs/crustacean zooplankton/bacteria. D, dark; L, light.

*Culture strains of heterotrophic organisms were inferred from phylogenetic analysis using their 18S rDNA sequences.

Table S2. Absorption maxima (λ_{abs}) and emission properties of Chl-a, cPPB-aE, and other naturally occurring Chl-a derivatives in THF and anisole

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Abbreviations</th>
<th>Soret (nm)</th>
<th>Q_y (nm)</th>
<th>λ_{ex}* (nm)</th>
<th>λ_{em}† (nm)</th>
<th>Quantum yield‡ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>THF</td>
<td>Anisole</td>
<td>THF</td>
<td>Anisole</td>
<td>THF</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>Chl-a</td>
<td>435.6</td>
<td>432.4</td>
<td>664.0</td>
<td>665.0</td>
<td>432.2</td>
</tr>
<tr>
<td>13(^2),17(^2)-Cyclophloroporphide a enol</td>
<td>cPPB-aE</td>
<td>455.0</td>
<td>485.6</td>
<td>688.4</td>
<td>691.0</td>
<td>409.0</td>
</tr>
<tr>
<td>Pheophytin a</td>
<td>Phe-a</td>
<td>412.2</td>
<td>415.8</td>
<td>667.8</td>
<td>669.8</td>
<td>409.0</td>
</tr>
<tr>
<td>Pyropheophytin a</td>
<td>pPhe-a</td>
<td>412.6</td>
<td>416.0</td>
<td>668.0</td>
<td>669.8</td>
<td>408.8</td>
</tr>
<tr>
<td>Cholesteryl ester of pyropheophytin a</td>
<td>Cholesteryl pPPB-a</td>
<td>412.6</td>
<td>416.4</td>
<td>667.8</td>
<td>669.8</td>
<td>409.4</td>
</tr>
<tr>
<td>(13(^R))-Hydroxychlorophyllone a (R)-hCPL-a</td>
<td>(R)-hCPL-a</td>
<td>411.4</td>
<td>416.2</td>
<td>673.0</td>
<td>675.8</td>
<td>409.4</td>
</tr>
<tr>
<td>(13(^S))-Hydroxychlorophyllone a (S)-hCPL-a</td>
<td>(S)-hCPL-a</td>
<td>412.6</td>
<td>416.8</td>
<td>667.0</td>
<td>672.6</td>
<td>409.6</td>
</tr>
</tbody>
</table>

*Maxima at the Soret region in fluorescence excitation spectra detected at the major emission peak (λ_{em}).
†Maxima (λ_{ex}) in fluorescence emission spectra excited at the Soret maxima (λ_{abs}).
‡Fluorescence quantum yields were measured by excitation at the Soret maxima (λ_{abs}).
§Fluorescence bands are tentatively ascribed to a trace amount of any fluorescent compound generated during the experiments (SI Text).
Table S3. Molar extinction coefficients at Qy maxima of various Chl-a derivatives in anisole as well as other organic solvents

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Anisole</th>
<th>tert-Butyl methyl ether</th>
<th>Diethyl ether</th>
<th>Chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl-a</td>
<td>82.4 (665.2)</td>
<td>90.4 (661.6)</td>
<td>89.8* (660.8)</td>
<td>—</td>
</tr>
<tr>
<td>cPPB-aE</td>
<td>31.7 (691.0)</td>
<td>31.2 (687.2)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phe-a</td>
<td>48.4 (669.4)</td>
<td>—</td>
<td>52.6* (667.0)</td>
<td>—</td>
</tr>
<tr>
<td>pPhe-a</td>
<td>51.6 (669.7)</td>
<td>55.1 (666.9)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cholesteryl pPPB-a</td>
<td>51.8 (669.9)</td>
<td>56.3 (666.9)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(R)-hCPL-a</td>
<td>46.5 (672.6)</td>
<td>—</td>
<td>—</td>
<td>48.3† (673.8)</td>
</tr>
<tr>
<td>(S)-hCPL-a</td>
<td>48.0 (667.4)</td>
<td>—</td>
<td>—</td>
<td>50.9† (668.2)</td>
</tr>
</tbody>
</table>

*Data from the work by Kobayashi et al. (1).
†Data from the work by Ma and Dolphin (2).

Table S4. List of samples with their settings, localities, and dates of sampling

<table>
<thead>
<tr>
<th>Environments</th>
<th>Sample identification</th>
<th>Locality</th>
<th>Date of sampling</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelagic ocean</td>
<td>Kumano-Nada in the Pacific Ocean</td>
<td>33°40′ N 136°21′ E</td>
<td>November 10, 2011</td>
<td>—</td>
</tr>
<tr>
<td>Coastal oceanic basin</td>
<td>Ise Bay</td>
<td>34°45′ N 136°44′ E</td>
<td>November 8, 2011</td>
<td>—</td>
</tr>
<tr>
<td>Coastal oceanic basin</td>
<td>Tokyo Bay</td>
<td>35°37′ N 139°46′ E</td>
<td>June 19, 2011</td>
<td>—</td>
</tr>
<tr>
<td>Freshwater lake</td>
<td>Lake Biwa</td>
<td>35°23′ N 136°06′ E</td>
<td>October 14, 2011</td>
<td>—</td>
</tr>
<tr>
<td>Minor waterpool</td>
<td>Dammed creek in BKC</td>
<td>34°59′ N 135°58′ E</td>
<td>October 25, 2011</td>
<td>—</td>
</tr>
<tr>
<td>Minor waterpool</td>
<td>Paddle in a garden rock</td>
<td>36°10′ N 136°13′ E</td>
<td>February 13, 2012</td>
<td>—</td>
</tr>
</tbody>
</table>

Table S5. Gradient programs for binary solvent system used in the HPLC analyses

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
<th>C (%)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−37</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>Preconditioning by TFA</td>
</tr>
<tr>
<td></td>
<td>−7</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−6</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>Purging by initial mobile phase</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>Actual analysis</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Solvent A, acetonitrile/ethyl acetate (90:10), 50 mM imidazole; solvent B, acetonitrile/ethyl acetate (10:90), 50 mM imidazole; solvent C, acetonitrile/ethyl acetate/trifluoroacetic acid (90:10:0.5).