

Photobiological Aspects of the Mutualistic Association Between *Paramecium bursaria* and *Chlorella*

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Abstract The main benefit involved in the mutualistic relationship between *Paramecium bursaria* and *Chlorella* has been traditionally interpreted as an adaptation to the struggle against starvation and nutrient limitation. However, other benefits such as the minimization of mortality and protection against damage by sunlight have been proposed recently. Here, we explore the photobiological adaptations and responses of *P. bursaria* and its algal symbionts when exposed to photosynthetically active and ultraviolet radiation, as well as the role of (photo-)oxidative and antioxidative defenses in the symbiosis. We conclude that the benefits are multiple and should be considered as a whole when assessing the selective advantage of living in mutualistic symbiosis.

1 Introduction

The study of mutualistic interactions in natural communities has become an important research area to understand the environmental adaptation and coevolution of species (Doebeli and Knowlton 1998; Herre et al. 1999). Symbiotic relationships

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where each species benefits are mutualistic, but depending on whether the species involved are able to survive or not in the absence of each other can be considered obligate or facultative. The symbiosis between the ciliate *Paramecium bursaria* and representatives of the green algae *Chlorella* is a good example of a facultative mutualistic trophic interaction and is one of the best studied. Indeed, the fact that it is possible to obtain aposymbiotic cell lines of *P. bursaria* in the laboratory and to infect them again with *Chlorella* (Niess et al. 1982; Meier and Wiessner 1988, 1989; Summerer et al. 2007) has largely contributed to the understanding of some basic principles and benefits in algae-ciliate mutualism.

Paramecia are ubiquitous freshwater organisms found in almost all kinds of freshwater habitats (Landis 1988). Whereas natural populations of aposymbiotic *P. bursaria* are known (Tonooka and Watanabe 2002), their *Chlorella*-bearing counterparts are commoner in natural habitats. This brings us to the central question of what kind of cost-benefit balance or selective advantage is involved in the *P. bursaria*–*Chlorella* mutualism and in general for other species of *Chlorella*-bearing ciliates. In the past, this question attracted the interest of many researchers (see Dolan 1992; Jones 1994; Stoecker 1998), but it is still relevant nowadays because we know little about the molecular, cellular, and organismal adaptations that made possible the establishment of this successful mutualism. Further, the recent evidence that ciliates may once have been photosynthetic (Reyes-Prieto et al. 2008) adds new material to the debate on the origin of mutualism between phototrophic and heterotrophic protists.

In this chapter, we first give an overview about the known benefits in algae-ciliate symbiosis, then we explore adaptations and responses of *P. bursaria* and its algal symbionts when they are exposed to photosynthetically active radiation (PAR; 400–700 nm) and UV radiation (UVR; 280–400 nm), and, finally, suggest future research topics to advance the understanding of the evolutionary and ecological adaptation of this mutualistic relationship. We think it is worth scrutinizing what other benefits are thought to form the basis of the mutualism, but we stress the idea of Lobban et al. (2007) that all benefits are important to the well-being of the symbiotic association, making it difficult to assert a “primary” one.

1.1 The “Classical” View of Mutual Benefits

Certainly, the “classical” understanding of the physiological basis for the existence of a mutualistic relationship between ciliates and *Chlorella* is the efficient transfer of inorganic elements from the ciliate to the algae and of photosynthate leaking from the endosymbionts to the host. Since the pioneering work of Muscatine et al. (1967), we know that endosymbiotic *Chlorella* excrete large concentrations of carbohydrates used by *P. bursaria* to maintain its metabolism. In this way, the ciliate becomes partially or totally independent of external food supply (Reisser et al. 1984). In return, the ciliate provides respiratory CO₂ that can be photosynthetically fixed by *Chlorella* (Reisser 1980). When light is sufficient to saturate the photosynthetic apparatus, the oxygen demand of the ciliates is covered entirely by their symbiotic algae and oxygen is also released into the medium.

The ultimate factor and the most accepted benefit of this type of mutualistic association as well as in plastid enslavement is undoubtedly the higher chance of surviving starvation and nutrient limitation. In oligotrophic marine and freshwater ecosystems, inorganic nutrients needed by photoautotrophs are scarce, as are prey for heterotrophs (Dolan 1992; Dolan and Pérez 2000). Mixotrophy (i.e., the nutrition mode combining both phagotrophy and phototrophy) is generally considered as an adaptation allowing exploitation of oligotrophic environments (Norris 1996; Dolan and Pérez 2000). In fact, there are many other examples of mutualistic associations that illustrate the widespread occurrence of phototrophic–heterotrophic symbioses such as those of reef-building corals with the so-called zooxanthellae, sea anemones and jellyfish with zooxanthellae or zoochlorellae, and the freshwater solitary coelenterate *Hydra viridis* and sponges from shallow freshwaters with *Chlorella*.

Pringsheim (1928) was among the first to recognize that starved green symbiotic *P. bursaria* perform better than aposymbiotic populations of the same species. Detailed quantitative experiments with symbiotic and aposymbiotic *P. bursaria* and other ciliates such as *Coleps* sp. have confirmed this observation (Görtz 1988; Stabell et al. 2002). For example, when food is abundant, the growth rates of symbiotic and aposymbiotic *Coleps* sp. are not significantly different. Stabell et al. (2002) found that the gross growth rate of endosymbiotic *Chlorella* is always close to maximum, making it possible for the host to receive an increasing fraction of the total carbon supply from the algae with increasing food limitation. Direct measurements of the relative importance of photosynthate production by algal endosymbionts versus heterotrophic food uptake in *Stentor* spp. also suggest a relationship with food supply levels (Woelfl and Geller 2002). For example, photosynthate production by *Stentor araucanus* and *Stentor amethystinus* exceeds food uptake in autumn and winter, but not in summer at the time of higher food supply (Woelfl and Geller 2002). Berk et al. (1991) reported that ingestion rates on bacteria by “green” *P. bursaria* increase in relation to PAR levels (range 1–90 $\mu\text{mol m}^{-2}\text{s}^{-1}$). As this was not observed in the aposymbiotic counterparts, the authors suggested that the endosymbiotic *Chlorella* control the feeding rates of the host.

Though a plethora of laboratory experiments support the idea of the *Chlorella*-ciliate symbiosis as an adaptation to survive starvation at least during certain periods of time, results from natural aquatic habitats show that this is not always the case and that the factors controlling the relative contribution of mixotrophic species to the ciliate assemblage remain unclear (Dolan 1992). As argued by Stoecker (1998), “the cost and benefits of mixotrophy in different taxa and environments are still largely a subject of speculation” and this still applies nowadays.

Yet, though symbiosis in algae-bearing ciliates provides an advantage in the struggle against starvation, the occurrence of these organisms also in sunlit waters of eutrophic environments suggests the existence of other benefits in this mutualistic association (Dolan and Pérez 2000). For example, the common finding of high numbers of algae-bearing ciliates at the oxic–anoxic interface in eutrophic lakes has been related with the supply of oxygen by algal endosymbionts to the ciliate’s metabolic demand (Berninger et al. 1986; Finlay et al. 1996). This hypothesis has been challenged by Stabell et al. (2002), however, who argued that at oxic–anoxic boundaries, light is usually limiting and net diel oxygen production is close to zero. Occupation

of deep water layers at the limit of the euphotic zone has also been observed in the colonial *Chlorella*-bearing ciliate *Ophrydium naumanii* living in well-oxygenated oligotrophic waters (Queimaliños et al. 1999). High food levels in eutrophic systems do not necessarily exclude the possibility of food limitation because the dominant prey cell size available may lie outside the range ingested by mixotrophic ciliate species, particularly if they are size-selective (Dolan 1992). Thus, it seems that surface avoidance by *Chlorella*-bearing ciliates may have different explanations, including higher availability of inorganic nutrients and reduction of metazoan predation pressure (e.g., in poorly oxygenated waters) as argued by Finlay et al. (1996).

Indeed, after a symbiosis has been established, mortality factors for the respective partners or for the whole symbiosis can be substantially minimized. For example, the presence of symbiotic *Chlorella* prevents the infection of *P. bursaria* by bacteria and yeasts which are not able to entirely support the ciliate's growth (Görtz 1982). Moreover, though *Chlorella* from other algae-bearing planktonic ciliates are ingested by *P. bursaria* and, further, stable symbioses are established (Niess et al. 1982), *P. bursaria* prefer their own *Chlorella* from a mixture of three strains (Summerer et al. 2007). In addition, *Chlorella*-bearing *P. bursaria* seems to be less predated than its aposymbiotic counterpart by other ciliates such as *Didinium nasutum* (Berger 1980). Similar results have been obtained with the *Chlorella*-bearing *Climacostomum virens* in experiments with the predatory ciliate *Dileptus margaritifer* (Miyake et al. 2003). The exact mechanism for the lower vulnerability of mixotrophic ciliates is unknown, but higher swimming speed and strong escape reactions have been argued as possible mechanisms (Pérez et al. 1997).

On the *Chlorella* side, the mutualistic association with a host represents a physical refuge and a way to avoid mortality, for example, by lytic chloroviruses that are widespread in freshwater systems (Van Etten 2003). Though generally less discussed in the scientific literature, the establishment of a symbiotic relationship between *Chlorella* and *P. bursaria* and other ciliates could have been driven by the additional selective pressure of mortality in an environment hostile at least to the symbiont. The information available on *Chlorella* viruses suggests they have a long evolutionary history of more than 1.2 billion years (Kang et al. 2003).

1.2 Photoprotection in *Chlorella*-Ciliate Symbiosis

To obtain the benefits of living in symbiosis with *Chlorella*, ciliate hosts need to expose themselves to solar radiation, which includes not only PAR but also damaging UVR. UVR is more rapidly attenuated in the water column than PAR; thus, exposure of organisms will be significant in shallow and transparent aquatic systems or in populations thriving in the upper water column. As evidenced by the several strategies developed among different forms of life to obtain protection and to repair damage, solar UVR has been an important selective factor during evolution of life on Earth, particularly for photosynthetic organisms (Cockell 1998). However, physiological and ecological work on *Chlorella*-bearing ciliates has largely ignored the effects of

these potentially harmful wavelengths of the natural solar spectrum. In fact, UVR can exert negative effects on several cell targets such as DNA, proteins, pigments, and membranes that may be caused by direct absorption of UV-B radiation (280–315 nm) or indirectly through the generation of reactive oxygen species (photo-oxidative stress) induced by both UV-B and UV-A radiation (315–400 nm). Whether UV-B or UV-A is more effective in causing a certain type of damage will finally depend on the presence of endogenous photosensitizers and the absorption maximum properties of the chromophore(s) involved. Further, other characteristics, such as the presence of DNA rich in A + T, can make organisms particularly susceptible to UV damage owing to the higher probability of production of cyclobutane thymine dimers. We are not aware of A + T values for *P. bursaria*, but in *Paramecium tetraurelia* and *Paramecium primaurelia* the macronuclear DNA has a very high content of 72–75% A + T (McTavish and Sommerville 1980; Aury et al. 2006), suggesting a high susceptibility to UVR of this genus. By contrast, values for other ciliate species such as the marine representatives *Dysteria procera* (56%) and *Hartmannula derouxi* (55%) are lower (Li and Song 2006) and in the range of other species (Schlegel et al. 1991).

The occurrence of negative effects of UVR on ciliates has been known since the pioneering work of Giese (1945) with species of *Paramecium*. Interestingly, the few studies available on UV sensitivity of mixotrophic ciliates suggest that they are resistant. For example, Modenutti et al. (1998) reported that *S. araucanus*, a symbiotic planktonic freshwater ciliate widely distributed in the southern hemisphere, is resistant to exposure to surface solar UVR. The authors, however, attributed the resistance to the protection given by the dark ciliate pigment stentorin. By contrast, Lobban et al. (2007) cast doubt on hypericin-like compounds such as stentorin having any direct role in protecting cells against UVR. UV-exclusion experiments performed with natural ciliate assemblages including the photosynthetic species *Mesodinium rubrum* and the kleptoplast-bearing *Laboea strobila*, collected from a shallow transparent area (George Bank) on the continental shelf in the Northwest Atlantic, indicate a lower UV-B sensitivity for these species (Martin-Webb 1999). Summerer et al. (2009) experimentally tested the sensitivity against UVR of symbiotic *P. bursaria* compared with *Chlorella*-reduced (about half symbiont density) and aposymbiotic *P. bursaria*. Their results indicate that under UVR exposure, aposymbiotic *P. bursaria* had significantly higher mortalities than *Chlorella*-bearing individuals.

Most of our knowledge on the interaction between UVR and mutualistic associations is based on studies on algae–invertebrate symbiosis, particularly on scleractinian corals and their zooxanthellae of the genus *Symbiodinium* (Shick et al. 1996; Shick and Dunlap 2002). Several studies have shown that UVR severely depresses photosynthetic rates in freshly isolated zooxanthellae from corals or other reef organisms, but this effect is small or absent *in hospite* (Dionisio-Sese et al. 2001). The different sensitivity between free and *in hospite* forms appears to be related to protection given by the host through the accumulation in its tissues of sunscreen compounds known as mycosporine-like amino acids (MAAs) produced by the symbionts. MAAs are intracellular water-soluble compounds with high molar extinction coefficients having absorption maxima between 309 and 360 nm.

Until now, approximately 22 different MAAs have been identified in marine and freshwater organisms (Karentz et al. 1991; Dunlap and Shick 1998; Sommaruga and Garcia-Pichel 1999). The biogenesis of MAAs presumably originates in the shikimate pathway that is known to be only present in bacteria, fungi, and algae (Dunlap and Shick 1998; Shick et al. 1999). Independently of whether MAAs are synthesized or obtained from other sources, they will protect to a certain extent (depending on concentration and pathlength) important cell targets from deleterious effects of UVR and, thus, increase the overall resistance of phototrophic and heterotrophic organisms (Shick and Dunlap 2002).

Recently, the existence of MAAs in symbiotic ciliates has been reported for marine and freshwater species (Tartarotti et al. 2004; Sommaruga et al. 2006; Sonntag et al. 2007; Summerer et al. 2008). One of those species is the giant marine heterotrich ciliate *Maristentor dinoferus* inhabiting coral reefs of Guam (Lobban et al. 2002). *M. dinoferus* is a sessile species that hosts 500–800 *Symbiodinium* cells (Lobban et al. 2002). Methanolic extracts of this ciliate analyzed by high-performance liquid chromatography showed the presence of three different MAAs (Sommaruga et al. 2006). The symbiotic origin of MAAs in several freshwater *Chlorella*-bearing ciliates has been demonstrated (Sonntag et al. 2007). Moreover, Summerer et al. (2008) have shown that the *Chlorella* strains isolated from two *Askenasia chlorelligera* populations inhabiting lakes having a tenfold difference in underwater UV transparency presented a distinct physiological trait, such as the ability to synthesize MAAs.

The occurrence of MAAs in several *Chlorella*-bearing ciliate species suggests the existence of multiple alternative benefits in this type of mutualistic association. However, the symbiotic *Chlorella* from *P. bursaria* have tested negative for the presence of MAAs (Summerer et al. 2009), indicating that this physiological trait is not ubiquitous in *Chlorella*. However, this is not the only mechanism possible to obtain photoprotection, as we discuss in the next section.

2 UV-Induced Accumulation Behavior of Symbiotic *P. bursaria*

When symbiotic *P. bursaria* are exposed to PAR, the phenomenon of “photoaccumulation” can be observed (Engelmann 1882; Pado 1972; Saji and Oosawa 1974). During photoaccumulation, the light stimulus induces a depolarization of the ciliates’ membrane potential and the individuals slow down or even come to a standstill (Machemer 1974; Nakaoka et al. 1987; Matsuoka and Nakaoka 1988). Generally, this behavior is a step-down photophobic response where the ciliates move from shaded regions into lighted areas where they are “trapped.”

Summerer et al. (2009) studied the aggregation behavior of symbiotic and aposymbiotic *P. bursaria* in the presence of UVR and PAR. Ciliate cultures exposed to artificial UVR + PAR and “high” PAR ($160 \mu\text{mol m}^{-2}\text{s}^{-1}$) showed an immediate aggregation of symbiotic *P. bursaria* into several dense “spots” of about 3 mm in diameter. However, this aggregation occurred only when the culture density was $500 \text{ cells ml}^{-1}$ or higher (Summerer et al. 2009). In contrast to the “classical” photoaccumulation,

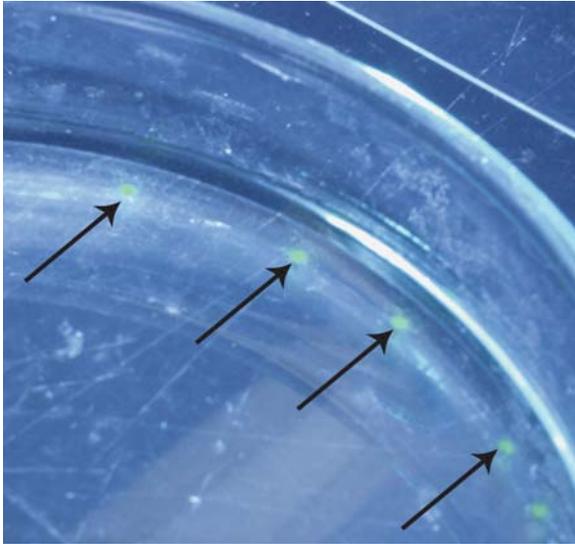


Fig. 1 Photograph of symbiotic *Paramecium bursaria* aggregated into dense spots of 1–3 mm diameter (arrows) during exposure to artificial UV radiation (UVR)

this aggregation behavior of *P. bursaria* occurs within the completely illuminated experimental vessel (Fig. 1). Summerer et al. (2009) suggested that aggregation under UVR exposure is induced by chemical signaling and the encounter rate of individual *P. bursaria* cells.

In aposymbiotic *P. bursaria*, no spot-aggregation is observed under UVR and PAR exposure; thus, Summerer et al. (2009) argued that the *Chlorella* symbionts are responsible for the induction of the aggregation behavior in the symbiotic individuals. Because the pattern of photoaccumulation under PAR exposure is not observed in aposymbiotic or in *Chlorella*-reduced (fewer than 50 algae per ciliate), as well as in symbiotic *P. bursaria* treated with a photosynthesis inhibitor (dichlorophenyl dimethylurea), a light receptor is thought to be located in the algal symbionts (Iwatsuki and Naitoh 1981; Niess et al. 1981, 1982; Reisser and Häder 1984). Further, other *Chlorella*-bearing ciliates such as *Euplotes daidaleos* “photoaccumulate”, whereas *Climacostomum virens* do not, though the aposymbiotic forms of both species show a step-up photophobic response (photodispersal), suggesting that the photoreceptor is located in the ciliates (Reisser and Häder 1984). Direct evidence for the presence of photoreceptors in the ciliate has been given by Tokioka et al. (1991), who extracted retinal, a chromophore of the visual pigment, from aposymbiotic *P. bursaria* and by Nakaoka et al. (1991), who detected a rhodopsin-like protein in the ciliary and somatic membranes of *P. bursaria*.

At present, the mechanisms that lead to the accumulation (behavior) under different wavelengths and intensities of the solar spectrum are not clear and further research is needed.

3 Photoprotection of *P. bursaria* by *Chlorella* Self-Shading

Another phenomenon observed when symbiotic *P. bursaria* are exposed to UVR + PAR is that their algal symbionts are dislocated and move to the posterior cell region (Summerer et al. 2009; Fig. 2). When the ciliates are returned to culture conditions without UVR, they disperse again in the medium and the symbionts are relocated to a “normal” distribution. However, it is unclear whether the displacement of the symbionts is controlled by the algae or the host (Summerer et al. 2009). Nishihara et al. (1999) detected cytoplasmatic movements of the symbionts in *P. bursaria* in connection with microtubule filaments. Usually, *P. bursaria* cells bear several hundred unicellular *Chlorella* of approximately 3.5 μm in diameter (Karakashian et al. 1968). Typically, within the host, the symbionts are arranged directly under the periphery of the cytoplasm, close to the macronucleus and micronucleus and to the buccal field (Karakashian et al. 1968). According to these distributions, especially underneath the cell surface and close to DNA-containing cell organelles, it seems likely that several cell layers of *Chlorella* and many food vacuoles, i.e., absorbing cell matter, increase the photoprotection of the host, as well as of the symbionts themselves (Giese 1968; Garcia-Pichel 1994; Summerer et al. 2009). On the basis of a bio-optical model to estimate the internal photoprotective self-shading potential of cell matter (Garcia-Pichel 1994), Summerer et al. (2009) calculated the transmittance and self-shading under UV exposure at 320 nm for one to several *Chlorella* cell layers within *P. bursaria*. When the *Chlorella* are distributed regularly, the screening efficiency achieved is 59% for one layer, and 83 and 93% for two and three layers, respectively. In *P. bursaria* with dislocated symbionts, 100% protection from UVR at 320 nm can only be achieved when at least eight layers are present (Fig. 3). The results of Summerer et al. (2009) indicate that *P. bursaria* can obtain protection against UV damage by accumulation, as well as by symbiont dislocation (Fig. 3).

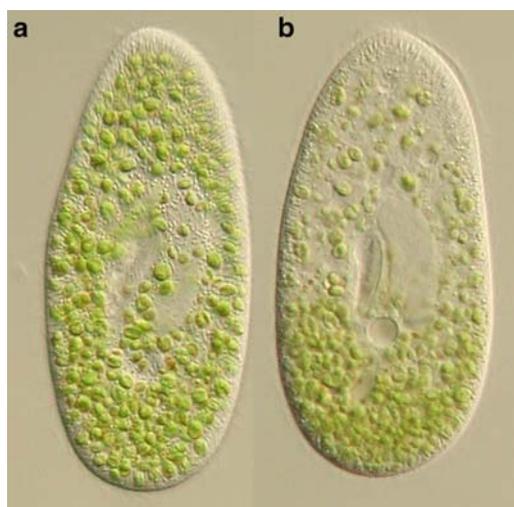


Fig. 2 *P. bursaria* showing the distribution of *Chlorella* before (a) and after (b) exposure to UVR

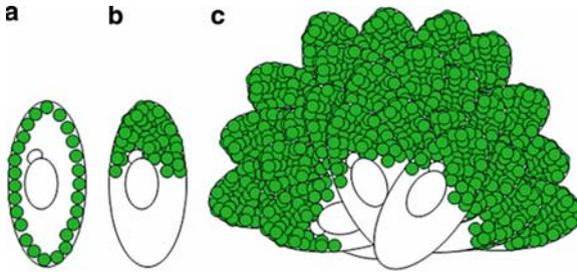


Fig. 3 The *Chlorella* distribution within *P. bursaria* under PAR (a) and UVR (b) exposure. Hypothetical assemblage of *P. bursaria* with dislocated symbionts within dense spots ("collective shield") formed under UVR exposure (c)

4 Role of Endosymbiotic *Chlorella* in the (Photo-)oxidative Stress Balance of *P. bursaria*

Detailed consideration of oxidative stress is found in a number of reviews (Apel and Hirt 2004; Lesser 2006). It is therefore necessary to consider here only some basic aspects. Oxidative stress is defined as the production and accumulation of reactive oxygen species (ROS) beyond the capacity of an organism to quench these strong oxidants to a safe level (Halliwell and Gutteridge 1999; Lesser 2006). Oxidation of membrane lipids, DNA, proteins, and pigments and cellular death are the consequences of oxidative stress (Fridovich 1998). The formation of ROS is basically a consequence of redox reactions with O_2 in all photosynthetic and respiring cells. However, the basal level of oxidative stress can be increased by exposure of organisms to several types of photosensitizers (e.g., certain pollutants), as well as to excessive PAR and UVR. In aquatic ecosystems, extracellular and intracellular photo-oxidative production of ROS is known to impose a physiological burden on many organisms (Kieber et al. 2003; Lesser 2006).

The photosynthetic activity of endosymbiotic *Chlorella* leads not only to the fixation of carbon dioxide but also to the release of molecular oxygen, which because of its two unpaired electrons has a limited potential to react with organic molecules. However, the univalent reduction of molecular oxygen produces different partially reduced short-lived ROS, such as superoxide radical ($O_2^{\bullet-}$), which is the first free radical normally produced in aerobic cells, singlet oxygen (1O_2), and hydroxyl radical ($HO\bullet$). Hydrogen peroxide (H_2O_2), which is not a radical, is produced by the dismutation of superoxide and its half-life is substantially longer than that for other ROS (Lesser 2006). In the chloroplast, the photosynthetic production of hydrogen peroxide, superoxide, and singlet oxygen proceeds through photorespiration, Mehler reaction (i.e., reduction of O_2 to O_2^-), and photodynamic actions, respectively (Asada 1999, Fig. 4a)

Similarly to other phototrophic symbioses, respiration of the ciliate and endosymbiotic algae at night and photosynthesis during daytime probably leads to dramatic changes in oxygen concentration within the host cell. Whereas photosynthetic organisms have efficient mechanisms to reduce oxidative stress, heterotrophic single cells

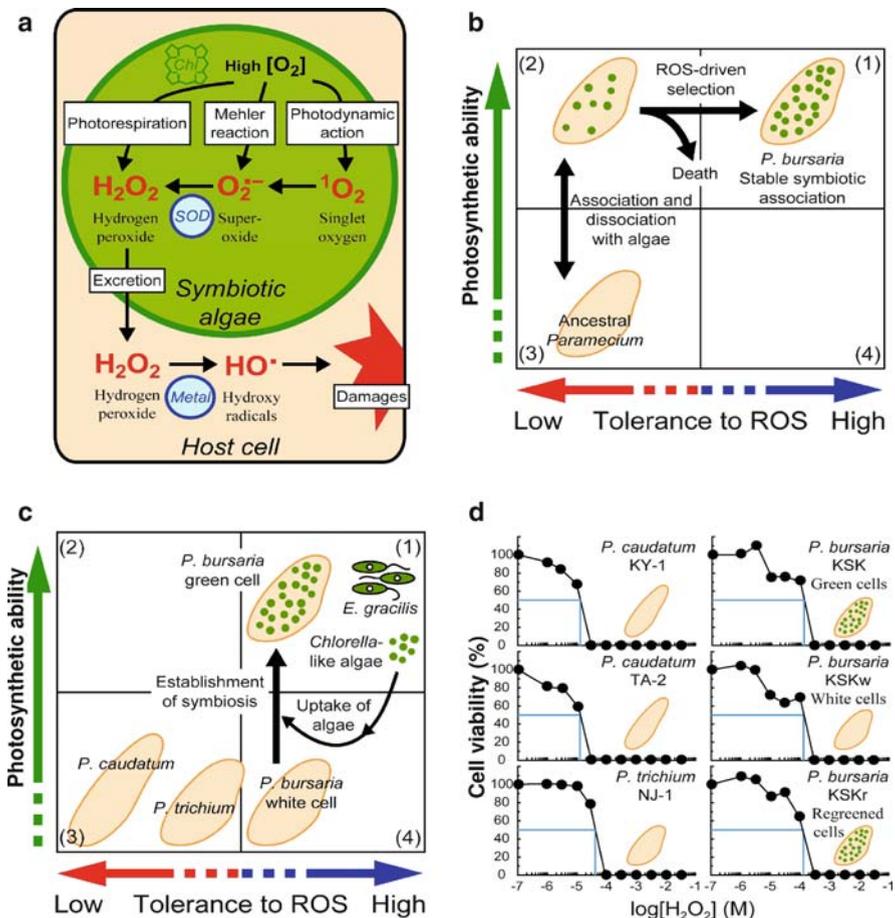


Fig. 4 Oxidative stress in symbiotic *P. bursaria*. **a** Potential sources of reactive oxygen species (ROS) in symbiotic *P. bursaria*. In this scheme, singlet oxygen, superoxide, and hydrogen peroxide (H_2O_2) are produced mainly by symbiotic *Chlorella*'s photosynthetic activity. H_2O_2 may be excreted by *Chlorella* to the host and highly reactive hydroxyl radicals may be formed via H_2O_2 dissociation, which can damage the host. **b** Hypothetical model illustrating how cohabitation of the ancestral paramecia with algae was followed by the selection of ROS-tolerant species. **c** In this alternative model, emergence of ROS-tolerant paramecia enabled the symbiosis with algae. **d** Viability of different *Paramecium* species to the oxidative burst of H_2O_2 . Data for *P. caudatum* (KY-1 and TA-2 strains), *P. trichium* (NJ-1 strain), and three cell lines derived from *P. bursaria* KSK-103 strain are shown. (Modified from Kawano et al. 2004)

may have had limited capacity to manage the excess of ROS caused by endosymbiotic algae. Kawano et al. (2004) presented two models to explain how the symbiosis between *P. bursaria* and *Chlorella* acquired the ability to cope with oxidative stress (Fig. 4b, c). In the first one, the symbiosis started by infection of *P. bursaria* cells having high tolerance to ROS that successfully acquired endosymbiotic *Chlorella*.

In the second model, the additional oxidative stress caused by the infection of the host by *Chlorella* selected for ROS-tolerant species during evolution. As Kawano et al. (2004) argued, in the first model the relation between the (re)greening ability of the paramecia and the tolerance to ROS is stressed, whereas in the second one evolutionary selection leading to emergence of ROS-tolerant *Paramecium* species is emphasized. The authors also discussed the possibility that ciliates could select for endosymbiotic *Chlorella* that are not so photosynthetically active and thus have lower oxidative stress potential. However, there is some circumstantial evidence suggesting that *P. bursaria* favor the symbiosis with active (i.e., high chlorophyll *a* and photosynthetic rates) *Chlorella* (Geraschenko et al. 2000).

Kawano et al. (2004) tested the tolerance to exogenous oxidative stress of diverse photosynthetic organisms including exsymbiotic and free-living *Chlorella* and different strains of green *P. bursaria*, aposymbiotic counterparts, as well as three other nonsymbiotic paramecia species. Cell viability of those organisms was measured in stationary cultures grown with PAR levels of approximately 33 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ after 12-h incubation in a range of hydrogen peroxide concentrations to establish the threshold lethal to 50% of the population (LD_{50}).

The results of those experiments (Fig. 4d) clearly demonstrated that free-living and exsymbiotic *Chlorella* have high tolerance (LD_{50} 300–600 $\mu\text{M H}_2\text{O}_2$) to this extreme oxidative burst. Similarly, among the paramecia tested, the “green” *P. bursaria* was the most tolerant (LD_{50} 120 μM), whereas the nonsymbiotic species *Paramecium trichium* (LD_{50} 43 μM) and *Paramecium caudatum* (LD_{50} 12 μM) were the most sensitive and cells burst out shortly after low H_2O_2 concentrations were added. Remarkably, the aposymbiotic *P. bursaria* strains and those reinfected with *Chlorella* had tolerance similar to the symbiotic ones. This result led Kawano et al. (2004) to conclude that endosymbiotic *Chlorella* do not contribute to the higher resistance observed in “green” *P. bursaria*. However, it is intriguing that the LD_{50} these authors found for free-living *Chlorella* and exsymbiotic ones is 2.5–5 times higher than for the symbiotic *Chlorella-P. bursaria* association.

Hörtnagl and Sommaruga (2007) studied whether oxidative stress and UV-induced photo-oxidative stress are greater in *Chlorella*-bearing *P. bursaria* than in its aposymbiotic counterpart. In this study, the level of oxidative stress was directly determined by assessing ROS with two fluorescent probes (hydroethidine and dihydrorhodamine 123) by flow cytometry.

Dihydrorhodamine 123 is an indicator of hydrogen peroxide levels, whereas hydroethidine is a more general proxy for ROS because it reacts with intracellular superoxide and also with hydrogen peroxide. This approach makes possible the rapid detection of oxidative levels in a population of cells and also the visualization by epifluorescence microscopy of oxidative burst within cells (Fig. 5). These experiments indicated that oxidative stress (PAR 160 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) is higher in aposymbiotic ciliates than in “green” *P. bursaria* (Fig. 6). This oxidative stress was higher during the exponential growth of both populations, but particularly in the aposymbiotic *P. bursaria*. During exponential growth, high cell division rates are accompanied by an increase in metabolic activity that usually results in higher oxidative stress levels (Hörtnagl and Sommaruga 2007). Interestingly, no significant

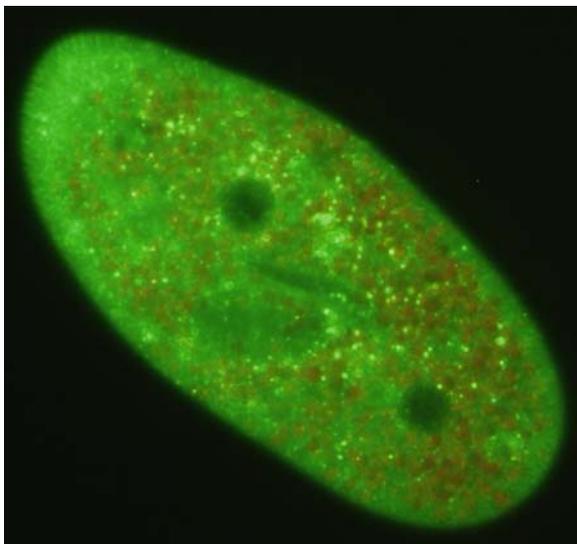


Fig. 5 *P. bursaria* showing spots of oxidative stress (brilliant green) as detected by the fluorescent probe 2', 7'-dichlorodihydrofluorecein diacetate under the epifluorescence microscope. The autofluorescence of the chlorophyll pigments of *Chlorella* appears in red. The two dark spots are the contractile vacuoles. Note that oxidative spots are found in the ciliate's cell but not inside *Chlorella*

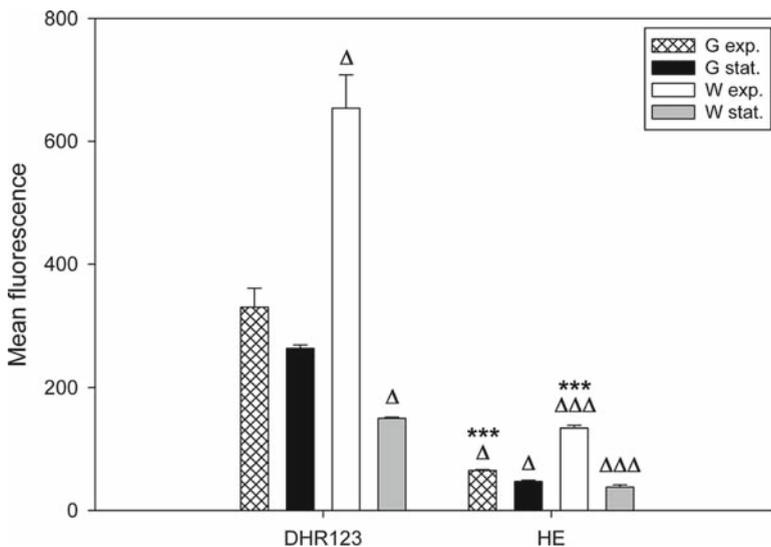


Fig. 6 PAR-induced oxidative stress in aposymbiotic and endosymbiotic strains of *P. bursaria* expressed as mean fluorescence ($n = 3 \pm$ standard error, SE) using two ROS detection methods. Stars denote significant differences between endosymbiotic (G) and aposymbiotic (W) strains, whereas triangles indicate significant differences between exponential (exp) and stationary (stat) growth phases. $*/\Delta p < 0.05$, $***/\Delta\Delta\Delta p < 0.001$. (From Hörtnagl and Sommaruga 2007)

difference in oxidative stress is found within growth phases of free-living *Chlorella vulgaris* (Malanga and Puntarulo 1995). However, it is questionable whether this result applies to endosymbiotic *Chlorella* too.

The results obtained by Hörtnagl and Sommaruga (2007) contradict somewhat the conclusion drawn by Kawano et al. (2004) that “green” and “white” *P. bursaria* are not different regarding their response to oxidative stress. However, it is important to note that the response measured by Kawano et al. (2004) relates mainly to the enzymatic defenses of the “green” and “white” cell lines of *P. bursaria*, particularly of the levels of catalase, which is one of the enzymes responsible for deactivating hydrogen peroxide. Hörtnagl and Sommaruga (2007), however, measuring the endogenous levels of oxidative stress, concluded that endosymbiotic *Chlorella* do not burden the host and there are clear differences between these two types of cell lines. As discussed below, the reason for the higher oxidative stress in aposymbiotic *P. bursaria* partially relates to differences in their antioxidative defenses. However, Gu et al. (2002) studying the physiological changes that occur after 1 month of dark cultivation of *P. bursaria* found an increase in the activity of several enzymes (notably of acid phosphatase and ATPase, but also of glucose 6-phosphatase and succinate dehydrogenase) compared with light-cultured cells. These results suggest that the aposymbiotic strain increases its metabolic activity (and oxidative stress) to compensate for the lack of endosymbiotic *Chlorella*.

Hörtnagl and Sommaruga (2007) also measured the response of “green” and “white” cell lines of *P. bursaria* to artificial UVR exposure (i.e., photo-oxidative stress). As mentioned in the introduction of this section, UVR stimulates the generation of ROS in both heterotrophic and photoautotrophic organisms. Again, photo-oxidative stress caused by UVR exposure was higher in “white” than in “green” *P. bursaria* (Fig. 7).

Hörtnagl and Sommaruga (2007) argued that the lower UV-induced photo-oxidative stress in the “green” strain of *P. bursaria* is related to the screening of UV wavelengths by several layers of *Chlorella* (see Sect. 3).

5 Antioxidative Capacity of the *P. bursaria*–*Chlorella* Symbiosis

Aquatic organisms have different defense mechanisms to minimize the harmful effects of ROS that include various antioxidative enzymes and nonenzymatic antioxidants. In fact, ROS trigger a series of responses that provide increased protection against the damaging effect of these strong oxidants. Among the antioxidative enzymes, superoxide dismutase (SOD; EC 1.15.1.1) is the first line of defense removing superoxide but producing hydrogen peroxide, which is eliminated by catalase (EC 1.11.1.6) and several peroxidases. Another antioxidative enzyme, glutathione reductase (EC 1.6.4.2), is involved in the conversion of oxidized glutathione to its reduced form with consumption of NADPH (Dunlap et al. 2000). The activity of catalase is crucial in keeping low oxidative damage within cells because hydrogen peroxide is uncharged, making possible its easy diffusion across biological membranes (Asada 1999; Lesser 2006). Consequently, significant damage can occur in different cell

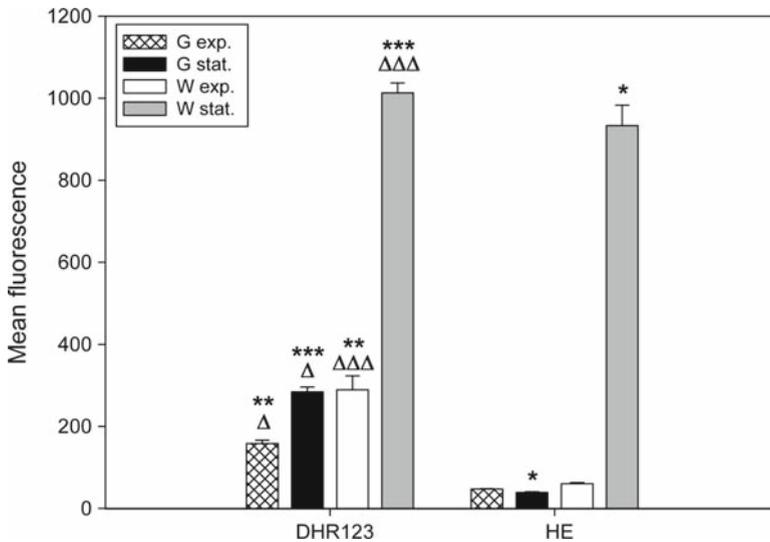


Fig. 7 UV-induced oxidative stress in endosymbiotic and aposymbiotic *P. bursaria* strains expressed as mean fluorescence ($n = 3 \pm \text{SE}$) for two different ROS detection methods. Stars denote significant differences between endosymbiotic (G) and aposymbiotic (W) strains, whereas triangles indicate significant differences between exponential (exp) and stationary (stat) growth phases of the ciliates from the endosymbiotic or the aposymbiotic strain. $^{*/\Delta}p < 0.05$, $^{**/\Delta\Delta}p < 0.01$, $^{***/\Delta\Delta\Delta}p < 0.001$. (From Hörtnagl and Sommaruga 2007)

compartments because the reaction of hydrogen peroxide with different cell targets is not restricted to its place of synthesis (Lesser 2006). In fact, Kawano et al. (2004) argued that hydrogen peroxide levels within symbiotic *Chlorella* are probably reduced to safe levels by diffusion, but the host ciliate will have to cope with oxidative stress. In addition to antioxidative enzymes, glutathione, ascorbic acid, α -tocopherol, β -carotene, and uric acid can protect many cellular constituents from ROS because these substances interrupt the spreading of autocatalytic radical reactions (Lesser 2006).

Hörtnagl and Sommaruga (2007) screened the antioxidant defenses of “white” and “green” *P. bursaria* by measuring the activity of catalase, SOD, and glutathione reductase in cells exposed to either UVR + PAR or just PAR. Whereas glutathione reductase activity was undetectable in all cases, UVR strongly decreased catalase activity during the exponential and stationary growth phases of both strains (Fig. 8). Catalase structure and function appears to be very sensitive to UVR (Zigman et al. 1996) and similar negative effects have been observed in other aquatic organisms, but this is not a general pattern. For example, catalase activity is not reduced by UVR in free-living *C. vulgaris* (Malanga and Puntarulo 1995). However, the enzymatic activity measured by Hörtnagl and Sommaruga (2007) should be regarded as that from the symbiosis, since the authors did not differentiate between symbiont and host sources. However, the catalase gene has been found in *P. bursaria* (Tonooka et al. 2008) and gene expression analysis in host paramecia in response to oxidative stress has been proved positive (Y. Tonooka, personal communication).

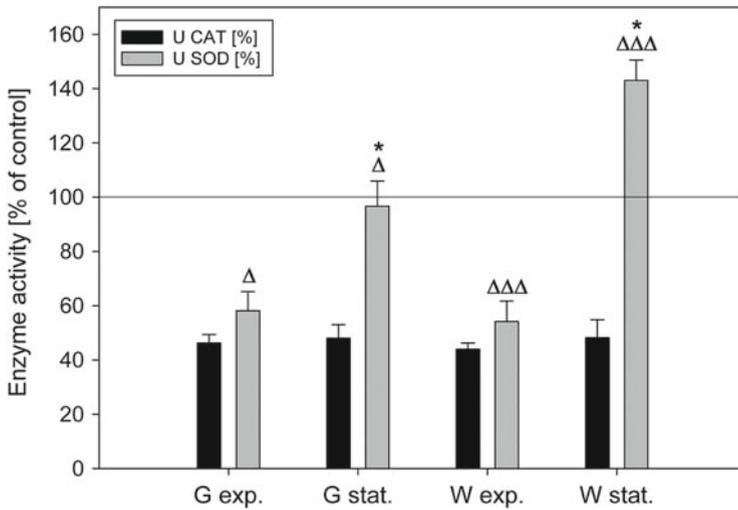


Fig. 8 Relative activity of the antioxidant scavenging enzymes catalase (CAT) and superoxide dismutase (SOD) in endosymbiotic (G) and aposymbiotic (W) strains of *P. bursaria* exposed for 2 h to PAR + UVR. Enzyme activity ($n = 3 \pm SE$) is expressed as the percentage of the PAR control (horizontal line reference) for the respective strains and growth phases. Stars denote significant differences between endosymbiotic and aposymbiotic strains, whereas triangles indicate significant differences between exponential (exp) and stationary (stat) growth phases of the ciliates from the endosymbiotic or the aposymbiotic strain. $*/\Delta p < 0.05$, $***/\Delta\Delta\Delta p < 0.001$. (From Hörtnagl and Sommaruga 2007)

A negative effect of UVR was also detected on SOD activity but only during the exponential growth phase of both strains (Hörtnagl and Sommaruga 2007). By contrast, SOD activity significantly increased in the aposymbiotic strain during the stationary growth phase (Fig. 8). As discussed by Hörtnagl and Sommaruga (2007), the increase in SOD activity did not counteract the direct and indirect (i.e., ROS) damaging effects of UVR on aposymbiotic ciliates as suggested by the authors' observation of an erratic swimming behavior after 2-h exposure. Further, they concluded that the higher tolerance of the symbiotic strain of *P. bursaria* can also be attributed to the antioxidant defenses from the algae.

6 Conclusions and Perspectives

In this chapter, we have shown new aspects on the *P. bursaria*–*Chlorella* association in relation to the photoprotective potential of the symbiosis against direct and indirect (photo-oxidative stress) damaging effects of solar irradiation. The distinct responses of symbiotic *P. bursaria* when they are exposed to artificial UVR and PAR include physical protection by spot-accumulation together with the phenomenon of symbiont dislocation, as well as physiological adaptations in the oxidative

stress balance between the algae and the host. At present, the exact mechanisms that trigger these reactions are largely unknown. Despite *P. bursaria* certainly being the best studied *Chlorella*-bearing ciliate species for over more than one century, possible effects of solar radiation and associated photoprotective strategies have been considered only marginally. However, their exploration would significantly contribute to the mostly unknown ciliate's photobiology and to the understanding of the environmental adaptation and (co-)evolution of symbiotic species, as well as of the establishment of mutualistic associations.

Many gaps in the photobiological knowledge of these protists can hopefully be filled in future studies when answering questions such as the following: What is the (chemical?) signal that induces the accumulation behavior of symbiotic *P. bursaria* when exposed to UVR and PAR? Do the algae or does the host trigger the symbiont displacement? Would *Chlorella*-bearing ciliate species from highly transparent habitats have different numbers of symbiotic algae than those from less transparent ones?

We hope this short review will stimulate the study of at least some of these and other photobiological aspects in the near future to better understand the specific relationship between the algae and the host in *P. bursaria* but also in other symbiont-bearing ciliate species.

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End Note

The original light intensity in the paper by Kawano et al. (2004) was given in lux, which can only be converted to micromole photons per square meter per second when the wavelength dependency is considered. Here a factor of 0.0165 was applied to facilitate comparison with other studies.

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