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The 'stress' concept in microalgal biology—homeostasis, acclimation and adaptation

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Abstract
The term 'stress' is widely used in the algal literature, usually in the context of the response of algae to changed abiotic and biotic factors. 'Stress' is seen as the cause of changes in algal metabolism and composition and often as a factor inducing the overproduction of particular desirable secondary metabolites. However, 'stress' is used differently by different authors and is often ill-defined, with no clear separation of cause and effect. This lack of a defined stress concept leads to poor experimental design, miscommunication of results and potentially erroneous conclusions. This paper reviews the stress concept as it applies to algae, especially microalgae. Here, stress is defined as the disruption of homeostasis due to a stressor and the stress response represents the changes in cell metabolism during acclimation and the restoration of homeostasis. Once homeostasis is restored the cell is no longer stressed. The stages of the stress response, i.e. alarm, regulation, acclimation and adaptation, are described. The well-studied responses of the green halophilic alga *Dunaliella* to changes in salinity are used as an example to illustrate the stress response and acclimation to the changed salinity.

Keywords Stress · Regulation · Acclimation · Adaptation · Stress signalling · Reactive oxygen species · Homeostasis

Introduction

> *Everybody knows what stress is and nobody knows what it is* (Selye 1973).

The term 'stress' is widely used in the context of growing microalgae, especially in the context of the overproduction of secondary metabolites such as carotenoids and triacylglycerols. However, from reading the phycological literature, it is quite clear that different authors use 'stress' in various ways and very often without any definition. 'Stress' is used both as the cause of the changes in algal metabolism observed as well as the effect caused by a change in growth conditions, or both cause and effect simultaneously. This has the potential to bias the design of experiments, the communication and interpretation of experimental results and the conclusions reached. Furthermore, in many papers 'stress' is implicitly assumed to have detrimental effects on the cells, although there may be no evidence to support this supposition.

Definitions of stress

In mechanics, the term 'stress' is defined as a function the force applied to a body per unit area leading to 'strain' (deformation) of the body, i.e. stress is a stimulus leading to a response. In biology, however, 'stress' sometimes is also used in the sense of 'strain' of mechanics, i.e. stress is the response to a stimulus or stressor. 'Stress' in biology also has a wide range of (often ill-defined and subjective) meanings and these meanings depend in part on the discipline (medicine, psychology, ecology, physiology, etc.), the organism(s) and the type of stressor under consideration (i.e. physical, nutritional, biochemical). Extensive discussions of these various biological 'stress' concepts in different fields of biology and medicine can be found in the following publications: Selye (1973); Hinkle (1974); Larcher (1987); Strasser (1988); Grime (1989); Lichtenthaler (1996, 1998); Gaspar et al. (2002); Goldstein and Kopin (2007) and Kranner et al. (2010). The purpose of this paper is not to review all the variants

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of the stress concept in biology, but rather to try to attempt to provide a coherent concept of stress as it relates to the biology of algae, especially microalgae, and the roles of homeostasis, regulation, acclimation and adaptation in the stress response of algae. Examples of specific stress responses are given in order to illustrate the concepts, but this paper is not a review of all possible algal stress responses.

The only attempt to comprehensively discuss and define the stress concept in algae was by Fogg (2001) although he found himself unable did not provide a clear definition of stress. Fogg considers stress as an inherent characteristic of any living organism that responds to stimulus, in other words, to any change, large or small, in its environment which affects biological processes. As he points out, organisms are almost continuously exposed to varying stimuli to which they respond without any dislocation of normal functions, as well as to more powerful stimuli which seriously disorganize vital activities. These stimuli intergrade imperceptibly, but when one reads the experimental literature, it is those stimuli which disorganize vital activities which are most commonly considered as 'stressors'.

Other authors have provided more restricted definitions of stress with respect to algae, including microalgae, often differentiating between different types of stress. For example, Davison and Pearson (1996), working on intertidal seaweeds, defined stress as reduction in growth rate and differentiated between 'limitation stress' [reductions in growth rate that occur because of an inadequate supply of resources] and 'disruptive stress' [reductions in growth rate which result from damage due to adverse conditions (or the allocation of resources to prevent damage)]. Limitation stresses include low light or insufficient nutrients, whereas disruptive stresses include conditions such as freezing or desiccation that either cause cell damage and/or require the allocation of resources to prevent and/or repair damage. In the context of mass cultures of microalgae, Torzillo and Vonshak (2013) have defined stress as 'an environmental condition that results in a metabolic imbalance that requires biochemical and metabolic adjustments before a new steady state of growth can be established'. They differentiate stress from a limiting factor which they define as 'one that determines the rate of growth or biochemical reaction, and that a change in its level will result in a change in the rate without any requirement for an acclimation process'. Thus, their 'limiting factor' is equivalent to the 'limiting stress' of Davison and Pearson (1996).

The word stress, not surprisingly, has negative connotations and these are either explicitly or implicitly contained in many definitions. For example, Grime (1989), an evolutionary ecologist, defined stress as 'external constraints limiting the rates of resource acquisition, growth or reproduction of organisms'. Similarly, Slaveykova et al. (2016) defined stress as 'any harmful environmental factor that induces cellular physiological changes, disturbing the homeostasis of an organism'. However, stress may not always be harmful as pointed out by Larcher (1987) in his definitions of stress in plants:

> 'Every organism experiences stress, although the way in which it is expressed differs according its level of organisation. From the botanist's point of view, stress can be described as a state in which increasing demands made upon a plant lead to an initial destabilization of functions, followed by normalization and improved resistance. If the limits of tolerance are exceeded and the adaptive capacity is overtaxed, permanent damage or even death may result. Stress thus contains both destructive and constructive elements: it is a selection factor as well as a driving force for improved resistance and adaptive evolution.'

This definition was further elaborated by Lichtenthaler (1988) whose main focus was on higher plants and who differentiated between 'eu-stress' (*eu* in Greek means good) and 'dis-stress', where eu-stress is an activating, stimulating stress and a positive element for plant development, whereas dis-stress (as seen in the English word distress) is a severe and a real stress that causes damage and thus has a negative effect on the plant and its development. This distinction is interesting, but in practice, it is difficult to discriminate between these two forms of 'stress' with respect to algae.

Related to this is the important and largely unresolved issue whether every environmental change that causes a response in an organism represents a stressor (see Table 1 for definition) and whether it is possible to clearly define a level of intensity and duration of exposure to this change that results in an environmental factor being classified as either stressful or non-stressful (Schulte 2014). Furthermore, there is the question of whether there is a difference between a normal homeostatic response to an environmental change and a stress response, and if so, how can we, *or should we*, draw a dividing line between them? For example, in nature (and large-scale outdoor cultures), algae are exposed to a constantly changing environment, especially diurnal and longer term changes in irradiance, but also changes in temperature, pH, nutrient availability, etc. These changes continuously expose the algal cells to multiple stressors of varying magnitude and duration. In practice, it appears that the algae acclimate to an 'average' state if these environmental changes are more or less regular. An example of this is the acclimation of outdoor algal cultures to the prevailing diurnal light and temperature pattern (Moheimani and Borowitzka 2007).

Furthermore, the change in environmental conditions (temperature, salinity, etc.) may actually mean that the new conditions actually may be closer to the optimal conditions for the

Table 1 Definitions of key terms

Term	Definition
Stressor	Stimulus impacting on algal cell (light, temperature, nutrients, shear force, etc.)
Stress	Disturbance of homeostasis due to the action of a stressor (Once cells have restored homeostasis by acclimation and possibly adaptation, they are no longer stressed)
Homeostasis	The equilibrium of the composition and physiological processes of an organism in balance with its environment
Regulation	Changes in the functioning of pre-existing catalysts and metabolites that do not require changes in the expressed proteome (occurs over short time periods of seconds to minutes)
Acclimation	The change of the macromolecular composition of an organism that occurs in response to a change in the environment and requiring changes in gene expression
Adaptation	A change in genotype in response to a change in the environment
Limitation	Reduction in growth rate caused by an inadequate supply of a resource (e.g. light, nutrient, temperature)
Balanced growth	Steady-state growth with constant growth rate and with no changes in cell composition, i.e. as occurs in chemostat continuous cultures
Unbalanced growth	Non-steady-state growth, i.e. as occurs in batch cultures

algae than the ones it had been growing under, resulting in higher growth rates.

When considering the effect of a stressor (or stressors) on an algal cell and the cell response, one must consider the following:

1. The optimum conditions for a particular organism/genotype

   For example, for freshwater algae, a change to seawater salinity would be very stressful and normally leads to cell death, whereas for marine algae, a salinity near that of seawater is optimal for growth. Similarly, for the halophilic alga *Dunaliella salina* salinities, some 5–7 x higher than seawater are optimum resulting in higher growth rates and lower salinities may cause 'stress'.

2. The magnitude of the stress, i.e. how far does it deviate from the status quo

3. The duration of the action of the stressor—short or long, temporary or permanent

   The magnitude and the duration interact and thus affect the level of 'stress' the alga is exposed to and the ability of the alga to respond to this stress. For example, a slow rise in salinity will be less 'stressful' than a sudden large rise as the cells have time to acclimate. A combination of stressors acting at the same time or repeatedly over a short time period is also likely to be more stressful than a single stressor acting at any one time as greater resources are required for acclimation.

4. The physiological state of the alga at the time the stressor is applied

   This includes factors such as the growth stage (logarithmic, linear, stationary) and whether the alga has been growing under near optimal conditions or under suboptimal conditions. The physiological state influences the capacity of the cell to respond to a stressor.

Unfortunately, some of these points, especially points 1 and 4, often cannot be answered.

In the definitions outlined above, stress often is being used for both the environmental factor causing the response and/or the response itself. In this paper, I define stressors as those environmental factors that disturb homeostasis and stress as the response to these stressors (indicated by the shaded portion in Fig. 1). In this sense, the term 'stressor' is equivalent to the term 'stimulus' as used by Fogg (2001). Stressors include abiotic factors such as temperature, light, nutrients and toxicants, as well as biotic factors such as the presence of predators and infection with pathogenic organisms. Stress is then a function of the magnitude of the stressor and the duration of the action of the stressor acting on the organism. However, not all responses to a stressor should be considered as stress. As Raven and Geider (2003) point out, algae can respond to environmental changes by adjusting catalytic efficiencies without net synthesis or breakdown of macromolecules as, for example, the photosystem state transitions in response to short-term excess light (Minagawa 2011). They call this 'regulation' (Table 1) and regulation cannot really be considered a stress response. Regulation operates at time scales of seconds to minutes, whereas acclimation and adaptation operate at longer time scales. Stress occurs when regulation is inadequate to maintain homeostasis.

Responses to stressors

Organisms respond to stressors in a number of ways, the response depending on the type of stressor, its magnitude and

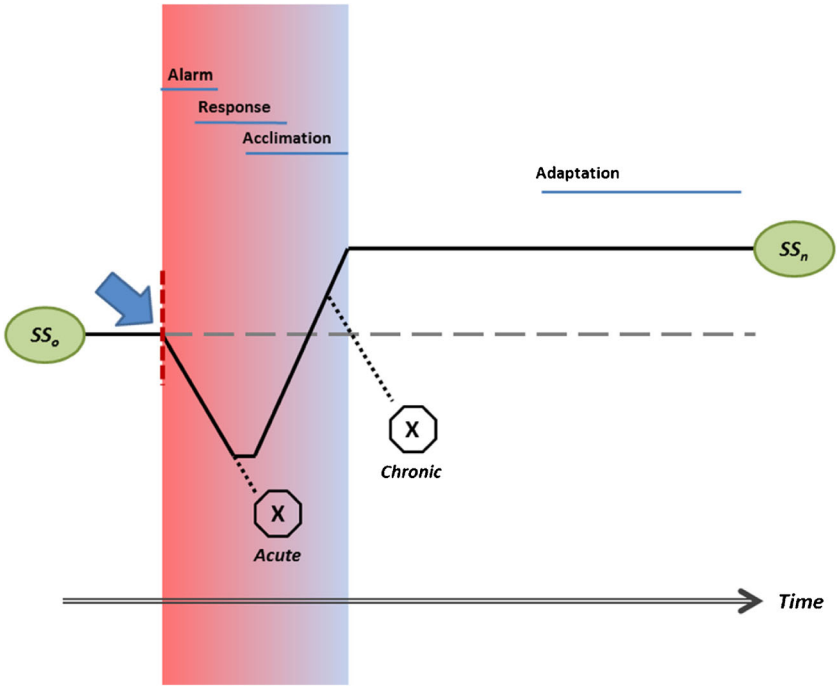

Fig. 1 Simplified diagrammatic representation of the response to a stressor over time. In response to a stressor (blue arrow), the original steady state (SS_o) or homeostasis of the cells is disrupted initiating an alarm' signal leading to a 'response' which initiates 'acclimation' processes to restore homeostasis, i.e. a 'stress response' (reddish bar). Initially, there is a decline in metabolism often seen as a cessation of motility in motile cells, a reduction on photosynthesis etc., and this is followed by a recovery of these processes during acclimation. Once the cells have fully acclimated (i.e. homeostasis is restored), a new steady state (SS_n) is achieved, meaning the cells are no longer under stress [Note: the new steady state (SS_n) is shown as being higher than the original steady state (SS_o) for convenience; however, it may also be lower than the SS_o]. If the new conditions persist long enough, the cells also may adapt to the new conditions by genetic changes. If the initial stressor is too disruptive (acute stress), acclimation may not be possible and cell death occurs. Similarly, if the acclimation response requires more resources than are available to allow full acclimation, cell death may occur (chronic stress)

duration and on the current physiological state of the organism.

Figure 1 is an idealised schematic diagram of the sequence of responses of an organism to an external stressor. When an organism growing in steady state with its environment (SS_O in Fig. 1) is faced with a change in that environment (a stressor or multiple stressors), homeostasis is disrupted. This initially leads to a decline in one or more physiological functions initiating an alarm response. The cell may be able to maintain homeostasis through regulation (not shown in Fig. 1); however, if homeostasis cannot be maintained by regulation and the decline in functions continues, a stress response is initiated. The decline in functions (stress) triggers a cellular response to restore homeostasis through acclimation. Depending on the magnitude and duration of the stressor(s), the acclimatory processes can eventually restore cell homeostasis of the cell. Furthermore, the duration of the stressor(s) will determine whether the cell is able to achieve a new homeostatic state (SS_N in Fig. 1). If the magnitude of the stressor(s) exceeds the capacity of the cell to acclimate (acute stress), then the cells die (unregulated cell death) or undergo suicide (regulated cell death or apoptosis) (Galluzzi et al. 2016). If the new environmental conditions persist long enough and the metabolic/energetic cost to achieve a new homeostatic state allows for long-term survival, the cells may acclimate to this new environment and they will no longer be stressed. However, if the metabolic/energetic cost to achieve the new homeostatic state exceeds the capacity of the cell (chronic stress), then cell death may occur (Berges and Falkowski 1998; Timmermans et al. 2007). Finally, if the new environmental conditions persist, genetic changes and selection in the algal population may ultimately lead to a new genotype adapted to these new conditions (Lakeman et al. 2009).

Homeostasis and acclimation

The fundamental response of a cell to a stressor which disrupts metabolism (i.e. stress) is to try to restore homeostasis. Homeostasis, a term coined by Cannon (1932), can be broadly defined as 'the equilibrium of the composition and physiological processes of an organism in balance with its environment' (Table 1). Biological systems tend to try and maintain homeostasis in the face of changes in the external milieu. Organisms respond to any deviation from homeostasis by regulation, acclimation and adaptation (Giordano 2013).

Once homeostasis is restored (i.e. the cells have fully acclimated), are the cells still under stress? The answer is no.

If we take the example of nutrient limitation, one can differentiate between two states: nutrient starvation (non-steady state or unbalanced growth) and acclimated nutrient limitation (steady-state or balanced growth). One commonly used indicator of the physiological state of algae is the maximum PSII photochemical efficiency F_v/F_m. In batch cultures under nutrient (N) limitation, the F_v/F_m decreases over time, whereas in continuous cultures in steady state, the F_v/F_m remains constant irrespective of the level of nutrient supply (Parkhill et al. 2001; Remmers et al. 2017), indicating that the cells are fully acclimated to the prevailing nutrient concentration. This would indicate that the acclimated cultures cannot really be considered to be stressed.

Avoidance

Some algal cells have alternative ways to avoid chronic stress by forming stress-resistant life cycle stages. For example, some species of *Dunaliella* respond to large, but non-lethal, changes in salinity by forming non-motile cells embedded in a polysaccharide matrix and known as palmellae (Borowitzka and Siva 2007). Alternatively, *Dunaliella* can also form thick-walled resting stages (aplanospores) at low salinity and low temperatures (Borowitzka and Huisman 1993). High salinity also induces palmella formation in *Chlamydomonas reinhardtii* (Khona et al. 2016). Cyst formation under unfavourable conditions such as low temperature or low nutrients has been reported in many species of microalgae (Borowitzka et al. 1991; Imai and Itakura 1999; Bravo and Figueroa 2014).

The initiation of sexual reproduction appears to be another form of stress avoidance (see for example Nedelcu (2005)). In those species of microalgae where sexual reproduction is known, an almost universal feature is that sexual reproduction is initiated by a stressor, usually nutrient limitation often combined with suboptimal temperature. Where studied, nitrogen limitation (stress?) is particularly efficient in inducing gamete formation and release in algae. A common feature of the formed zygote (zygospore, planozygote, etc.) is that this cell has a thick cell wall and can be very resistant to extreme environmental conditions such as low nutrients, salinity, pH, low and high temperatures, high light, UV, and desiccation. Only when conditions once again become favourable for growth will the zygote germinate releasing the new daughter cells.

The link between stress and sexual reproduction has been well illustrated by work on algae which grow in temporary water bodies which periodically dry out. For example, in the haploid colonial green alga *Volvox carteri*, sexual reproduction is triggered by environmental stress such as increased temperature via a 30-kDa glycoprotein sexual inducer produced by the somatic cells of both females and males. This sexual inducer acts on the asexual reproductive cells (gonidia) of both sexes altering their development so that in the next generation, sexual females, bearing eggs, and males bearing sperm packets will be produced. Following fertilisation of the eggs, the desiccation-resistant diploid zygotes are formed (Starr 1970; Kirk and Kirk 1986). This induction of sexual reproduction also involves overproduction of reactive oxygen species (ROS), a common feature of cells under stress (Nedelcu et al. 2004; Nedelcu 2005). Similarly, either hot or cold temperature, stress will induce gametogenesis in the intertidal multicellular green alga, *Ulva* (Strain et al. 2006; Carl et al. 2014).

Death

If the magnitude of the stressor is too great, the cells may be unable to acclimate. The impact of large magnitude stressors may result in almost instant cell death as, for example, a too large change in salinity may cause the cells of *Dunaliella* to burst. This is called an acute stress (Fig. 1). Similarly, acclimatory metabolic changes may begin following the action of a stressor; however, the energy requirement for successful acclimation is greater than the energy available so that the cell dies before becoming fully acclimated to the new conditions. This is called chronic stress (Fig. 1). For example, the induction of apoptosis by chronic stress has been shown for nutrient stress in several diatoms (Brussaard et al. 1997; Klaas et al. 2007) and by salt stress in the freshwater chlorophyte *Micrasterias denticulata* (Affenzeller et al. 2009).

Adaptation

If the conditions originally stressed the culture and to which it successfully acclimated, there is the possibility that the cultures will adapt to these conditions over time, i.e. genetic changes arising by mutations and selection will 'fix' the acclimated phenotype. Several studies have shown adaptation to changed salinity, temperature and CO_2 concentrations in laboratory cultures grown under constant conditions for hundreds of generations (Collins and Bell 2004; Lohbeck et al. 2013; Perrineau et al. 2014; Lachapelle et al. 2015). Although these studies have assessed only changes in the phenotype, they clearly reflect major changes in gene expression (Lohbeck et al. 2014) and possibly also the genotype. Genotypic changes following long-term acclimation to different temperatures have been shown in the marine diatom *Thalassiosira pseudonana* (Schaum et al. 2017).

Responses to a stressor—stress and acclimation

In the following section, each of these steps is illustrated in more detail using the example of the widely studied response of the green alga *Dunaliella* to changes in salinity. This example serves to illustrate the complexity of the stress response and acclimation. Of course, different stressors will elicit different processes and these are likely also to vary between different algal taxa.

Example: *Dunaliella* and salinity stress

Alarm response

The immediate effect of a stressor is the disruption of various cell functions, the level of disruption depending on the magnitude and nature of the stressor. For example, in the green wall-less alga *Dunaliella salina*, a sudden increase in salinity leads to a rapid (time scale = seconds) efflux of water from the cell due to the osmotic differential between the external medium and the cytoplasm causing cell shrinkage (Weiss and Pick 1990). This water transport is a passive process. This cell shrinking can continue for several minutes resulting in changes in cytoplasmic pH and ionic concentrations. In particular, the cell concentration of Na^+ rises rapidly via the action of a H^+/Na^+ antiporter activated by the pH change (Weiss and Pick 1990; Katz et al. 1992). Alternatively, a sudden decrease in salinity leads to water influx and an increase in cell size and volume (Maeda and Thompson 1986). Another, immediately visible, sign of the effect of the changes in salinity is that the cells stop swimming. If the rapid salinity change is large enough, the cells may also lose their flagella.

Following these rapid changes in cell volume, the cells slowly recover to their original cell volume and ionic composition over a period of minutes to hours. This recovery of cell volume is associated with a rebalancing of the internal osmotic environment with that of the external environment by either glycerol accumulation in the case of hyper-osmotic shock, or a reduction in the cellular glycerol content in the case of hypo-osmotic shock. The recovery of the cell volume after hyper-osmotic shock is preceded by a recovery in the cell Na^+ concentration by Na^+ extrusion via a plasmalemma Na^+-ATPase (Weiss and Pick 1990; Pick 1992; Popova et al. 2005). Following hypo-osmotic shock, glycerol accumulation begins within 2–5 min of the stress and proceeds linearly with time reaching a maximum after about 1–2 h, depending on the magnitude of the stress (Brown and Borowitzka 1979; Lilley et al. 1987).

Growth also ceases after a salinity shock and only resumes once the osmotic and ionic equilibrium of the cells is restored.

Exactly how the cell senses the osmotic change in the external environment is still unknown, but sensing appears to occur at the level of the plasma membrane. Two types of sensors located in the plasma membrane have been hypothesised. Tsukahara et al. (1999) found that the stretch-activated Ca^{2+} channel blocker, Gd^{3+}, inhibited glycerol dissimilation in *Dunaliella tertiolecta* following a hypo-osmotic shock. Mechanosensitive ion channels have also been shown to be associated with osmoregulation in yeasts and chloroplasts (Hamilton et al. 2015). However, mechanosensitive ion channels are unlikely to be able to respond to hyper-osmotic changes and Hill and Shachar-Hill (2015) have suggested that aquaporins may act as osmosensors in such situations.

Regulation

The potential occurrence of a regulation stage following disruption of homeostasis was proposed by Giordano (2013). Regulation occurs within seconds to minutes by changes in the functioning of pre-existing catalysts and metabolites that do not require changes in the expressed proteome, e.g. post-translational modification by phosphorylation-dephosphorylation of light-harvesting complexes, enzymes or transporters and/or the activation-deactivation of existing enzymes. Regulation is best demonstrated by the fast response of plants and algae to changes in the light environment (Lavaud 2007; Derks et al. 2015).

Acclimation

The action of the 'sensors' of osmotic changes such as changes in medium salinity triggers one or more signal cascades which ultimately result in changes in translational control of protein synthesis and of gene expression, the first stages of acclimation. In order to establish full cell function and homeostasis following an osmotic shock, *Dunaliella* cells need to re-establish the osmotic and ionic balance of the cell. As glycerol is the main compatible osmoregulatory solute in *Dunaliella*, much attention has been paid to the mechanisms by which the algal cells regulate glycerol metabolism.

Calcium clearly has a role in the response to osmotic stress in *Dunaliella* (Issa 1996). It appears that Ca^{2+} signalling is involved in early signal transduction associated with glycerol synthesis in *D. salina* (Chen et al. 2011). Under a hyper-osmotic up-shock (from 2.0 to 4.5 M NaCl), intracellular Ca^{2+} increases slowly initially for the first 200 s and then more rapidly, whereas under a hypo-osmotic shock (from 2.0 to 0.5 M NaCl), there is an initial rapid increase in intracellular Ca^{2+} in the first 110 s followed by a gradual decline after that. This increase in intracellular Ca^{2+} is due to the influx of Ca^{2+} via Ca^{2+} channels. Ca^{2+} signalling in response to osmotic stress has also been shown in *Chlamydomonas reinhardtii* (Bickerton et al. 2016). Interestingly, hyper-osmotic stress induced a single Ca^{2+} increase that was modulated by the strength of the stimulus and originated in the apex of the cell, spreading as a fast Ca^{2+} wave. On the other hand, hypo-

osmotic stress induced a series of repetitive Ca^{2+} increases in the cytosol that were spatially uniform.

In *D. tertiolecta*, two different protein kinases were found to be activated on osmotic shock, one following hyper-osmotic shock and the other one after hypo-osmotic shock (Yuasa and Muto 1996). A Ca^{2+}-dependent protein kinase has also been isolated and characterised from this alga (Yuasa and Muto 1992; Yuasa et al. 1995). The likely participation of protein kinases of the p38 and JNP families during signal transduction under hyper-osmotic, heat and UV stress in *Dunaliella viridis* has been demonstrated by Jiménez et al. (2004). They identified a 57-kDa protein in *D. viridis* that cross-reacted with p38 and Hog1p specific antibodies and was transiently phosphorylated after a saline hyper-osmotic stress, suggesting that signalling through mitogen-activated protein kinase (MAPK) (Jiménez et al. 2004). Lei et al. (2008) have also reported the response of a MAPK gene (*DsMPK*) following hyper-osmotic shock. Recently, in an elegant study, Zhao et al. (2016) showed that in *Dunaliella tertiolecta* after hyper-osmotic shock, *DtMAPK* and *DtGPDH* gene expression increased within 0.5 and 1 h, respectively. *DtMAPK* is a yeast Hog1 homologue MAPK encoding gene and *DtGPDH* is the glycerol 3-phosphate dehydrogenase (GPDH) gene. GPDH is an important rate-limiting enzyme for glycerol synthesis and, in yeast, Hog1 triggers signalling and transcription events which promote transcription of the GPDH enzyme and synthesis of glycerol (Saito and Posas 2012). Zhao et al. (2016) also found that glycerol production and *DtGPDH* expression level paralleled the expression of *DtMAPK* under different osmotic stress conditions suggesting a close correlation between the expression of these two genes and glycerol production. Moreover, suppressed transcription of *DtGPDH* and delayed accumulation of intracellular glycerol were observed in *DtMAPK* knock-down cells upon hyper-osmotic shock, providing further evidence that *DtMAPK* is involved in the regulation of *DtGPDH* expression and thus glycerol synthesis. These results demonstrate that a MPK-mediated signalling pathway similar to the High-Osmolarity Glycerol (HOG) pathway of yeast (Hohmann 2009) may exist in *D. tertiolecta*.

In *Dunaliella*, as in yeast, glycerol is synthesised via the glycerol cycle. Glycerol biosynthesis starts from the Calvin-Benson cycle or the glycolysis intermediate dihydroxyacetone phosphate (DHAP). DHAP is converted into glycerol 3-phosphate by a DHAP/glycerol 3-phosphate dehydrogenase. Glycerol 3-phosphate is then dephosphorylated by a specific phosphatase to produce glycerol. In hypo-osmotically shocked cells, glycerol can be converted back into DHAP via dihydroxyacetone using dihydroxyacetone/glycerol dehydrogenase and then a specific glycerol kinase. DHAP/glycerol 3-phosphate dehydrogenase is the critical enzyme for glycerol accumulation, and its activity is stimulated in vitro by increasing the concentration of NaCl in enzyme assay mixtures. In *D. tertiolecta*, three isozymes for this enzyme have been characterised, one in the cytoplasm and two in the chloroplast (Gee et al. 1993), one of which was stimulated by NaCl. This NaCl stimulation means that glycerol synthesis can occur immediately after the salt shock even in the presence of the increased Na^{+} which occurs immediately after the salt shock. The drop on ATP concentration after a hyper-osmotic shock (Ehrenfeld and Cousin 1984; Belmans and van Laere 1987) is also consistent with the ATP requirement for glycerol synthesis. In *D. salina*, five different isozymes of DHAP/glycerol 3-phosphate dehydrogenase whose activity varies with growth salinity have been detected (Chen et al. 2009). De novo protein synthesis is apparently not required in the early stages of acclimation to hyper-osmotic shock (Sadka et al. 1989). Since photosynthesis is inhibited by a hyper-osmotic shock (Goyal 2007a), it is also important that the DHAP for glycerol synthesis is mainly derived from starch catabolism rather than from photosynthesis (Goyal 2007b; Fang et al. 2017) enabling glycerol synthesis even while photosynthesis is inhibited (Kessly and Brown 1981).

The emphasis on the restoration of the osmotic and ionic balance of the cells is to ensure the protection and renaturation of damaged proteins, nucleic acids and membrane lipids. However, a number of secondary responses also are needed to ensure successful and complete acclimation to the salt stress. These include the scavenging of liberated free radicals (but see below the likely role of ROS as signalling compounds), an increase in energy-supplying reactions and other changes in gene expression and enzyme activity.

For example, Sadka et al. (1991) observed the induction of a salt resistant 150-kDa plasmalemma membrane protein once growth resumed after a hypersaline shock (1.5 to 3.5 M NaCl) in *D. salina*. This protein was later identified as a transferrin-like protein (Ttf) which has high specificity and affinity for Fe^{3+} ions, strict dependence on carbonate/bicarbonate ions and very low activity in acidic pH (Fisher et al. 1998). Fisher et al. (1996) have also identified a salt-tolerant plasmalemma carbonic anhydrase following hyper-osmotic shock whose concentration increases with increasing salinities. The latter is particularly important as the solubility of CO_2 decreases with increasing salinities and temperatures. Many other salinity-dependent changes to the plasma membrane proteome (Katz et al. 2007) and lipid composition (Azachi et al. 2002) of *D. salina* have been demonstrated to be part of the process of acclimation to higher salinities.

Reactive oxygen species and redox state

One common feature of stress responses is the production of reactive oxygen species (ROS) and the cell redox state as well as changes in the activity of antioxidant enzymes and antioxidants. In analogy with higher plants, these ROS are also very likely to be a component of the signalling and acclimation

processes in microalgae. In higher plants, ROS have been shown to play a key role in the acclimation process to abiotic stress. Reactive oxygen species ($O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, $^1O_2$) are partially reduced or activated forms of atmospheric oxygen (O_2), and each subcellular compartment of plant and algal cells (chloroplasts, mitochondria, peroxisomes, etc.) contains its own set of ROS-producing and ROS-scavenging pathways. Furthermore, each ROS species has a different mode of action and a distinct half-life ranging from 1 ns for hydroxyl radicals to > 1 ms for hydrogen peroxide (see Fig. 1c in Mittler 2017). The steady-state levels of ROS, as well as the redox state of each compartment, are different at any given time resulting in a distinct signature of ROS levels at the different compartments of the cell (Noctor and Foyer 2016). In the past decade, it has become clear that, although ROS can be toxic by-products of stress metabolism, they primarily function as signal transduction molecules that regulate different pathways during plant acclimation to stress (see the following recent articles for details: Choudhury et al. (2016); Dietz et al. (2016); Mignolet-Spruyt et al. (2016); Mittler (2017); Raja et al. (2017)). For example, the release of chloroplast-produced ROS and oxidation products, envelope permeabilisation (for larger molecules) and metabolic interference with mitochondria and peroxisomes produce an intricate ROS and redox signature, which controls acclimation processes. This photosynthesis-related ROS and redox information interact with various signalling pathways (e.g. the mitogen-activated protein kinase and OXI1 signalling pathways) and control processes such as gene expression and translation (Dietz et al. 2016).

Almost all the work on ROS signalling so far has been done on higher plants, and little actual detail is known about their role and function in acclimation to environmental changes in algae as yet (Mittler et al. 2011). However, there is evidence that ROS have a role in algal stress responses such as, for example, hypo- and hypersaline stress in *Dunaliella* species (Tammam et al. 2011), or UV (Zhang et al. 2017) or nitrogen limitation in the diatom *Phaeodactylum* (Rosenwasser et al. 2014). However, most research in algae to date has been focused on the detoxification of the produced ROS via the action of antioxidant enzymes such as superoxide dismutase, glutathione reductase, catalase, ascorbate peroxidase and glutathione S-transferase, as well as antioxidants such as glutathione, ascorbic acid and β-carotene (e.g. El-Baky et al. 2004; Nowicka et al. 2016) rather than on the role they play in the stress response.

Summary

Here, I have attempted to show that 'stress' in an algal cell occurs in the period between the exposure of the cell to a stressor (or stressors) which disrupts homeostasis, until the time the cell is fully acclimated to the changed conditions, having achieved a new homeostasis. Stress is therefore defined as the disruption of homeostasis, and the stress response represents the changes in cell metabolism during acclimation and the restoration of homeostasis. If the new conditions persist long enough, there may also be also lead to changes in the genome (i.e. adaptation) following the initial acclimation to these new conditions. The actual stress responses are varied, complex and still little understood. They will vary between different taxa, the type(s) of stressors, both biotic and abiotic, and the magnitude and duration of the stress. The ability to successfully acclimate will depend on the genetic capacity (genotype) of the cells and the available resources, especially energy, which are required for the acclimation process. Acclimation is not the only option available to algal cells; some may escape stress by sexual reproduction and/or the formation of resistant cysts or spores. However, if the acclimation process requires more resources, especially energy, than are available, cell death will occur

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