Christian E.W. Steinberg

Stress Ecology

Environmental Stress as Ecological Driving Force and Key Player in Evolution

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ISBN 978-94-007-2071-8 e-ISBN 978-94-007-2072-5
DOI 10.1007/978-94-007-2072-5
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2011936021

Preface

I dedicate this book to our "crazy" animals who voluntarily migrate into chemically stressful environments and spend energy to overcome this situation. Yet, they even benefit from this stress and thereby teach us that several stress paradigms are outdated and must be re-considered.

In my classes on "Stress Ecology" in Berlin (Germany), Wuhan and Kunming (China), and Rio de Janeiro (Brazil), I probably stressed many young scientists and, nevertheless, hope that this stress was as positive to them as the stress to our "crazy" animals was. Finally, I gratefully acknowledge the help, stimulation, discussion, and inspiration of so many friends, colleagues, and students: Ralph and Steffi Menzel, Nadine Saul, Kerstin Pietsch, Yvonne Pörs, Hanno Bährs, Rihab Bouchnak, Ramona Rauch, Ramona Henkel, Sylva Hofmann, Nadia Ouerghemmi, Steffen Hermann, Laura Vinćentić, Shumon Chakrabarti, Antonia Engert, Sandra Euent, Maxim Timofeyev, Darya Bedulina, Marina Protopopova, Elena Sapozhnikova, Zhanna Shatilina, Vassily Pavlichenko, Albert Suhett, and, last but not least, Stephen Stürzenbaum.

Furthermore, I particularly thank Dawn M. Allenbach, University of New Orleans, for carefully checking the manuscript and commenting on many parts of it. Her work and thoughts have substantially improved the quality of several chapters of the book.

Even to a book, space limitation applies. Due to this circumstance, I would like to apologize in advance to all individuals whose research was not cited or whose papers have not been discussed in full but whose work has certainly advanced the understanding of this complex field of research and education.

Many thanks are due to the staff of Springer, Dordrecht, The Netherlands, particularly Paul Roos, Suzanne Mekking, and Martine van Bezooijen, for their understanding and their continuous help in preparing my book.

Contents

Chapter 1
Why a Small Worm Is Not Crazy

Usually, stress is considered adverse: too much work load, or, conversely, unemployment; lack of success; unsolved family problems, etc. More scientifically, Selye (1936) discovered in his fundamental study by challenging rats that "if the organism is severely damaged by acute non-specific nocuous agents such as exposure to cold, surgical injury, production of spinal shock (transcision of the cord), excessive muscular exercise, or intoxications with sublethal doses of diverse drugs (adrenaline, atropine, morphine, formaldehyde, etc.), a typical syndrome appears, the symptoms of which are independent of the nature of the damaging agent or the pharmacological type of the drug employed, and represent rather a response to damage as such".

In ecological terms, stress may therefore be defined as any internal state in an organism resulting from placing it outside its fundamental ecological niche, whereby the niche may be defined in terms of gene expression profiles under normal or ideal operating conditions (van Straalen 2003). Selye (1936) showed that a stress response includes three different phases: the bipartite alarm phase, the resistance phase, and the *exhaustion phase* (Fig. 1.1).

The *alarm phase* corresponds to modifications of biochemical and genetic parameters in the absence of reduced vital activities and growth. These physiological reactions terminate a primary disturbance and enable restitution. An exposure that is too strong or fast will result in acute damage and cell death. The *resistance phase* is characterized by the activation of defense mechanisms (e.g., antioxidant defense, protein repair, biotransformation) that are concomitant with first signs of reduced vital activity and growth. The *exhaustion phase* becomes apparent by a collapse of vital cellular functions (e.g. photosynthesis, membrane integrity, reproduction), leading to chronic damage and ultimately to death.

This model implies that stress is something that happens to organisms, something that is fate and cannot be avoided (if the organisms cannot escape the situation), something that must be tolerated instead. But what about organisms that actively look for stressful environments, migrate into them, and suffer from symptoms of stress such as loss of energy, activation of oxygen, induction of stress genes, etc.? Organisms

C.E.W. Steinberg, *Stress Ecology: Environmental Stress as Ecological Driving Force and Key Player in Evolution*, DOI 10.1007/978-94-007-2072-5_1,

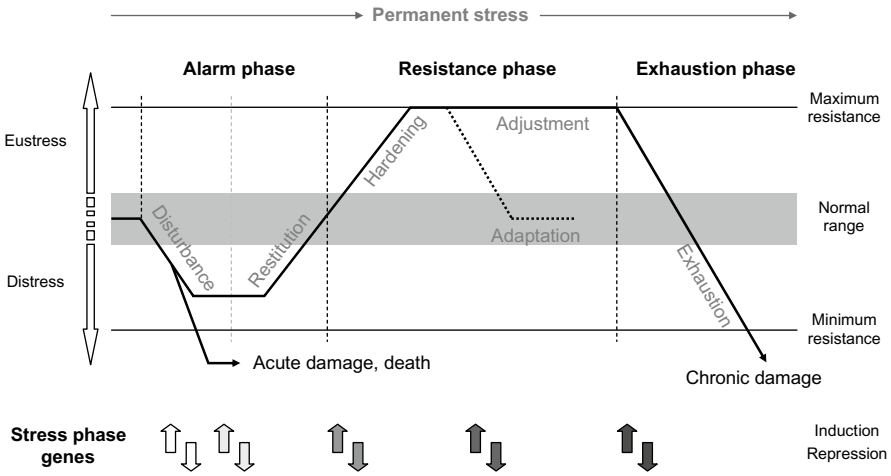

Fig. 1.1 The classical stress phase model based on Selye (1936) and amended by several authors. Shades of *grey* of arrows represent different genes specifically expressed during the individual stress phases (From Steinberg et al. 2008a, with permission from Elsevier)

demonstrating this seemingly crazy behavior do exist. For countless generations, the nematode *Caenorhabditis elegans* has been cultured in solutions or on agar plates completely free of humic substances, a biogeochemical matrix of soils and aquatic systems. These substances recently have been demonstrated to cause many stress defense reactions, such as oxygen activation and eventually lipid peroxidation, expression of stress proteins, and modulation of biotransformation enzymes. Many of these responses are transcriptionally controlled and require a great deal of energy (Steinberg et al. 2008b). In a simple laboratory test, *C. elegans* was offered the choice to stay in humic-free environments or to migrate to humic-rich environments (Fig. 1.2). The individuals were allowed to feed on bacteria either with or without concomitant humic substances. The majority of the animals decided to feed on bacteria **with** humic substances present – despite the aforementioned far-reaching consequences. The nematodes were able to sense the presence of humic substances, because several olfactory and chemosensory genes were induced (Menzel et al. 2005a).

The nematode *C. elegans* may appear to be a rather peculiar organism that is an "exception to the rule" that species prefer a stress-free environment. Yet, a look into recent literature shows that it is by no means an isolated case. For instance, the bacterium *Herminiimonas arsenicoxydans* behaves as strangely as the worm. It is a species of ultramicrobacteria and was first been reported in 2006 as an isolate of industrial sludge. Aside from multiple biochemical processes such as arsenic oxidation, reduction, and efflux, *H. arsenicoxydans* – most astonishingly – also exhibits positive chemotaxis and motility towards arsenic (Muller et al. 2007), a metalloid, which is commonly classified as "toxic" and "dangerous for the environment". Yet, Fig. 1.3 shows increased swimming rings with increasing arsenic and ferric iron concentration. No such effect occurred with other toxic elements tested, such as cobalt.

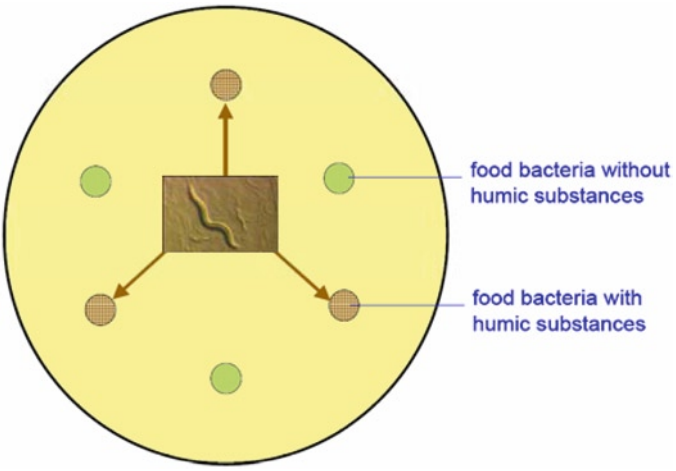

Fig. 1.2 *Caenorhabditis elegans* attraction test with humic substances (Modified and redrawn from Menzel et al. 2005a)

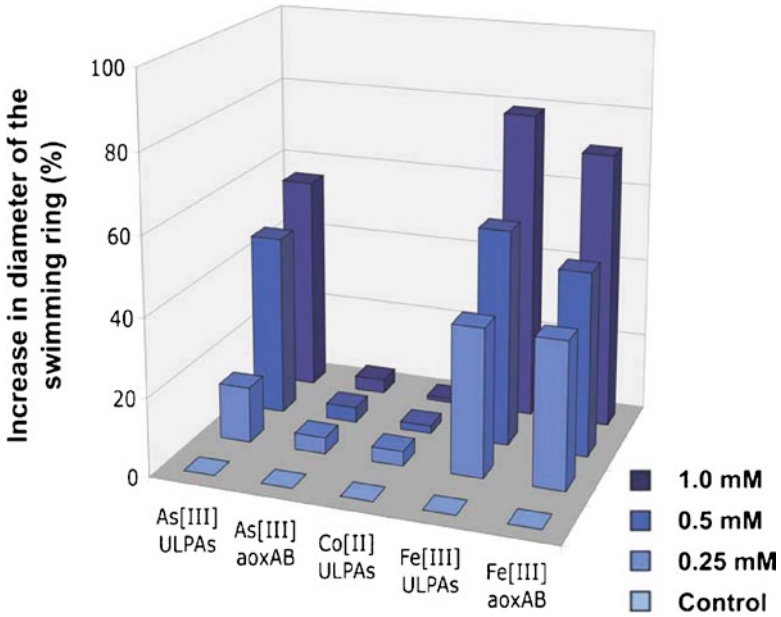

Fig. 1.3 Effect of metal and metalloid concentration on swimming properties in *Herminiimonas arsenicoxydans*. Motility assays were performed in the presence of an increasing concentration of As[III], Co[II], or Fe[III]. The level of motility of wild-type strain (*ULPAs*) and of its *aoxAB* knockout derivative was evaluated as the diameter of the swimming ring expressed in millimeters (From Muller et al. 2007; courtesy of Public Library of Science). The knockout mutants do not significantly respond to As[III] exposure

The hypothesis that arsenic contributes to the metabolism of *H. arsenicoxydans* was further supported by its positive chemotactic response toward arsenic, demonstrating that the bacterium is able to sense and respond to the presence of arsenic in the medium. Muller et al. (2007) concluded that the genome of *H. arsenicoxydans* contains 12 methyl-accepting chemotaxis protein–encoding genes. As most of these genes have no predicted function, it is tempting to speculate that at least one of them plays a role in this mechanism.

Why do both the worm and bacteria behave so strangely? Surely, according to current (eco)-toxicological paradigms, they must be crazy or masochistic. However, the worm and bacteria do not know these paradigms and demonstrate that our knowledge must be incomplete. In fact, several consecutive and detailed studies with *C. elegans* revealed that the worm is by no means crazy, but rather smart, because they increase their number of offspring under the stressful conditions (Höss et al. 2001) and prolong their individual lifespans (Steinberg et al. 2007) – provided that the exposed humic material had certain qualities and the overall chemical stress remained in the mild range.

The presence of natural endogenous and exogenous chemical stressors have been instrumental for, and in fact have driven, the development of various stress defense systems. In addition, anthropogenic chemical stressors, though sometimes severe or even lethal, also can impact organismal stress defense systems. The example of *H. arsenicoxydans* demonstrates the existence of a strategy to efficiently colonize seemingly hostile environments and may have played a crucial role in the occupation of ancient ecological niches on Earth (Muller et al. 2007).

The purpose of this book is to elucidate the background, basic mechanisms, and benefits of various stress defense mechanisms. In the beginning, its structure follows the signaling pathway of stresses in organisms, then covers the potential and actual stress responses, shows beneficial effects on the individual level which include modulation of life traits and development of stress resistances, discusses shifts in population structures, and tries to find footprints of stress in communities. In particular, the book is comprised of several topics:

Activation of oxygen: multipurpose tool:
To most biomolecules, elemental oxygen is inert. Under energy consumption, it has to be activated. If it is activated, it is multipurpose tool. Some organisms steel structures to activate oxygen from others by feeding them; others have to accomplish this task with external help.

Defense means against pathogens and parasites: reactive oxygen species:
Activated oxygen is also a universal tool against and particulate invaders.

Arms race between plants and animals: biotransformation system:
The biotransformation system started as an arm race between plants and animals. Plants produce secondary plant metabolites to defend against herbivory, and animals try to cope with this chemical challenge by enzyme systems of low specificity. Due to this low specificity, organisms can even handle many, but not all, synthetic chemicals without being intoxicated.

Heat shock proteins: the minimal, but universal stress response:
The coined term "heat shock protein" is misleading, since these protein families have a fundamental function, not only after various external or internal stresses. Their energy consuming stress response is as universal as the activation of oxygen. Organisms in stable environments have lost this stress response pathway.

Heavy metals: defense and ecological utilization:
Most organisms developed after heavy metals were buried beneath the biosphere. Yet, where both co-occur organisms are forced to handle the stress, to develop strategies to survive and to pass the adverse challenge to competitors or predators.

The basis of stress response: ecological transcriptomics.
Transcription is the initial step in gene expression and gives the first indication of cellular response potentials. Yet, such molecular biological data should be combined with further "omics" techniques.

Not all lies in the genes: microRNAs and epigenetics.
The translation of transcription products into proteins can be strongly modulated as the readability of the genetic information itself. The post-genetic era has overcome the genetic bias and opens new fields of investigations.

The actual response: ecological proteomics and metabolomics.
The stress response is formed by proteins and their metabolites. We are beginning to understand that each environmental stress appears to have a proteomic and metabolomic fingerprint.

Whatever doesn't kill you might make you stronger: hormesis.
It seems that the hormesis concept is more than a fashionable concern. To avoid a zero-sum game, from an ecological viewpoint this concept has to be considered more comprehensively than many current laboratory studies do.

Multiple stressors as environmental realism: synergism or antagonism.
A central belief is that organisms living under conditions close to their environmental tolerance limits appear to be most vulnerable to additional stress. Yet, there is increasing body of evidence that multiple stressors do not necessarily act additively or synergistically, but antagonistically. The mechanisms behind remain obscure in many instances.

One stressor prepares for the next one to come: cross-tolerance.
Subsequent or even simultaneous stressors induce cross-tolerances and prepare for the next stressor. This phenomenon is essential for organisms and populations to survive under suboptimal or fluctuating environmental conditions.

Longevity: risky shift in population structures.
The modulation of lifespan and reproduction under stresses shifts the population structure and bears the intrinsic risk of extinction.

Footprints of stress in communities.
The stress defenses translate into changes in community structures, which can be assessed by various phenotypic approaches and one theory-based approach. The gap between molecular and cellular responses and these approaches remains open.

Environmental stresses – ecological driving force and trigger of evolution.

We will see that not all stress is stressful – in contrast, it appears that mild chemical stress in the environment, below the mutation threshold, is essential for many subtle manifestations of population structures and biodiversity and may indeed have played a key role in the evolution of life in extreme environments. Even without any anthropogenic chemical discharge into the environment, ecosystems are loaded with natural chemicals which may have served as triggers for the evolution of some defense systems. Due to the long period of co-existence between stressors and organisms, the latter have not merely adapted, but have instead developed biochemical and molecular biological strategies to convert an adverse stress into a benefit for their individual integrity, for individual health and longevity, for the potential extension of the realized ecological niche, and for biodiversity and evolution. We are only just beginning to understand the subtle impacts on and the underlying mechanisms of stress in organisms; however, it does not seem fallacious to state that several ecological phenomena which are attributed to other factors, such as climate, nutrients and food, or competition, are at least influenced by factors that triggered the evolution of defense systems.

This book is not a textbook on ecotoxicology, environmental genetics, environmental physiology, ecological parasitology, or chemical ecology. Rather, it is simply an attempt to examine how stress in general affects organisms in beneficial ways. We hope that it will find its way into the scientific community and, finally, that the readers will not suffer from stress.

Chapter 2
Activation of Oxygen: Multipurpose Tool

To most biomolecules, elemental oxygen is inert since it usually does not oxidize them without prior activation either inside or outside of organisms. Atmospheric oxygen in its ground state is distinctive among the gaseous elements because it is a bi-radical. This means it possesses two unpaired electrons with parallel spins which make it paramagnetic. In this constitution, it is very unlikely to participate in reactions with organic molecules unless activated. Activation of oxygen can be facilitated by two different mechanisms:

- absorption of sufficient physical energy to reverse the spin on one of the unpaired electrons and to form the diamagnetic form of molecular oxygen, the so-called singlet oxygen $^1O_2$, or
- stepwise monovalent reduction.

Both pathways of oxygen activation are energy dependent (Fig. 2.1).

In the environment, photoactivation of oxygen may take place whenever light is absorbed by chromophores (pigments, humic substances). This process is termed *photodynamic* or *photosensitized reaction*. Inside phototrophs, this process is central in the photosynthesis. Externally, this process is of major ecological significance. Other pathways, such as superoxide dismutation or electron donation by $^{\bullet}O_2^-$ to an oxidized election acceptor, are not likely to occur in nature (Elster 1982).

2.1 Oxygen Activation in Ecosystems

In natural systems, the majority of chromophoric substances are comprised of humic substances. These are brownish materials which mainly derive from plant debris that leach into freshwater systems and ultimately into the oceans. Whenever they interact with light, a series of chemical reactions occur. They absorb both ultraviolet (UV) and visible light (VIS) in the wavelength range (290) 300–600 nm. These chromophores are activated many times a day. One calculation says that on a sunny

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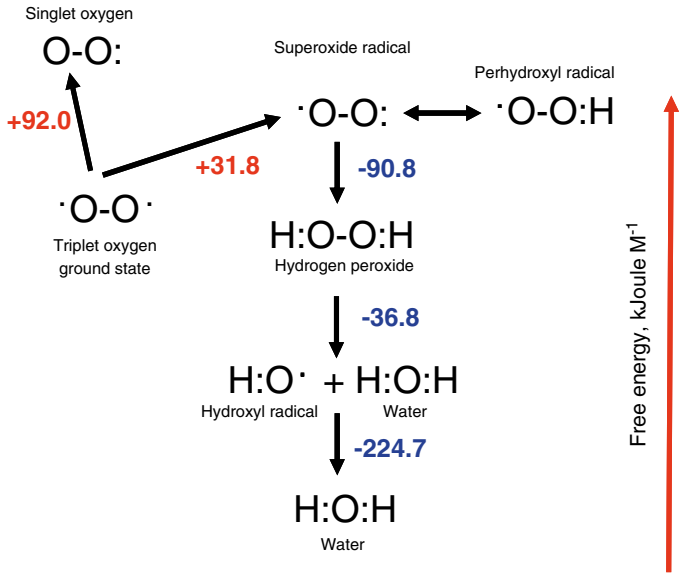

Fig. 2.1 Nomenclature of the various forms and activation pathways of oxygen. *Left*: absorption of energy (92 kJ M^{-1}) to activate the triplet state into the singlet state. *Right*: After the endergonic (31.8 kJ M^{-1}) reduction of O_2 to $^{\bullet}O_2^{-}$, the subsequent reduction steps are exergonic and occur spontaneously, either catalyzed or uncatalyzed. *Red* figures denote endergonic reactions, *blue* figures indicate exergonic reactions

day in Lake Greifensee (Switzerland), each chromophore in the lake's epilimnion is activated 270 times, that is, ten times or more per hour (Schwarzenbach et al. 1993). The light-absorption capacity is, in most cases, linked to the presence of π-electron systems that are available from heteroatoms, aromatic rings, or conjugated double bonds. These are the so-called 'chromophores'. With energy absorption, the outermost electron orbitals gain energy, and electrons are elevated from their lowest energy state to a higher energy state. Molecules in excited states are more reactive than in their ground states.

Direct photochemical reactions are immediate chemical changes to the chromophore such as isomerization, bond cleavage, or degradation of larger molecules into smaller molecules because of electron transfer reactions. In the presence of oxygen, photochemical decarboxylation and formation of CO_2 are observed in HSs, which are usually enhanced by the presence of iron in HS complexes.

The different reaction products are called reactive oxygen species (ROS). The individual ROS have very different half-lives, from only a few microseconds for $^1O_2$ to well over 1 h for H_2O_2. Depending on production rates and half-lives, average steady-state concentrations for ROS from 10^{-18} to 10^{-2} M are found in natural waters (Steinberg and Paul 2008).

Production and gross ecological effects of ROS are summarized in Fig. 2.2. The light-induced formation of ROS is called *sensitization*, and the photo-excited molecule itself is the *sensitizer*. Although the sensitizer molecule returns without

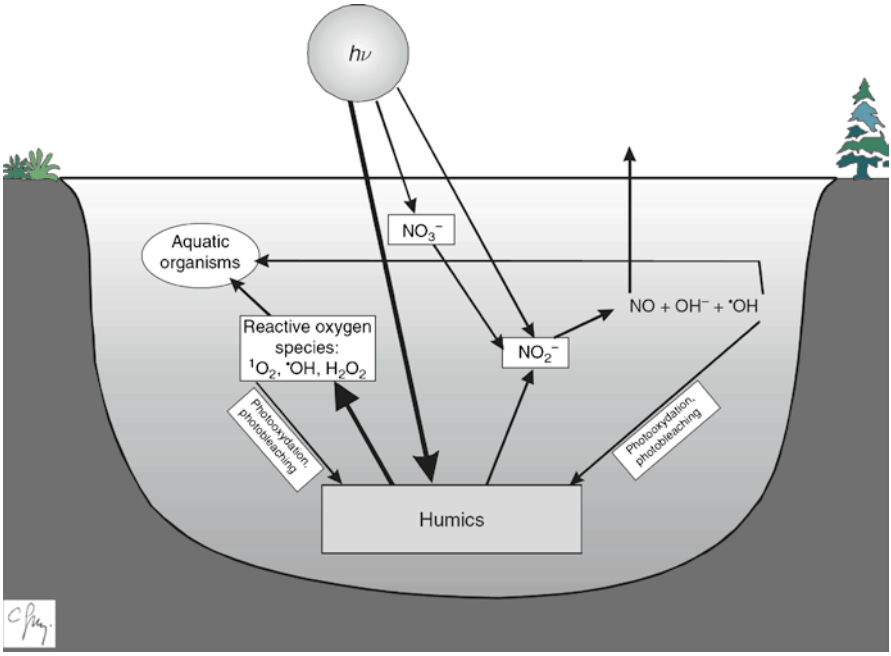

Fig. 2.2 Schematic presentation of photolytic production of reactive oxygen species (*ROS*) in an aquatic ecosystem. The major process is their release from illuminated dissolved chromophoric organic carbon. The ROS may interact with a great variety of water constituents, including organisms and dissolved organic compounds (From Steinberg and Paul 2008, with permission from Elsevier)

modification to the ground state, the photogenerated reactive species can attack any suitable target in its neighborhood, including the sensitizer itself. In fact, ROS account for the majority of photodegradation reactions observed with HSs. Any photosensitized reaction involves the transfer of energy, hydrogen atoms, protons, or electrons. The importance of oxygen in the photooxidation of natural organic matter is evident from oxygen consumption studies dating back to the early days of limnology. Oxygen plays a pivotal role as the initial scavenger of radicals that are produced during irradiation of water. This leads to the generation of alkoxy and peroxy radicals that decay to stable oxygenated species.

2.1.1 Effects on Organisms

Photolysis of various chromophoric dissolved compounds results in the production of ROS, of which H_2O_2 is long-lived and $^{\bullet}O_2^-$ as well as $^1O_2$ have the highest reactivity. All ROS may attack organisms. H_2O_2 easily penetrates membranes and contributes to internal oxidative stress which may be detrimental to the organism. For instance, leachates from aquatic macrophytes, which release the highest concentrations

of H_2O_2, support microbial growth least. In addition, the predominantly adverse effect of internal oxidative stresses, for instance from UV irradiation or processing of xenobiotic chemicals, is well documented and comprises induction and modulation of stress response proteins and enzymes, reduction of photosynthetic activity, and increased membrane (lipid) peroxidation. However, oxidative stress as a potential ecological driving force has not yet gained the attention it deserves (Steinberg and Paul 2008). Only very recently, Glaeser et al. (2010) studied the short-term as well as long-term effects of $^1O_2$ on bacterioplankton composition in a humic lake. The authors artificially increased the natural rate of $^1O_2$ formation in short-term (~4 h) *in situ* and long-term (72 h) laboratory incubations of surface water samples from a humic acid-rich lake. The analysis of abundant bacterioplankton phylotypes upon $^1O_2$ exposure showed that a moderate increase in $^1O_2$ exposure led to similar changes in different years, indicating the establishment of bacterial communities adapted to $^1O_2$ exposure. Bacterioplankton phylotypes favored under these conditions belonged to *Betaproteobacteria* of the beta II cluster (e.g. *Polynucleobacter necessarius*) and the beta I cluster related to *Limnohabitans* (R-BT subcluster) as well as *Alphaproteobacteria* affiliated to *Novosphingobium acidiphilum*. In contrast, *Actinobacteria* of the freshwater acI-B cluster were sensitive to even moderate $^1O_2$ exposure. Overall, the authors demonstrated that $^1O_2$ exposure due to photolysis of dissolved organic matter represents an important natural selective factor affecting bacterial species dynamics in aquatic ecosystems.

2.2 Activation of Oxygen in Organisms

2.2.1 Using "Stolen" Structures

In organisms, the activation of oxygen in principle does not differ from the abiotic processes in the environment. Oxygen has to be activated for any aerobic heterotrophic process to occur. Oxidative burst and a subsequent potential oxidative stress is a universal phenomenon experienced by both aerobic and anaerobic organisms from all domains of life (Imlay 2003).

Solar irradiation has the potential to activate oxygen by forming singlet oxygen. For organisms, the necessary energy is provided free of charge. Heterotrophic reduction of oxygen, however, is energy demanding, and the energy has to be deducted from other processes, such as body maintenance (growth, repair, and longevity) or reproduction. Consequently, smart animals should be able to save energy for heterotrophic and reproductive processes. And they do, probably much more frequently than is addressed in the literature.

"Stolen chloroplasts" (= kleptochloroplasts) convert a heterotroph into a mixtotroph organism. This occurrence is typical of dinoflagellates, such as *Gymnodinium* sp. and *Cryptoperidiniopsis* sp. who take the kleptochloroplasts generally from cryptophytes, their preferred phytoplankton prey (Jakobsen et al. 2000). After ingestion, chloroplasts may remain photosynthetically active for some time (Schnepf and Elbrächter 1999;

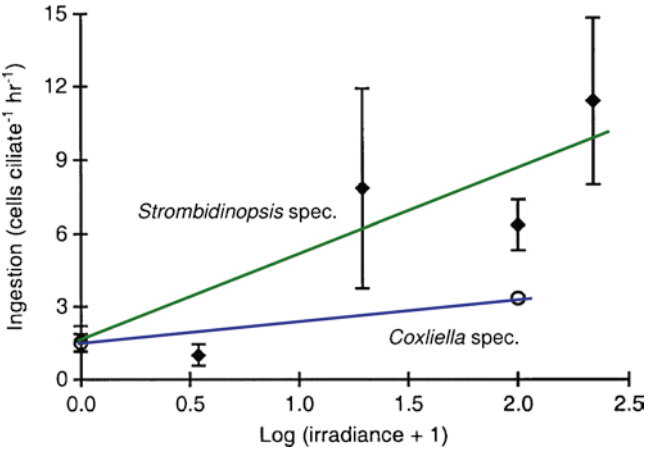

Fig. 2.3 Ingestion rates as a function of irradiance (μmol photons m^{-2} s^{-1}) for the ciliates *Strombidinopsis acuminatum* fed the pigmented *Prorocentrum minimum* and *Coxliella* sp. fed the non-pigmented *Gymnodinium simplex* (From Strom 2001, courtesy of Inter-Research Science Center). The increased ingestion efficiency of pigmented prey is obvious. For sake of clarity, straight lines are drawn by CS

Eriksen et al. 2002). In this respect, Skovgaard (1998) showed that their photosynthetic activity is lost within a few days. In his detailed study, he showed that light had a positive effect on growth kinetics of *Gymnodinium* cf. *gracilentum* in that growth and ingestion rates are higher at a high light intensity than at a low light intensity. He concluded that this effect was due to factors other than photosynthetic activity of kleptochloroplasts, since a control experiment with a supposed strictly heterotrophic dinoflagellate also showed a dependence of growth kinetics on light intensity.

More recent work (Strom 2001, 2002) also showed that some strictly heterotrophic protists digest phytoplankton at a higher rate in the light than in the dark and provided some mechanistic explanations. The light-dependent digestion differences translated into substantially higher rates of protist feeding and population growth, so that grazing potential may be linked to light intensity. In fact, chloroplast-sequestering dinoflagellates grow well in the light, but only when food is available (Jakobsen et al. 2000), which means that the gain of photosynthetic capability is not significant.

Light-aided digestion in protists has been seen only for phytoplankton prey, and was not observed when prey was heterotrophic. The phenomenon is mediated by visible light, which includes photosynthetically active wavelengths. These observations suggest that the digestive mechanism involves the photosynthetic apparatus of ingested prey cells. The hypothesis on the mechanism is that active oxygen compounds, whose formation should be promoted by photosensitization reactions involving chlorophyll, directly decomposed lipids and proteins of the ingested phytoplankton cell once the cell was enclosed in the degradative environment of the protist food vacuole. The light-aided digestion is not restricted to dinoflagellates, but has been shown also with ciliated protozoans (Fig. 2.3) and applies most likely to all transparent heterotrophs in a euphotic zone.

2.2.2 *Using Own Structures*

2.2.2.1 Balancing ROS and RNS – The Redox Homeostasis

Traditionally, ROS and reactive nitrogen species (RNS) were considered to be toxic by-products of aerobic metabolism, which were disposed of using antioxidants. However, in recent years, it has become apparent that plants actively produce ROS and RNS as signaling molecules to control processes such as programmed cell death, abiotic stress responses, pathogen defense, and systemic signaling (Mittler 2002).

Oxygen Activation

In the presence of photosynthetic pigments, which become excited by light absorption, the inert triplet state is transformed into the reactive singlet oxygen by absorbing energy from the excited pigment. This happens in the light-harvesting complex of both photosystems. In the case of photosynthetic electron transport, O_2 uptake associated with photoreduction of O_2 to $^{\bullet}O_2^-$ is called the *Mehler reaction*. Although photoreduction of oxygen is an important alternative sink for the consumption of excess energy, it is always associated with the generation of toxic ROS.

The major process of oxygen activation in all organisms is the stepwise reduction of triplet oxygen. The first univalent reduction step is energy demanding; the subsequent one-electron reduction steps are not energy dependent and can occur spontaneously or require an appropriate e^-/H^+ donor. In biological systems, heavy metal ions (Fe^{2+}, Cu^+) and semiquinones can act as e^- donors. Four-electron reduction of oxygen in the respiratory electron transport chain is always accompanied with a partial one- to three-electron reduction, yielding the formation of ROS: superoxide radical ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). Although H_2O_2 is less reactive than $^{\bullet}O_2^-$, in the presence of reduced heavy metals such as Fe^{2+} in a chelated form (which is the case in biological systems), the formation of $^{\bullet}OH$ can occur in the Fenton reaction (Blokhina et al. 2003). Ferrous iron is oxidized by hydrogen peroxide to ferric iron, a hydroxyl radical, and a hydroxyl anion. Ferric iron then is reduced back to ferrous iron, peroxide radical, and a proton by the same hydrogen peroxide (dismutation):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \tag{2.1}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + {}^{\bullet}OOH + H^+. \tag{2.2}$$

The recycling of iron from ferric to ferrous form by reducing agents facilitates the permanent generation of $^{\bullet}OH$ and maintains the Fenton reaction; hence, it is a self-catalyzing chain reaction with damage of cellular structures and biomolecules far in excess of the initial ROS concentration. In biological systems, the availability

of ferrous ions (and other redox-sensitive metals, such as Cu, Zn, Mn, and recently discovered: Ni) limits the rate of the Fenton reaction. Consequently, it is one major strategy of cells and organisms to reduce the availability of redox-sensitive metals in case of an oxidative stress, with phenols central in this termination of the Fenton reaction (see below).

Mechanisms for the generation of ROS in biological systems are represented by both non-enzymatic and enzymatic reactions. Non-enzymatic one-electron O_2 reduction can occur at low oxygen concentrations. Among enzymatic sources of ROS, xanthine oxidase (XO), an enzyme responsible for the initial activation of dioxygen, should be mentioned. As electron donors, XO can use xanthine, hypoxanthine or acetaldehyde. The next enzymatic step is the dismutation of the superoxide radical by superoxide dismutase to yield H_2O_2. Due to its relative stability, the level of H_2O_2 is regulated enzymatically by an array of catalases (CAT) and peroxidases localized in almost all compartments of the cell. Peroxidases, besides their main function in H_2O_2 elimination, can also catalyze $^{\bullet}O_2^-$ and H_2O_2 formation by a complex reaction in which NADH is oxidized using trace amounts of H_2O_2 first produced by the non-enzymatic breakdown of NADH. Next, the $NAD^{\cdot}$ radical reduces O_2 to $^{\bullet}O_2^-$, some of which dismutates to H_2O_2 and O_2. Thus, peroxidases and catalases play an important role in the fine regulation of ROS concentration and signaling in the cell through activation and deactivation of H_2O_2. Lipoxygenase (LOX, linoleate:oxygen oxidoreductase) reaction is another possible source of ROS and other radicals. It catalyzes the hydroperoxidation of polyunsaturated fatty acids (PUFA). The hydroperoxyderivatives of PUFA can undergo autocatalytic degradation, producing radicals and thus initiating the chain reaction of lipid peroxidation (LPO). In addition, LOX-mediated formation of singlet oxygen or superoxide radicals is feasible (Blokhina et al. 2003).

Most cellular compartments have the potential to become a source of ROS. Most ROS are formed in the chloroplasts via reduction to $^{\bullet}O_2^-$ or via excitation. Another potential source of ROS, namely H_2O_2, is the oxidation of glycolate or fatty acids in the peroxisomes (Fig. 2.4, Table 2.1). In the apoplast, several enzymes may also lead to ROS production under normal and stress conditions by oxidation of amines and oxalate. The mitochondrial electron transport system is also a source of ROS (Fig. 2.4, Table 2.1), including $^{\bullet}O_2^-$, H_2O_2, and $^{\bullet}OH$. In general, ROS are generated in mitochondria, an undesirable side product of oxidative energy metabolism (Dröge 2002). Direct reduction of O_2 to $^{\bullet}O_2^-$ takes place in the flavoprotein region of NADH dehydrogenase segment of the respiratory chain. Several observations reveal ubiquinone as a major H_2O_2 generating location of the mitochondrial electron transport chain *in vitro* with $^{\bullet}O_2^-$ as a major precursor (Fig. 2.4). It is calculated that in animals, approximately 1.5% of electrons flowing through the electron transport chain can be diverted to form $^{\bullet}O_2^-$ (Novo and Parola 2008).

Superoxide radicals are known to be produced during NADPH-dependent microsomal electron transport. Two possible loci of $^{\bullet}O_2^-$ production in microsomes are auto-oxidation of oxycytochrome-P450 complex that forms during microsomal mixed function oxidase (MFO) reactions and/or auto-oxidation of cytochrome P450 reductase, a flavoprotein that contains both flavin adenine

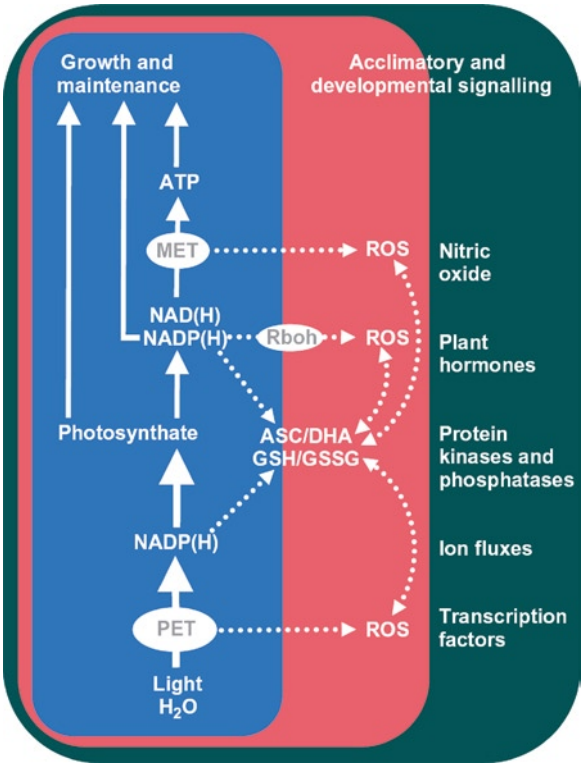

Fig. 2.4 Simplified scheme situating redox reactions in plant metabolism and their relationship to signaling. ROS are produced by many reactions, notably photosynthetic and respiratory metabolism, including photorespiration (not shown), and by homologs of mammalian respiratory burst oxidases (*Rboh*). ROS are processed by dismutases (superoxide dismutase, catalases) and reductive systems in which NAD(P)H, ascorbate and glutathione play a key part. Interactions between ROS, ascorbate and glutathione are important in acclimatory signaling mechanisms by which the plant perceives and responds to environmental change. These mechanisms involve interplay with many other cell signaling components, some of which are indicated in the outer green frame. Redox signals other than ROS are also produced by photosynthetic and mitochondrial electron transport chains. *ASC* ascorbate; *GSH* glutathione; *MET* mitochondrial electron transport; *PET* photosynthetic electron transport (From Noctor 2006, courtesy of Blackwell)

dinucleotide (FAD) and flavin mononucleotide (FMN, or riboflavin-5′-phosphate) (Bhattacharjee 2005).

Cell wall peroxidase is able to oxidize NADH and in the process catalyze the formation of $^{\bullet}O_2^-$. This enzyme utilizes H_2O_2 to catalyze the oxidation of NADH to NAD^+, which in turn reduces O_2 to $^{\bullet}O_2^-$. Superoxide radicals subsequently dismutate to H_2O_2 and O_2. Other important sources of ROS in plants that have received little attention are detoxification reactions catalyzed by cytochrome-P450 in cytoplasm and endoplasmic reticulum (ER). In plants, ROS are also generated at the plasma membrane or extracellularly in the apoplast. Plasma membrane NADPH-dependent oxidase (NADPH oxidase) has recently received a lot of attention as a

Table 2.1 Producing, scavenging, and avoiding reactive oxygen species (ROS) in plants and animals; *PS* = photosystem

Mechanism	Localization		Primary ROS
	In plants	In animals	
Production			
Photosynthesis	Chloroplast (water-splitting site in PSII, reduction by ferredoxin in PSI)		$^{\bullet}O_2^-$
Excited chlorophyll	Chloroplast (light harvesting complexes)		$^1O_2$
Respiration	Mitochondria (reduction by bioquinones)	Mitochondria (reduction by bioquinones)	$^{\bullet}O_2^-$, H_2O_2, $^{\bullet}OH$
Lipoxygenase	Membranes	Membranes	$ROO^{\bullet}$
Glycolate oxidase	Mitochondria, peroxisomes	Mitochondria, peroxisomes	H_2O_2
Fatty acid β-oxidation			
Further oxidases			
Xanthine oxidase	Peroxisomes	Peroxisomes	$^{\bullet}O_2^-$
Nitric oxide synthase			
Cyclooxygenase			
Other NAD(P)H dependent oxido-reductases			
NADPH oxidases	Plasma membrane	Plasma membrane of phagocytic and non-phagocytic cells	$^{\bullet}O_2^-$
Oxalate oxidase	Apoplast		H_2O_2
Amine oxidase	Apoplast		H_2O_2
Peroxidases, Mn^{2+} and NADH	Cell wall		H_2O_2, $^{\bullet}O_2^-$
Detoxification	Endoplasmic reticulum, cytoplasm	Endoplasmic reticulum, cytoplasm	$^{\bullet}O_2^-$
Scavenging			
Superoxide dismutase	Chloroplast, cytosol, mitochondria, peroxisomes, apoplast	Mitochondria, peroxisomes	$^{\bullet}O_2^-$
Ascorbate peroxidase	Chloroplast, cytosol, mitochondria, peroxisome, apoplast	Mitochondria, peroxisomes	H_2O_2
Catalase	Peroxisomes	Peroxisomes	H_2O_2
Glutathione peroxidase	Cytosol, membranes	Cytosol, membranes	H_2O_2, $ROO^{\bullet}$
Peroxidases	Cell wall, cytosol, vacuole	Cytosol	H_2O_2
Thioredoxin peroxidase	Chloroplast, cytosol, mitochondria	Cytosol, mitochondria	H_2O_2

(continued)

Table 2.1 (continued)

Mechanism	Localization		Primary ROS
	In plants	In animals	
Ascorbic acid	Chloroplast, cytosol, mitochondria, peroxisomes, apoplast	Cytosol, mitochondria, peroxisomes	H_2O_2, $^{\bullet}O_2^-$
Glutathione	Chloroplast, cytosol, mitochondria, peroxisomes, apoplast	Cytosol, mitochondria, peroxisomes	H_2O_2
α-Tocopherol	Membranes	Membranes	$ROO^{\bullet}$, $^1O_2$
Carotenoids	Chloroplast		$^1O_2$
Proline	Chloroplast, cytosol, mitochondria	Cytosol, mitochondria	$^1O_2$
Mycosporine-like amino acids, phlorotannins			$^1O_2$, $ROO^{\bullet}$
Alternative oxidases	Chloroplast, mitochondria	Mitochondria	$^{\bullet}O_2^-$
Avoidance			
Anatomical adaptations	Leaf structure, epidermis		$^{\bullet}O_2^-$, H_2O_2, $^1O_2$
C_4 or CAM metabolism	Chloroplast, cytosol, vacuole		$^{\bullet}O_2^-$, H_2O_2
Chloroplast movement	Cytosol		$^{\bullet}O_2^-$, H_2O_2, $^1O_2$
Suppression of photosynthesis	Chloroplast		$^{\bullet}O_2^-$, H_2O_2
Photosystem and antenna modulations	Chloroplast		$^{\bullet}O_2^-$, $^1O_2$

source of ROS for oxidative burst, which is typical of incompatible plant–pathogen interaction. In phagocytes, plasma membrane localized NADPH oxidase was identified as a major contributor to their bacteriocidal capacity. In addition to NADPH oxidase, pH-dependent cell wall-peroxidases, germin-like oxalate oxidases and amine oxidases have been proposed as a source of H_2O_2 in apoplast of plant cells. pH-dependent cell-wall peroxidases are activated by alkaline pH, which in the presence of a reductant produces H_2O_2. Alkalization of apoplast upon elicitor recognition preceding the oxidative burst and production of H_2O_2 by a pH-dependent cell wall peroxidase has been proposed as an alternative pathway of ROS production during biotic stress (Bhattacharjee 2005).

Reactive Nitrogen Species

Reactive nitrogen species (RNS) are a family of reactive molecules derived from nitric oxide ($^{\bullet}NO$) and $^{\bullet}O_2^-$ produced via the enzymatic activity of inducible nitric oxide synthase 2, NOS2, and NADPH oxidase respectively. RNS act together with

ROS to damage cells, causing nitrosative stress (Pauly et al. 2006). Therefore, these two species are often collectively referred to as ROS/RNS. Reactive nitrogen species also are continuously produced as by-products of aerobic metabolism or in response to stress.

Nitric oxide exerts physiological effects by controlling vascular tone, cell adhesion, vascular permeability, and platelet adhesion. Furthermore, $^{\bullet}NO$ is able to react rapidly with $^{\bullet}O_2^-$ to form the much more powerful oxidant peroxynitrite ($ONOO^-$). $^{\bullet}NO$ is not particularly toxic *in vivo* because $^{\bullet}NO$ is removed because of its rapid diffusion through tissues. $ONOO^-$ is a strong oxidant and produces nitrite and a hydroxide ion rather than isomerizing to nitrate. Like the other oxidants, it can react with proteins, lipids, and nucleic acids. $ONOO^-$ can also interact with mitochondria, reaching them from extra-mitochondrial compartments or being locally produced through the interaction of $^{\bullet}NO$ (generated by the mitochondrial NOS) and $^{\bullet}O_2^-$. Mitochondrial toxicity of $ONOO^-$ results from direct oxidative reactions of principal components of the respiratory chain or from free radical-mediated damage. Persistent generation of significant levels of $ONOO^-$ can lead to the induction of cell death, either by apoptosis or necrosis (Novo and Parola 2008).

Scavenging of ROS

Major ROS-scavenging mechanisms include superoxide dismutase (SOD), ascorbate peroxidases (APX), and catalase (CAT) (Table 2.1). The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide. Together with sequestering of metal ions, this balance is important to prevent the formation of the highly toxic hydroxyl radical via the Fenton reaction. The different affinities of APX (μM range) and CAT (mM range) for H_2O_2 suggests that they belong to two different classes of H_2O_2-scavenging enzymes: APX might be responsible for the fine modulation of ROS for signaling, whereas CAT might be responsible for the removal of excess ROS during stress, which most likely enables plants particularly to distinguish between different challenges (for details, see below).

The major ROS-scavenging pathways that are well summarized by Mittler (2002) (Fig. 2.5) are:

- The water–water cycle in chloroplasts (Fig. 2.5a),
- The ascorbate–glutathione cycle in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes (Fig. 2.5b),
- Glutathione peroxidase (GPX; Fig. 2.5c), and
- CAT in peroxisomes (Fig. 2.5d).

The water–water cycle (Fig. 2.5a) draws its reducing energy directly from the photosynthetic apparatus. Thus, this cycle appears to be autonomous with respect to its energy supply. However, the source of reducing energy for ROS scavenging by the ascorbate–glutathione cycle (Fig. 2.5b) during normal metabolism and particularly during stress, when the photosynthetic apparatus might be suppressed or