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Proteomics in Seaweeds: Ecological Interpretations

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1. Introduction

Macro and micro-algae are fundamental components of coastal benthic ecosystems and are responsible for a large part of the coastal primary production (Lobban & Harrison, 1994). Adverse effects on these groups caused by natural or anthropogenic phenomena, can affect directly or indirectly organisms of higher trophic levels and the integrity of entire ecosystems. In this context, both the ecological and economic importance of many algal species justifies the need to expand our knowledge on the molecular biology of these organisms.

The distribution and abundance of algal species occurring in the marine zone results from the interplay of biotic (i.e. competition and herbivore pressure) and abiotic (i.e. tolerance to extreme and fluctuating environments) factors (Abe et al., 2001; Burritt et al., 2002; Davison & Pearson, 1996; Pinto et al., 2003; van Tamelen, 1996). For example, the distribution of macroalgal species at the upper limit of the rocky intertidal zone is principally determined by abiotic factors such as UV radiation, light, salinity, temperature changes, nutrient availability and desiccation (e.g. Aguilera et al., 2002; Burritt et al., 2002; Cabello-Pasini et al., 2000; Contreras-Porcia et al., 2011a; Véliz et al., 2006). On the other hand, the microalgae diversity is maintained by a combination of variable forces - environmental oscillations (e.g. habitat instability), more severe disturbances and recovery from catastrophic forcing - backed by the powerful dispersive mobility of this group (Reynolds, 2006). The richness, relative abundance and occasional dominances of the phytoplankton in successive years, depends on water movements, thermal stress and carbon fluxes, but mainly on nutrient enrichment of the sea (Hodgkiss & Lu, 2004; Holm-Hansen et al., 2004; Reynolds, 2006; Wang et al., 2006; Zurek & Bucka, 2004).

Superimposed on the natural abiotic oscillations, algae are also exposed to various other sources of stress, particularly those resulting from human industrial, urban and agricultural activities. Among these is copper mining, whose wastes have reportedly caused severe and negative effects on the coasts of England (Bryan & Langston, 1992), Canada (Grout & Levings, 2001; Marsden & DeWreede, 2000), Australia (Stauber et al., 2001) and Chile (Correa et al., 1999). Although copper is a micronutrient for plants and animals, occurring naturally in coastal seawater at levels at or below 1 μg L^{-1} (Apte & Day, 1998; Batley, 1995; Sunda, 1989), at higher concentrations it becomes highly toxic. The phenomenon of toxicity

in algae is strongly influenced by the speciation of this metal (Gledhill et al., 1997), and within the cell it likely operates through the Haber-Weiss reaction, characterized by a heavy metal-catalyzed production of hydroxyl radicals from hydrogen peroxide (Baker & Orlandi, 1995). For example, in northern Chile, mine wastes originated at a copper mine pit are disposed of directly into the sea. The rocky intertidal zone along the impacted coasts shows a severe reduction in species richness, and the macroalgal assemblage is reduced to the opportunistic algae *Ulva compressa* (Plantae, Chlorophyta) and *Scytosiphon lomentaria* (Chromista, Ochrophyta) (Medina et al., 2005). This negative effect on the biota has been widely recognized as the result of the persistent high levels of copper in the water, by far the most important metal brought into the system by mine wastes (Medina et al., 2005). Many macroalgae species are absent, such as *Lessonia nigrescens* complex (Chromista, Ochrophyta), which are key components in structuring the intertidal zone (Ojeda & Santelices, 1984). As for microalgae, an example is a mine effluent that contained high levels of copper, which was disposed in a reservoir named Venda Nova in northern Portugal. There, a phytoplankton survey was carried out between the years 1981-1982. A shift in the dominant species was demonstrated when compared with an uncontaminated area, Alto Rabagão. More than 50% of the algal species developed lower populations. Also, at the most polluted zone, phytoplankton density, biomass and richness were strongly reduced (Oliveira, 1985).

In macro and micro-algae it is possible to determine that under natural abiotic factors, a common cellular response could involve the over-production of reactive oxygen species (ROS) (Andrade et al., 2006; Contreras et al., 2005, 2007b, 2009; Contreras-Porcia et al., 2011a; Kumar et al., 2010; Lee & Shin, 2003; Liu et al., 2007; Rijstenbil, 2001). ROS are ubiquitous by-products of oxidative metabolism that are also involved in intracellular signalling processes (e g. Blokhina & Fagerstedt, 2010; Rhee, 2006). ROS are produced directly by the excitation of O_2 and the subsequent formation of singlet oxygen, or by the transfer of one, two or three electrons to O_2. This results in the formation of superoxide radicals, hydrogen peroxide or hydroxyl radicals, respectively (Baker & Orlandi, 1995). Oxidative damage to cellular constituents such as DNA/RNA, proteins and lipids may occur (e g. Contreras et al., 2009; Vranová et al., 2002) when ROS levels increase above the physiological tolerance range. However, a coordinated attenuation system can be activated in order to eliminate this ROS over-production, and therefore, the oxidative stress condition (e. g. Burritt et al., 2002; Ratkevicius et al., 2003; Rijstenbil, 2001). For example, in the coastal zones of northern Chile it has been demonstrated that the high copper levels in the seawater generate in sensitive species a high oxidative stress condition, which appears as the starting point for a series of molecular defense responses. In first place, the condition of oxidative stress has been demonstrated by the direct production of ROS and oxidized lipid in individuals living at an impacted site as well as in those transplanted from control sites to the impacted site (Contreras et al., 2005; Ratckevicius et al., 2003). Compared with high tolerant species such as *Ulva* and *Scytosiphon,* in low tolerant species such as *L. nigrescens* the ROS production by copper, specifically superoxide anions, is poorly attenuated, which is reflected in i) higher levels of oxidized lipids, ii) the generation of cellular alterations and iii) negative effects on early developmental stages of the life cycle (Andrade et al., 2006; Contreras et al., 2007a; 2009). Thus, ecophysiological differences are evident between diverse algal species. This is also true for microalgal species since there are species-specific responses to oxidative stress caused by high levels of copper. For example, it was demonstrated that 4 species of

phytoplankton under high concentrations of copper only grew up to 80-95% of that observed in the control condition (Bilgrami & Kumar, 1997). Furthermore, a study including two microalgae species exposed to copper stress showed significant differences between them. In the high tolerant species, *Scenedesmus vacuolatus,* in comparison to the low tolerant species, *Chlorella kessleri,* the chlorophyll a/chlorophyll b ratio was partially reduced. Likewise, both the antioxidant enzyme activity and protein content were progressively increased (Sabatini et al., 2009).

Another environmental factor that affects the abundance and distribution in macroalgae is desiccation. It is an important stress factor faced by living organisms because, as cells lose water, essential macromolecules are induced to form non-functional aggregates and organelles collapse (Alpert, 2006). Some animals (Clegg, 2005) and plants are well adapted to significant water losses, displaying full physiological recovery during rehydration (Alpert, 2006; Farrant, 2000). Compared to vascular plants or animals, in macroalgae the effects of desiccation on the physiology and the molecular mechanisms involved in its tolerance are poorly understood. For example, in one of the few reports available, the activation of different antioxidant enzymes, such as ascorbate peroxidase (AP) and glutathione reductase (GR) was recorded in the upper intertidal macroalga *Stictosiphonia arbuscula* (Plantae, Rhodophyta) (Burritt et al., 2002) as a response to desiccation-mediated oxidative stress. The remaining studies have focused on assessing the capacity to tolerate desiccation displayed by measuring the photosynthetic apparatus activity in *Porphyra*, *Gracilaria*, *Chondrus*, and *Ulva* species among others (Abe et al., 2001; Ji & Tanaka, 2002; Smith et al., 1986; Zou & Gao, 2002). Presently, the only study using molecular approaches to unravel the desiccation tolerance responses, found that genes encoding for photosynthetic and ribosomal proteins are up-regulated in *Fucus vesiculosus* (Chromista, Ochrophyta) (Pearson et al., 2001, 2010). Additionally, independent studies have shown that diverse physiological parameters are altered by desiccation including the lipid and protein levels (Abe et al., 2001), photosynthetic alterations (*Fv/Fm*) as well as cellular morphology and ontogenetic changes (e.g. Contreras-Porcia et al., 2011b; Varela et al., 2006). Moreover, in microalgae it has been shown that salt (i.e. changes in water osmolarity) and temperature stress can be highly stressful and may finally trigger a programmed cell death (PCD) (Kobayashi et al., 1997; Lesser, 1997; Takagi et al., 2006; Zuppini et al., 2010). In these species the effects of both types of stress have been widely studied, and have been reported to provoke photosynthetic alterations, ROS production and ultimately cell death (Liu et al. 2007; Lesser, 1996; Mishra & Jha, 2011; Vega et al., 2006).

Recently, the red species *Porphyra columbina* Montagne (Plantae, Rhodophyta) was recognised among the macroalgae that are highly tolerant to natural desiccation stress. *P. columbina* is highly seasonal and grows abundantly along the upper intertidal zone (Hoffmann & Santelices, 1997; Santelices, 1989). This alga is well adapted to the extreme fluctuating regimes of water/air exposure, as demonstrated by the formation of sporophytic thalli from monoecious fronds (*n*) during long daily periods of desiccation stress due to its position in the intertidal zone (Contreras-Porcia et al., 2012). Additionally, desiccation in *P. columbina* induces morphological and cellular alterations accompanied by a loss of ca. 96 % of the water content (Contreras-Porcia et al., 2011b). Specifically, under natural desiccation stress, the production of ROS (i.e. H_2O_2 and O_2) in *P. columbina* is significantly induced (Contreras-Porcia et al., 2011b). However, during the high tide, ROS quickly returned to basal levels because *P. columbina* displays an efficient antioxidant system. In addition, at

biomolecular level, only a low production of oxidized proteins is recorded during desiccation, due to the efficient antioxidant system of this alga.

The results mentioned above, indicate that desiccation in *P. columbina* causes an over-production of ROS, which is efficiently attenuated. Morphological and photosynthetic changes could be operating as tolerance mechanisms, due to the fact that these responses principally prevent biomolecular alterations, protein aggregation and cellular collapse. For example, it has been proposed that cell wall folding is a cellular strategy used to prevent tearing the plasmalemma from the cell wall during desiccation, ensuring cell integrity (Contreras-Porcia et al., 2011b). The activation of antioxidant enzymes and the photoinhibition of the photosynthetic apparatus help to explain the attenuation of ROS. Thus, ROS excess is buffered by the activation of several physiological and biochemical responses, which suggest a mechanism allowing this plant to tolerate desiccation (Contreras-Porcia et al., 2011b). The ecophysiological responses in this species help, in part, to account for its position and dominance at the highest level in the intertidal zone, and thereby, suggesting desiccation stress tolerance as a determinant trait for explaining that situation. In fact, our recent results demonstrate that the magnitude of the effects generated by desiccation in algae is related to the position of the species in the intertidal zone. Additionally, this work demonstrated the exceptional metabolism of *P. columbina* used to buffer this stress condition. Thus, the determinations of novel metabolic pathways are necessaries in order to fully understand the high desiccation tolerance in this species, for example at the proteomic level. In fact, in this time our forces are concentrated in resolving the proteomic profile of this species under natural hydration and desiccation stress.

Finally, the need to unravel the mechanisms associated with tolerance to different environmental factors by algal species opens the electrophoretic and proteomic approximations as important tools in comprehending and explaining the observed tolerances. However, little information regarding electrophoretic and proteomic analysis is available in algal species. Compared with other group of organisms (e.g. vascular plant or animals) protein extraction in macroalgae has been extraordinary difficult, due principally to the limited knowledge at biochemical and molecular levels. In this context, the present chapter aims to understand the different proteomic approaches utilized in this group of organisms in order to comprehend their ecophysiological behaviour.

2. Proteomic methodology in micro and macroalgae

Sample preparation, in particular the quality of protein extraction, is critical to the successful resolution of 2-DE patterns. In fact, when protein extraction protocols from higher plants are applied to algae, the 2-DE resolution is reduced (Contreras et al., 2008; Hippler et al., 2011). Due to the large variation in cellular biochemical composition among diverse organisms, which affects solubility and recovery of a complex mixture from the sample, there are no 2-DE sample preparation protocols accurate for all organisms. In macro and microalgae the protein extraction protocol must be optimized, due to the high concentration of photosynthetic pigments that are known to interfere with the resolution of the 2-DE gels (e.g. Contreras et al., 2008; Wang et al., 2003; Wong et al., 2006). Particularly, in macroalgae protein extraction is difficult due to a low concentration and the co-extraction of contaminants such as anionic polysaccharides, polyphenols and salts, which are highly concentrated in the tissue (Chinnasamy & Rampitsch, 2006; Cremer & Van de Walle, 1985;

Flengsrub & Kobro, 1989; Mechin et al., 2003). These contaminants pose a significant difficulty for 2-DE, as they cause horizontal and vertical streaking, smearing and a reduction in the number of distinctly resolved protein spots. Thus, the selection of the most appropriate protein extraction method is necessary in order to obtain high quality extracts, and therefore, a high quality 2-DE pattern. For a better understanding and explanation of the current techniques and methodology in algae proteomic, this chapter has been divided in two sections: microalgae and macroalgae methodology.

2.1 Microalgae methodology

It is important to highlight that due to the small size of microalgae, all of the protein extraction protocols for these organisms begin with a centrifugation step in order to pellet cells. This helps to concentrate cells, and consequently allows a correct extraction of the desired proteins.

2.1.1 Early proteomic studies

One of the first proteomics studies on microalgae dates from the year 1972, in which Mets & Bogorad showed alterations in the chloroplast ribosomes proteins of erythromycin-resistant mutants of *Chlamydomonas reinhardtii* (Plantae, Chlorophyta) compared to the wild-type. The ribosomal protein extraction performed on this work was the LiCl-urea method described by Leboy et al. (1971) that was developed for *Escherichia coli* (as cited in Mets & Bogorad, 1972). Thus, the Mets & Bogorad work was a precursor to microalgae proteomic studies. Here, ribosomes are disrupted and freed of RNA by adding LiCl. Then, the samples are centrifuged to precipitate total RNA and the supernatant, which contains the proteins, is retained.

Several studies in the same decade also focused their attention on characterizing ribosomal proteins (e.g. Götz & Arnold, 1980; Hanson et al., 1974). The Hanson et al. (1974) work based their protocols on the Mets & Bogorad (1972) research paper and also used *C. reinhardtii* as model species. Instead, in 1980 Götz & Arnold used a different ribosomal protein extraction after testing several protocols. The procedure chosen was the acetic-acid method in presence of $MgCl_2$ according to Kaltschmidt & Wittmann (1972), method that was also first developed for *E. coli*. In this method, $MgCl_2$ and glacial acetic acid are added to the ribosome suspension, and then the mixture is centrifuged to pellet RNA. For better mixture cleaning, the pellet can be extracted a second time in the same way.

Not all studies from this decade focused their attention on ribosomal proteins as was the case of the work of Piperno et al. (1977), in which the protein mixture came from *Chlamydomona* flagella and axonemes. It is important to highlight this research since the extraction method used was very rustic. After the flagella and axoneme separation, the proteins were dissolved only in SDS and kept for 2-DE analysis.

2.1.2 Current proteomic studies

Recent studies evaluate more complex protein mixtures, so the method chosen must be more accurate in extracting proteins with minimum contaminants and interferents. In fact, a work in *C. reinhardtii* that performed an analysis of all the thylakoid membranes proteins used a more complex protocol (Hippler et al., 2001) than the ones previously discussed in

this chapter. This method uses methanol in order to precipitate cell debris and retains proteins in the supernatant. Then, chloroform is added and the sample is vortexed and centrifuged. The upper phase containing DNA is discarded. Afterwards, methanol is added to the sample in order to pellet proteins and leave the RNA in the aqueous phase. Finally, the pellet is washed with methanol in order to remove contaminants.

In 2003, a study tested different protein extraction protocols in the microalga *Haematococcus pluvialis* (Plantae, Chlorophyta) in order to determine which ones yielded better results (Wang et al., 2003). After cell disruption, the samples were dialysed to remove any salt left in the samples, which are known to interfere in the IEF step. After the dyalisis, each sample was treated in three different ways: i) proteins were left to precipitate in a non-denaturing preparation, ii) a mixing of dialysate with acetone was kept at -20 °C o/n to allow complete precipitation and iii) a mixing of dialysate with TCA in acetone containing β-mercaptoethanol also kept at -20°C o/n. Methods ii) and iii) were denaturing procedures but it was procedure iii) the one that yielded 2-DE gels with higher resolution (detailed protocol in Appendix A).

The work by Kim et al. (2005) is interesting since the protein extraction protocol used is relatively simple when compared to others (e.g. Wang et al., 2003; Contreras et al., 2008) (detailed protocol in Appendix A). Proteins of *Nannochloropsis oculata* (Chromista, Ochrophyta) are obtained in very short time compared to the other microalgae protocols, however, not with the same quality as the more complex protocols. In another *C. reinhardtii* work, but this time conducting a whole cell proteomic study (Förster et al., 2006), a protocol described by Mathesius et al. (2001) that is suited for root proteins was used (detailed protocol in Appendix A). This procedure is denaturing and relatively simple, but includes washing steps that help to improve the quality of the protein extracts compared to the one used on *N. oculata* (Kim et al., 2005). A work from 2009 in the microalga *Haematococcus lacustris* also had a denaturing protocol in which pelleted cells were grounded to a fine powder in liquid nitrogen (Tran et al., 2009). Then, they are disrupted with a lysis buffer containing urea, thiourea, DTT, CHAPS, Tris-base and a plant protease inhibitor cocktail tablet. Samples are centrifuged to separate cell debris, and then the pellet is resuspended in acetone to precipitate proteins and remove contaminants. Finally, the samples are again centrifuged, acetone is removed by air-drying and pellet is clean and ready for 2-DE gels.

Chlamydomonas reinhardtii is one of the most studied microalgae worldwide and as noted in this chapter, proteomics studies are no exception. Another protocol for this algae dates from 2011, in this one the cells are disrupted with a lysis buffer containing urea, CHAPS and thiourea (Mahong et al., 2012). The sample is centrifuged and the supernatant retained. To eliminate possible photosynthetic pigments and other hydrophobic compounds, the samples are washed with ice-cold acetone. Then, each sample is centrifuged, and the pellet is ready for electrophoretic processes.

2.1.3 Gel loading: From proteins to gels

Another key step in obtaining 2-DE gels is gel loading and gel running. After protein extraction, the pellet must be resuspended in a rehydration buffer, which is generally the same in all works. Then, proteins are loaded in order to perform the IEF step for their

correct horizontal migration, however, the protocols varied according both to the biological model and the protein type extracted (i.e. soluble or membrane proteins). Finally, proteins separated in the IEF step are loaded in to the second dimension (SDS-PAGE). Thus, in this section rehydration buffers, IEF steps and second dimension gels will be analyzed.

2.1.3.1 Early proteomic studies

In the work of Mets & Bogorad (1972), ribosomal proteins were only run in the IEF step at 1.5 mA for 4 h but it was enough to separate them due to the low quantity of proteins that were obtained in this extraction. The second dimension was run at 25 mA, enough time to allow the protein migration, since the 2-DE gel patterns are very clear and well resolved. Also, no vertical or horizontal streaking is present, thereby, permitting clear protein detection. It is not astonishing to observe similar 2-DE patterns in quality terms in the work by Hanson et al. (1974), since both the ribosomal protein extraction and the two-dimensional gel electrophoresis were performed essentially as described by Mets & Bogorad (1972). Therefore, no vertical or horizontal streaking was found, resulting in gels with high resolution. Both protein extraction and gel electrophoresis proved to be very efficient and adequate for protein separation. However, it should be emphasized that the patterns from both works are easier to obtain, since the protein mixture is very simple since it only came from ribosome structures.

Unlike the ribosomal protein mixture, others do not generate 2-DE patterns with the same resolution. One case may be flagella and axonemes of *C. reinhardtii* in which a larger number of proteins are founded. Piperno et al. (1977) compared proteins of this structure from both wild-type and paralyzed mutants strains of this species. The IEF step was performed at 300 V for 18-19 h and followed by 400 V for 1.5 h. The second dimension was first run at 25 mA (initial voltage: 60 V) for 1 h and then it was raised to 50 mA. The run continued until the dye in the molecular weight standard had reached the bottom according to Ames and Nikaido (1976) (as cited in Piperno et al., 1977). The 2-DE gels had minimum vertical streaking, but lot of horizontal streaking and big stains regardless of the sample. The horizontal streaking could be due to a more complex protein mixture; however, the protein extraction protocol of this work is very deficient since it only uses SDS. Regardless of this, some spots were easily detected in the gels allowing for comparison between wild-types and mutant strains. Finally, in the work of Götz & Arnold (1980) ribosomal proteins from eight species were evaluated with two gels showing clear and well-resolved 2-DE patterns. The protein extraction was well suited for all species. Therefore, the $MgCl_2$-acetic acid method proved efficient in a large number of species, but again it was used to extract only ribosomal protein, so minimum contaminants are present.

2.1.3.2 Current proteomic studies

In more recent papers, such as those described in the previous section, the rehydration buffer used to resuspend the proteins prior to gel loading is key for the proper migration of proteins. The most commonly used buffer contains the reagents thiourea, urea, CHAPS, DTT, ampholytes and bromophenol blue. However, the concentrations of the reagents vary among the different works, so choosing the most accurate one is no easy task. As an example, we chose the protocol described by Wang et al. (2003) in which several reagents were tested to determine which one that yielded the best 2-DE pattern (i.e. no streaking and more defined spots) (see Appendix A). The majority of researchers state in their works that

after resuspending the proteins, the mixture must be left at room temperature for at least 1 h (e.g. Hippler et al., 2001; Kim et al., 2005; Tran et al., 2009). Likewise, the amount of proteins normally loaded is 500 μg, concentration enough to yield well resolved gels (e.g. Förster et al., 2006; Mahong et al., 2012; Wang et al., 2003).

The IEF profile contains several steps, which vary between the different works, so making comparisons is complicated and not very productive. Nowadays, researchers worldwide use IPG gel strips for a better protein migration, which leads to a better 2-DE pattern (e.g. Mahong et al., 2012; Wang et al., 2003). Having said that, all IPG gel strips must be first rehydrated for at least 10 h before setting the IEF profile. As an example we chose the IEF profile of Wang et al. (2003) which was initiated at 250 V for 15 min, and gradually ramped to 10,000 V over 5 h, and remained at 10,000 V for an additional 6 h.

After the IEF steps and prior to the second dimension, IPG gel strips must be incubated twice in an equilibration buffer containing Tris-HCl, urea, glycerol and SDS. The first time DTT is added to the equilibration buffer in order to denaturate proteins, whereas the second time iodoacetamide is added to alkylate the reduced cysteines and inhibit protein refolding. After equilibration, IPG gel strips are ready to be loaded on to the second dimensional SDS-PAGE for the vertical protein separation (i.e. according to their molecular weight). Gel thickness will vary in each experiment in order to allow the desired protein separation. Regardless of this, gels are run until the bromophenol blue reaches the bottom of the gel since it migrates faster than the proteins. The last step for obtaining the 2-DE gel is gel staining in which two principal stains are used: blue Coomassie and silver nitrate. Regardless of this, generally prior to staining, the gels are washed with deionised water. After staining, the excess of dye is removed with deionised water to obtain well-defined gels with minimum background noise.

Now with the gels stained, we are able to determine which protocol(s) yielded the best 2-DE gel(s) in terms of patterns quality (i.e. minimum or none streaking, spots with defined circles, a maximum spot number and high spot intensity). In the work by Kim et al. (2005) 2-DE gel images show smearing, some vertical streaking and high horizontal streaking specifically in the acidic side of the gel. Also, spots are not well-defined circles and are overlapped among them. Similar were the image gels by Tran et al. (2009), because smearing as well as vertical and horizontal streaking are present in the acidic part of the 2-DE gel. Also several spots were overlapped among them; nevertheless a few of them were well defined. These were the two protocols that yielded the worst results (e.g. poor gel resolution quality) and this must be to the simplicity of the protein extraction protocols used. The two protocols that follow in terms of 2-DE gel quality are those of Hippler et al. (2001) and Förster et al. (2006). In both works 2-DE gels are of high quality, which obviously obey more complex protein extraction protocols. In the oldest work, there are several traits that give this images high quality: i) minimum horizontal streaking, ii) well defined spots (i.e circle shaped), iii) highly stained spots and iv) high number of spots (since only thylakoid membrane proteins were extracted) (Hippler et al., 2001). The high quality of 2-DE gels is probably due to that only a portion of the cell proteins was extracted having less contaminants interfering in both IEF and second dimension. Förster et al. (2006) 2-DE gel images show a high number of spots and most of them are well define with almost no smearing. However, a lot of vertical streaking is observed in the gels, thus the problems must be found in the second dimension since minimum horizontal streaking is present.

Finally, the protocols that yielded the 2-DE images of higher quality were those developed by Wang et al. (2003) and Mahong et al. (2012). In both works, total proteins were extracted from two different microalgae, *H. pluvialis* and *C. reinhardtii* respectively. Highlight that both protocols are the most complex ones among all six analyzed. Gels from both works succeeded in having reduced streaking as well as defined, highly stained and high number of spots. Nevertheless, if one must choose between both, it is Mahong et al. (2012) protocol the one with the best results since gels in this work have minimum background allowing an easier spot detection.

2.2 Macroalgae methodology

The difficulty in obtaining high quality 2-DE gels from macroalgae was first highlighted by Wong et al. (2006), who obtained algal proteins from *Gracilaria changii* (Plantae, Rhodophyta) using four different extraction methods: 1) direct precipitation by trichloroacetic acid/acetone, 2) direct lysis using urea buffer, 3) tris buffer and 4) phenol/chloroform. However, only methods 3) and 4) were compared for their suitability to generate *G. changii* proteins for two-dimensional gel electrophoresis. It was stated in this work that the phenol/chloroform method (detailed protocol in Appendix B) was Ideal for obtaining well resolved 2-DE patterns. Nevertheless, the quality of the 2-DE profiles was poor due to the presence of high amounts of interfering substances accompanied by low protein yield and horizontal and vertical streaking along gels regardless the pH gradient. Thus, this method is not fully accurate for this algal species.

As part of an on-going work focused on unravelling the metabolic processes occurring in physiologically stressed brown macroalgae, a new method for protein extraction that minimizes the co-extraction of non-protein compounds using two structurally distinct brown algal species *Scytosiphon gracilis* (Chromista, Ochrophyta) (Contreras et al., 2007b) and *Ectocarpus siliculosu*s (Chromista, Ochrophyta) (Contreras et al., 2008) was developed. In order to do this, several protein extraction methods available in the literature were tested. However, neither of the previous protocols was ideal for obtaining a good quality algal protein extraction, due to high background noise, band distortion, and more importantly, very low protein dissolution. The protocol developed in this work allowed the use of a highly resolving 2-DE protein analyses, providing the opportunity to unravel potentially novel physiological processes unique to this group of marine organisms (see Table 1 and Results section). Specifically, the protocol uses an initial desalting step with Milli Q water – phosphate buffer in order to remove the salt from the algal tissues. Afterwards, the tissue is pulverized using liquid nitrogen and homogenized with sucrose, EDTA and CHAPS. The proteins are extracted using phenol and washed with ammonium acetate. Finally, the quality of the extracted proteins is improved by using the 2-D clean-Up Kit (GE Healthcare).

In another important proteomic work with macroalgae developed by Kim et al. (2008) and published contemporarily with the Contreras et al. (2008) work, using as models the red algae *Bostrychia radicans* and *B. moritziana* (Plantae, Rhodophyta), used a lysis buffer comprised principally by urea and thiourea (detailed protocol in Appendix B). Although these species belong to the same group of red algae like *G. changii*, the simplicity of this method utilized in comparison with the phenol one (Wong et al., 2006) is due to the morphological characteristics of this species (see image in Appendix B).

The work described by Yotsukura et al. (2010) presents a similar protocol to the one described by Contreras et al. (2008). Here, proteins are extracted from the brown alga *Saccharina japonica* (Chromista, Ochrophyta), important kelp described principally on the coastal areas of northern Japan. In this protocol, the protein extraction was improved by using phenol as the principal component in the lysis buffer (detailed protocol in Appendix B). This protocol was also used in *Ecklonia cava* (Chromista, Ochrophyta), other important kelp found on the coast along the Sea of Japan, and also good quality 2-D patterns were obtained (Yotsukura et al., 2012). The use of phenol in the protein extraction described by Contreras et al. (2008) has been recently used in the red alga *Porphyra columbina* in order to identify the proteins that are over-induced during desiccation stress tolerance responses. A highly resolved 2-DE protein was obtained using this method (Fig. 1), with minor modifications (detailed protocol in Appendix B), such as an important rinse of the protein pellet due principally to the over-production of phycocyanin and phycoerythrin. Thus, the phenol protocol developed by Contreras et al. (2008) could be used in macroalgae species from different taxonomic groups.

The first dimension of the 2-DE in the works mentioned above used approximately 200-500 µg of extracted proteins. However, for the isoelectric focusing (IEF) the protocols varied depending on the algal species used. For example, in *Bostrychia radicans* and *B. moritziana*, the voltage was linearly increased from 150-3,500 V during 3 h, followed by a constant 3,500 V, with focusing complete after 96 V. In *Scytosiphon gracilis* and *Ectocarpus siliculosu*s, on the other hand, the strips are actively rehydrated for 15 h in IEF buffer containing the proteins and focused at 20ºC with the following successive steps: a liner increase from 0 to 250 V for 15 min, a gradient phase from 250 V to 10,000 V for 4 h, and the a hold phase at 10,000 V for a total of 60 kVh. Using this protocol, the IEF for *Porphyra columbina* has some modifications, principally in a total operational voltage of 70 kVh.

3. Results and discussion: From gel to molecular/ecological interpretation

Proteomic analyses have proved to be an important molecular approximation that enables comparisons between species and/or cell variants, and understanding of cell function and stress tolerance (e.g. metals, high salinity, high temperatures, among others) (e.g. Contreras et al., 2010; Kim et al., 2005; Ritter et al., 2010). Due to the particularity of the cellular components (e.g. high content of polysaccharides) of this group of organisms, protein extraction has been the principal problem. However, as stated in the previous sections, some protocols have proved capable of producing high quality protein extracts for 2-DE electrophoresis (microalgae: Mahong et al., 2012; Wang et al., 2003 and macroalgae: Contreras et al., 2008). A high quality protein extract will yield high-resolved 2-DE patterns. Therefore, with a suitable protocol the use of a proteomic approximation appears to be of high importance for understanding various physiological responses in this group of organisms. However, it is imperative to highlight that proteomic works in micro and principally in macroalgae, are considerably lower in comparison with vascular plants and animals. Then, our effort in this chapter was concentrated in describing those important works utilizing as model the algal assemblage.

One of the first proteomic studies in microalgae characterized the chloroplastic ribosomal proteins of wild-type and erythromycin-resistant mutants of *Chlamydomonas reinhardtii* (Mets & Bogorad, 1972). In the mutant *ery-M2d* a protein of the 52S subunit was missing when compared to the wild-type. Nevertheless, low intensity proteins spots with the same

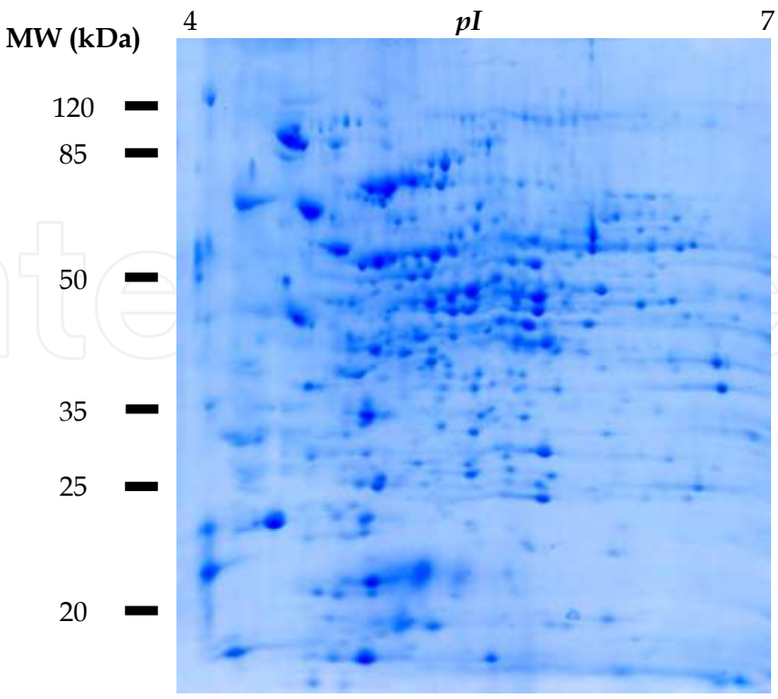

Fig. 1. 2-D proteome of *Porphyra columbina* under natural desiccation. First dimension was performed on a linear gradient IPG strip of pH 4-7 using 600 µg of total proteins. The 12.5% SDS-PAGE gel was stained with colloidal Coomassie blue.

pI but different molecular weight were found, indicating that the *ery-M2* gene is involved in determining the properties of that protein. Hanson et al. (1974) performed 2-DE gels in order to characterize cytoplasmic and chloroplastic ribosomal proteins. Their results showed that the number of proteins in both small and large subunits was higher in cytoplasmic than in chloroplastic ribosomes, indicating that cytoplasmic ribosomes are more complex. Another study did a comparison between the ribosomal proteins of 8 species including *C. reinhardtii* (Götz & Arnold, 1980). The results showed that the number of proteins in both subunits was similar among all species, and that the *Polytoma papillatun* (Plantae, Chlorophyta) proteins were the most similar to those of *C. reinhardtii* in terms of protein homology.

Piperno et al. (1977) analysed the flagella proteins of wild-type and paralyzed mutants of *C. reinhardtii* in order to identify the mutated protein that incapacitates the mobility in mutant strains. In the flagella of *pf* 14, which completely lack radial spokes and associated spokeheads, 12 polypeptides were missing. Also in *pf* 1 flagella, where spokes are clearly present but spokeheads appear to be absent, 6 polypeptides were missing. Then, protein electrophoretic studies confirmed the phenotypical characteristics displayed by both paralyzed mutants, where the missing proteins may be involved in spokes and spokeheads correct morphology. Another work in *C. reinhardtii,* used a proteomic approach to analyse photosynthetic thylakoid membrane proteins isolated from wild-type and mutant strains (Hippler et al., 2001). The two mutant strains were Δ*ycf4* (PSI-deficient) and *crd1* (which is conditionally reduced in PSI and LHCI under copper-deficiency). In this work more than 30 different LHCP spots were identified using a tandem quadrupole mass spectrometer,

Protein	Expression level[a]	nº of peptides analized[b]	Species, nº access[c]	*pI*, Mw (KDa)
Transferase	over	13	*Dechloromonas aromatica* (Q47F82)	5.6, 65
tRNA synthetase	over	24	*Helicobacter pylori* (P56126)	5.9, 60
Phosphomannomutase	over	24	*Schizosaccharomyces pombe* (Q9UTJ2)	5.3, 53
Proteosome, subunit α	over	24	*Oryza sativa* (Q10KF0)	5.6, 53
ATP synthase, subunit α	over	13	*Syntrophus aciditrophicus* (Q2LQZ7)	6.0, 67
Ribulose biphosphate carboxylase large chain	over	22	*Porphyra yezoensis* (Q760T5)	9.6, 65
Glyceraldehyde 3-phosphate dehydrogenase 1	over	22	*Gracilaria verrucosa* (P30724)	6.2, 43
Peptidase/Protease	over	19	*Methanothermobacter* (O27355)	6.3, 42
tRNA binding protein	over	23	*Anaplasma* (Q2GJX4)	6.2, 38
ATP binding protein	over	24	*Methanocaldococcus jannaschi* (Q58049)	6.4, 38
Transcriptional regulator	over	24	*Mesorhizobium loti* (CAD31581.1)	8.7, 29
Carbohydrate kinase	over	35	*Salmonella enterica* (YP_152740.1)	3, 18.2
RNA binding protein	over	28	*Bacillus phage* (P06953)	6.4, 25
ABC transporter subunit	over	40	*Theileria parva* (XP_764551.1)	8, 20.6
RNA polymerase, subunit α	over	19	*Francisella tularensis* (Q5NHU3)	9.2, 17.5
Peroxiredoxin	over	20	*Porphyra purpurea* (P51272)	9.5, 18
Chaperonine	over	10	*Caulobacter crescentus* (P48222)	5.6, 8
ABC transporter subunit	over	45	*Desulfitobacterium hafniense* (ZP_01371968.1)	6.2, 8.2
ABC transporter subunit	over	35	*Janibacter* sp. (ZP_00996449.1)	8.6, 7.3

Table 1. Proteins differentially expressed in *S. gracilis* exposed to copper excess. The analysis by MSMS allowed to obtain various protein peptides which were identified by BLASTP (NCBI). (a) Changes in expression level compared with controls. (b) Number of peptides analyzed by LC/MS/MS. (c) NCBI access number of the species with the highest identity obtained by BLASP.

thereby, permitting proteins with transmembrane domains to be separated with high resolution. Here, the results showed that LHCI spots were present on Δ*ycf4* and absent on *crd1* mutants.

Proteomics approaches have been helpful in understanding tolerance to naturally or anthropologically occurring environmental factors (e.g. high light, thermal stress and heavy metals respectively) in different species. Due to anthropological activities (e.g. industry and mining), heavy metals such as cadmium (Cd) and copper (Cu) are accumulating in the environment (Vermeer & Castilla, 1991; Medina et al., 2005). At high concentrations these metals are a source of abiotic stress, and can be highly toxic to organisms. In this matter, proteomic approaches are of high utility because they may provide new information regarding

mechanisms to cope with the stress induced by the high concentration of metals. For example, in the work developed by Contreras et al. 2010, the copper-tolerance capacity of the brown algae species *Scyotsiphon gracilis* was evaluated by means of the 2-DE approximation. In this work, using the protocol previously described by Contreras et al. 2008 in the Appendix B, 19 over-expressed proteins were identified, including a chloroplast peroxiredoxin, a cytosolic phosphomannomutase, a cytosolic glyceraldehyde-3-phosphate dehydrogenase, 3 ABC transporters, a chaperonine, a subunit of the proteasome and a tRNA synthetase, among others (Table 1). The possible involvement of these over-expressed proteins in buffering oxidative stress and avoiding metal uptake in *S. gracilis* exposed to copper excess is discussed considering this proteomic information. For example, the peroxiredoxine (PRX) is an enzyme involved in the detoxification of hydrogen peroxide and fatty acid hydroperoxides (Dietz et al., 2006). In plants, *prx* transcripts increase in response to different abiotic stresses such as salinity, drought and metals (Dietz, 2003; Wood et al., 2003). Furthermore, PRX in the microalga *C. reinhardtii* and the red macroalga *Porphyra purpurea* (Plantae, Rhodophyta) have shown high similarity with plant PRXs (Baier and Dietz, 1997; Goyer et al., 2002). The expression of PRX in *C. reinhardtii* seems regulated by light, oxygen and redox state (Goyer et al., 2002). Thus, the PRX identified in *S. gracilis* may play an important role in oxidative stress buffering and in lipoperoxides detoxification. In fact, we have recently demonstrated the active participation of this enzyme in copper tolerant species in comparison with sensitive ones, where the over-expression of this enzyme is localized in the cortical cells (Lovazzano et al., personal communication). The proteomic information obtained by Contreras et al. 2010 in *S. gracilis* opens the opportunity of understanding many biological/physiological processes in algae. Using this information and those obtained using a biochemistry approximation, it is possible to strongly suggest a cross-talk between different pathways to re-establish the cellular homeostasis distorted by copper-associated oxidative stress in this species as well as in other tolerant ones (Fig. 2). Thus, the differential ability of each species to deal with oxidative stress resulting from the high copper levels, explains the persistence of tolerant species and the absence of sensitive ones at copper contaminated zones.

Using the method described by Contreras et al. 2008, it was also possible to evaluate differential tolerance in *Ectocarpus siliculosus* strains, originated from habitats with contrasting histories of copper levels (Ritter et al., 2010). Here, the authors showed a differential stress tolerance between 50 and 250 $\mu g\ L^{-1}$ of copper. This difference was also observed at the level of the 2-DE proteome profile. For example, in the tolerant strains from a copper contaminated site (i.e. Chañaral, Chile) a specific expression of PSII Mn-stabilizing protein, fucoxanthine chlorophyll a-c binding protein and vanadium-dependent bromoperoxidase proteins, among others, was observed. Thus, the occurrence of the differential proteome profile among the strains could be strongly suggested by the persistence copper driving force in the evolution of *Ectocarpus siliculosus* from the copper contaminated sites (Ritter et al., 2010). In other brown macroalgae such as *Ecklonia cava*, it was possible to observe the effects of temperature on the proteomic profile (Yotsukura et al., 2012). Here, the authors define that the differential protein expression induced by temperature could be considered as an important biomarker of the health individuals in the culture conditions.

In *Saccharina japonica* it was possible to observe differences at the level of the proteome under seasonal variation and pH conditions (Yotsukura et al., 2010; 2012). Under seasonal

variation, the specific expression of different proteins was identified, among them the vanadium-dependent bromoperoxidase (Yotsukura et al., 2010). Comparatively, under different pH culture conditions the over-expression of several proteins was described such as: glyceraldehyde-3-phosphate dehydrogenase, actin, phosphoglycerate kinase, elongation factor Tu and ATP synthase subunit β, among others. Thus, different metabolic pathways could be induced in brown macroalgae according to the type of stress factor. In this context, the utilization of the 2-DE approximation has been extraordinarily important in unravelling the tolerance mechanisms associated with environmental variables from natural and anthropogenic sources. In fact, the identification of important enzymes, never before described in algae (i.e. Peroxiredoxine (Contreras et al., 2010) and vanadium-dependent bromoperoxidase (Ritter et al., 2010)), opens the opportunity to further understanding the biology of this group of organisms.

In microalgae, several works have also been reported. For example, Wang et al. (2004) studied the proteome changes of *Haematococcus pluvialis* under oxidative stress induced by the addition of acetate and Fe^{2+} and exposure to excess of high light intensity. About 70 proteins were identified in which 19 were up-regulated (e.g. antioxidant enzymes and sugar synthesis proteins) and 13 were down-regulated (e.g. metabolism and cell growth proteins). Also, transient regulated proteins were identified in which 31 were up-regulated (e.g. antioxidant enzymes) and only 8 were down-regulated (e.g. chloroplastic proteins). In 2006, Förster et al. performed a proteome comparison among wild-type and two very high light-resistant mutants (*VHL^R-S4* and *VHL^R-S9*) under different high light stress. About 1500 proteins were detected in the gel and 83 proteins from various metabolic pathways were identified by peptide mass fingerprinting. The results revealed complex alterations in response to the stress, where total proteins varied drastically in the wild-type compared to the mutants. Nevertheless, the mutant *VHL^R-S4* proved to have better adaptation to high light stress since a more controlled protein regulation was conducted (e.g. up-regulation of several chaperonins and down-regulation of energy metabolism proteins). Another work conducted in *H. pluvialis* analyzed the proteome under high irradiance, but combined with nitrogen starvation (Tran et al., 2009). In the gels, about 900 protein spots were detected of which 13 were down-regulated and 36 up-regulated. Among the up-regulated proteins, a glutathione peroxidase and a translocase from the outer mitochondrial membrane were matched to *C. reinhardtii*; therefore, these stress responses may be common among these microalgae. A study assessing a proteomic analysis on *C. reinhardtii* under a short-term exposure to irradiance revealed significant down regulation of several heat-shock proteins (HSPs) (Mahong et al., 2012) under differential times of exposition to this stress (0 h, 1.5 h, 3 h and 6 h of high light). Spot densities allowed the determination that early rearrangement of the light-harvesting antenna proteins occurs, where this was manifested by the up- and down-regulation of several protein spots identified as LHC-II polypeptides. Moreover, increased expression of proteins involved in carbohydrate metabolism was found, which could help accelerate the utilization of electrons generated, in order to minimize the risk of superoxide formation. Surprisingly, after 6 hours of high light several molecular chaperones were down-regulated and this could result in drastic effects on cell structure and function. Nevertheless, *C. reinhardtii* is normally light-sensitive which could be explained by the down-regulation of molecular chaperones.

In microalgae, the response of species to heavy metal contamination has also been evaluated. A proteomic analysis conducted on *N. oculata* showed differences between protein expression

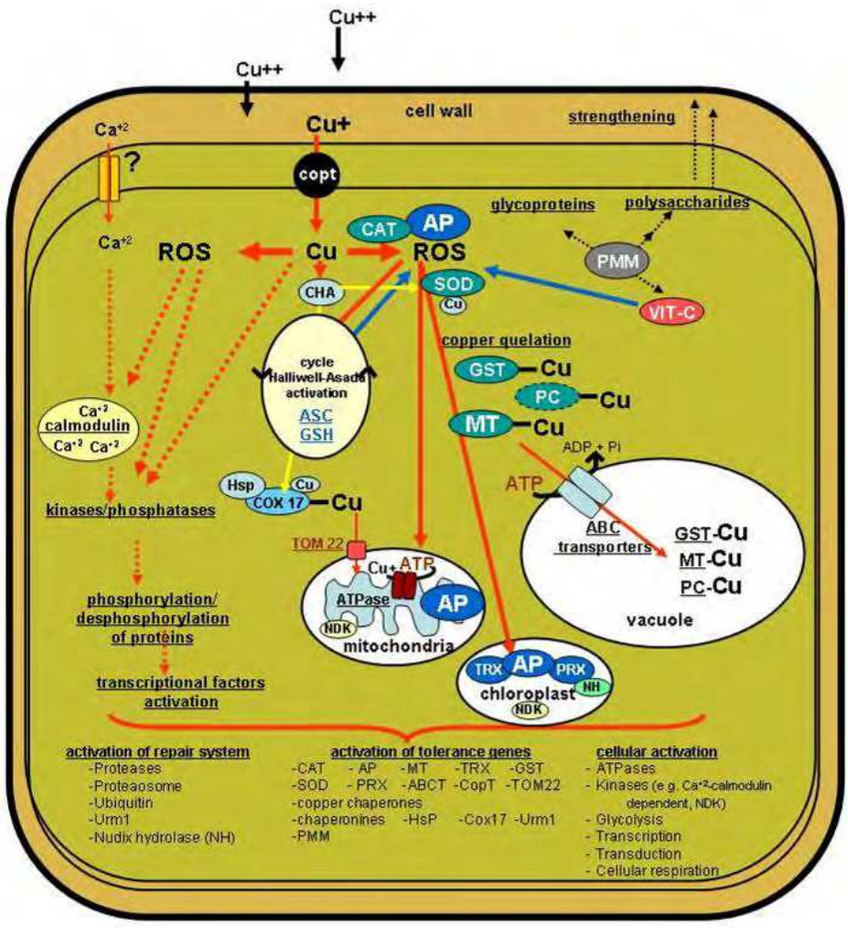

Fig. 2. Cellular events involved in the mechanisms of copper stress tolerance in algae. Dotted arrows indicate routes not directly evidenced in brown algae. The alteration of the state redox, cell damage, and the metal may trigger the antioxidant machine [i.e. compounds and antioxidant enzymes (activation of cycle Halliwell-Asada (MDHAR, DHAR and GP), CAT, SOD, AP, TRX, PRX)] as the activations of protein/genes that form part of various metabolic pathways. Proteins such as HSP or CHA may be involved in the protein protection as in the transport of the metal to proteins that use it as a cofactor, respectively. The sequestration of the metal by different proteins (i.e. MT, GST and PC) is an important homeostatic pathway of tolerance to the metal. The strengthening of the cell wall can increase the resistance to the entry of the metal to the cell. Copts, copper transporter; ROS, reactive oxygen species; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reducatase; GP, gluthathione peroxidase; AP, ascorbate peroxidase; SOD, superoxide dismutase; CAT, catalase; TRX, tioredoxine; PRX, peroxiredoxine; PMM, phosphomannomutase; VIT-C, vitamin C or ascorbic acid; CHA, copper chaperone; GST, glutathione-s-transferase; PC, phytochelatin; MT, metallothionein; ASC, ascorbate; GSH, glutathione; HSP, heat shock protein; TOM 22; cox 17 transporter; Urm1 (modifier protein type ubiquitin); NDK, nucleoside diphosphate kinase.

of treated (10 μM Cd for 4 days) and untreated (control) cells (Kim et al., 2005). The protocol used in this work, as was discussed before, yielded deficient 2-DE gels, resulting in few

proteins detected with only 11 of them with significant changes. Also, the Cd concentration was far from toxic levels suggesting that changes in the protein expression were not needed. This is a non-sequenced species, and therefore, cross-species protein identification was conducted in order to identify those expressed in *N. oculata*. The results showed that malate dehydrogenase and NADH-dehydrogenase were newly induced, whereas glyceraldehyde 3-phosphate dehydrogenase was suppressed. The induction of malate dehydrogenase could be a defense mechanism against Cd toxicity, since at least in *C. reinhardtii* this enzyme controls the malate valve system, which exports reducing power from the chloroplast. Another work assessing Cd toxicity evaluated the proteomic profiles of treated (150 μM Cd) and untreated (control) mutants lacking cell walls of *C. reinhardtii* (Gillet et al., 2006). These mutants are more sensitive to heavy metals due to the lack of a cell wall (Macfie et al., 1994 as cited in Gillet et al., 2006). It was observed that cadmium slowed down the growth rate, and furthermore, induced a 30-50% of growth inhibition. In this work, an elevated number of protein spots were detected and subsequently identified. In fact, 20 proteins were down-regulated in response to Cd stress. Among the down-regulated proteins were those that are involved in amino acid and nitrogen metabolism, chloroplast function and molecule biosynthesis to minimize ROS production. The most variable protein was the RubisCo large subunit, where the protein spot in the control treatment was 15.3 times more intense than in the Cd treatment. It was observed that enzymes with antioxidant properties, chaperonins, and enzymes involved in ATP and carbohydrate metabolism were up-regulated. In addition, in both works chloroplast proteins were found to be down-regulated and proteins involved in antioxidant response to be up-regulated. Therefore, the Cd tolerance mechanism may be similar among different species of microalgae.

4. Conclusions

Micro and macroalgae contain high levels of compounds that interfere with protein extraction. These compounds lead to precipitation of insoluble polymers where the proteome obtainment is almost impossible. However, many efforts have been made in the last years to minimize the coprecipitation of those compounds, and thus now important proteomic protocols are available. For example in macroalgae, it is highlighted the use of phenol during the protein extraction, resulting in consistent electrophoresis runs in several species, conciliating suitable quality and reliability for 2-DE gels and its downstream analysis. The advantage of using phenol as an extracting agent resides in its capacity to disrupt membranes, leaving most of the water-soluble molecules totality in the aqueous phase.

Compared to animals and vascular plants, there is limited information about the use of 2-DE in either micro or macroalgae, both at technical and proteomic level. In fact, low number of published information in the proteomic context can be registered in micro and macroalgae. For example, in this group of organisms only about 42 works can be founded in the www.ncbi.nlm.nih.gov data base, using as search the concepts proteomic or proteome. On the other hand, in vascular plants it is possible to find about 3,100 works in the same data base and ca. 13,100 in animals. Thus, insignificant information exists nowadays in the proteome involvement in algal species, independent of the taxonomic status, ecological importance and economic value of this group of organisms.

2-DE in algal species has allowed the identification of several pathways involved in tolerance mechanisms, associated principally to different abiotic factors. For example,

under copper stress the identification of proteins such as peroxiredoxine, enzyme involved in the detoxification of hydrogen peroxide and fatty acid hydroperoxide has Allowed to understand the differential degree of tolerance between Copper tolerant and sensitive species. In fact, using the proteomic protocol described in these species, which uses phenol in the protein extraction, a differential proteome profile in algal individuals between desiccation stress and normal hydration was founded. In this context, new tolerance mechanisms will be revealed using this approximation in order to understand the high desiccation tolerance that exists in this species in comparison with many others, including that from the same phylum. Thus, 2-DE approximation is an important tool that can be interconnected with those obtained to ecological level in order to understand mechanisms of stress tolerance, and therefore explanation of distribution patterns at local and latitudinal scale.

5. Appendix A: Microalgae methodology

Chlamydomonas reinhardtii **(Plantae, Chlorophyta)** (Förster et al., 2006). The figure correspond to the species mentioned.

This protocol is an adaption of the one described by Mathesius and co-workers (Grotewold, 2003) that are suited for plant material.

1. Collect *Chlamydomonas* cells by centrifugation at 5,000 x *g* for 5 min at 20° C. Determine fresh weight of cell pellets. Samples can be stored at -80°C for later use.
2. Grind pelleted *Chlamydomonas* cells to a fine powder in liquid nitrogen using a mortar and pestle after addition of 0.5 g of glass powder per 1 g fresh weight of pelleted cells.
3. Suspend the ground material in -20°C cold acetone containing 10% w/v TCA and 0.007% w/v DTT. Sonicate this suspension on ice six times for 10 s each with intermittent 1-min breaks using an ultrasonicator. Centrifuge samples at 35,000 x *g* for 15 min at 4°C.
4. Wash the pellet twice by resuspension in -20°C acetone containing 0.07% w/v DTT, placing it at -20°C for 30 min and centrifuging at 12,000 x *g* for 15 min at 4°C.
5. Lyophilize the pellet for 3 min and resuspend in sample buffer containing 9 M urea, 4% w/v CHAPS, 1% w/v DTT, 0.8% v/v ampholytes (ones suited for the desired 2D-gel), 25 mM Tris base, 1 mM PMSF and 5 mM EDTA.
6. Sonicate samples twice in a sonic bath in an ice-water mixture for 5 min and centrifuge them at 19,000 x *g* for 15 min at 20°C.
7. Determine protein concentration of the sample (e.g. with a Bradford assay or a BCA assay) and keep at –80°C until used for isoelectric focusing.

Haematococcus pluvialis **(Plantae, Chlorophyta)** (Wang et al., 2003). The figure correspond to the species mentioned.

In this study a number of key chemical reagents were evaluated, the protocol that yielded the best 2-DE results is detailed below.

1. Collect *H. pluvialis* cells by centrifugation at 3,000 x *g* for 5 min and wash the pellet three times with cold deionized water.
2. Resuspend cell pellet in one volume of 50 mM Tris-HCl buffer pH 8.0, 3 mM DTT, 5 mM MgCl2, 10% glycerol, 0.5% PVP, 5 mM Na_2-EDTA, 1 mM PMSF, 5 mM benzamidin, 5 mM acoproic acid and 1% v/v plant protease inhibitor cocktail.
3. Disrupt cells by one passage through a pre-cooled French Press Cell at a pressure of 20,000 psi. Centrifuge cell lysate at 3,000 *g* for 10 min to pellet cell debris.
4. Collect the supernatant and centrifuge at 100,000 *g* for 1 h.
5. Dialyze the supernatant from the previous centrifugation step against 250 mL of 85% w/v sucrose solution at 4°C for 2 h. Precipitate the dialysate with 9 volumes of ice-cold 10% w/v TCA in acetone containing 0.07% w/v β-mercaptoethanol at -20°C overnight.
6. Centrifuge samples at 15,000 x *g*. Discard supernatant and wash the pellet with acetone containing 0.07% w/v β-mercaptoethanol to remove TCA.
7. Then, remove residual acetone by air-drying.
8. Resuspend pellet in solubilization buffer containing 2 M thiourea, 8 M urea, 4% CHAPS, 2 mM TBP, and 0.2% ampholytes (ones suited for the desired 2-DE gel).
9. Determine protein concentration of the sample (e.g. with a Bradford assay or a BCA assay) and keep at –80°C until used for isoelectric focusing.

Nannochloropsis oculata **(Chromista, Ochrophyta)** (Kim et al., 2005). The figure correspond to the species mentioned.

In this study only one method for protein extraction was performed, the details are shown below.

1. Collect *N. oculata* cells by centrifugation at 12,000 x *g* for 10 min. Suspend cell pellet in PBS buffer pH 7.2.

2. Mix suspension with the same volume of sample buffer containing 0.3% w/v SDS, 1% w/v β-mercaptoethanol and 0.05 M Tris-HCl pH 8.0.
3. Denature solution at 100°C for 3 min, cool on ice and treat with DNase/RNase. Precipitate proteins with 10% TCA in 100% Acetone at -70°C for 3 h.
4. Wash the pellet with 100% acetone several times and then air-dry it at room temperature for 5 min.
5. Determine protein concentration of the sample (e.g. with a Bradford assay or a BCA assay) and keep at –80°C until used for isoelectric focusing.

6. Appendix B: Macroalgae methodology

Gracilaria changii **(Plantae, Rhodophyta)** (Wong et al., 2006). The figure correspond to the species mentioned.

In this study two protein extraction methods were analysed in two-dimensional gels. The best results were yielded by the phenol/chloroform method, which is detailed below.

1. Grind frozen seaweeds at -70°C into a fine powder with a mortar and pestle in liquid nitrogen. Put approximately 100 mg of the resulting powder into a 1.5 mL tube for a single extraction.
2. Add 1 mL of TRI reagent (containing phenol and guanidine-isothiocyanate) to 100 mg of seaweed powder and homogenize the mixture.
3. Store the homogenate for 5 min at room temperature to clarify phases. Reserve the phenolic phase and add 200 µL of chloroform per 1 mL of TRI reagent.
4. Cover the samples and shake vigorously for 15 seconds. Store the resulting mixture at room temperature for 2-15 min. Centrifuge mixture at 12,000 *g* for 15 min at 4°C.
5. Discard upper aqueous phase containing RNA, and retain interphase and lower red phenol-chloroform phase containing DNA and proteins.
6. Add ethanol to the reserved phases in order to precipitate DNA.
7. Retain phenol/ethanol supernatant and add 3 volumes of acetone to precipitate proteins, mix by inversion for 10-15 sec to obtain a homogenous solution.
8. Store sample for 10 min at room temperature and sediment the protein precipitate at 12,000 *g* for 10 min at 4° C.
9. Discard the phenol/ethanol supernatant and disperse the protein pellet in 0.5 mL of 0.3 M guanidine hydrochloride in 95% ethanol + 2.5% v/v glycerol.
10. Add another 0.5 mL aliquot of the guanidine hydrochloride/ethanol/glycerol solution to the sample and store for 10 min at room temperature. Centrifuge the proteins at 8,000 *g* for 5 min.

11. Discard the wash solution and perform two more washes in 1 mL each of the guanidine Hydrochloride/ethanol/glycerol wash solution.
12. Perform a final wash in 1 mL of ethanol containing 2.5% glycerol v/v. At the end of the 10 min of ethanol wash, centrifuge the proteins at 8,000 *g* for 5 min.
13. Discard the alcohol and air-dry the pellet for 7-10 min at room temperature.
14. Resolubilize protein pellet in 40 mM Tris buffer pH 8.8 containing 8 M urea, 4% CHAPS and 2 mM TBP.
15. Determine protein concentration of the sample (e.g., with a Bradford assay or a BCA assay) and keep at –80°C until used for isoelectric focusing.

Scytosiphon gracilis and *Ectocarpus siliculosus* **(Chromista, Ochrophyta)** (Contreras et al., 2008). The figures correspond to the species mentioned.

In this method, a major extraction of proteins was obtained in comparison with pervious macroalgae methods described.

1. Remove the excess salt by rinsing in Milli Q water and 50 mM Tris-HCl pH 8.8.
2. Freeze seaweed material at -80°C before pulverization.
3. Homogenize seaweed material using a mortar-driven homogenizer in sample lysis solution composed of 1.5% PVP, 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl pH 7.5, 250 mM EDTA, protease inhibitor cocktail, 2% v/v β-mercaptoethanol and 0.5% w/v CHAPS
4. Equal volume of Tris-HCl pH 7.5-satured phenol is added and the mixture homogenized at 4°C. Then, by centrifugation the upper phase is removed and the lower phase is re-extracted using the same volume of phenol.
5. The proteins in the phenol phase are precipitated by means of ammonium acetate (0.1 M in methanol). The protein pellet obtained by centrifugation is washed in 80% ice-cold acetone and cold acetone containing 20 mM DTT.
6. Determine protein concentration of the sample and keep at –20°C until used for isoelectric focusing.

Bostrychia radicans / B. moritziana **(Plantae, Rhodophyta)** (Kim et al., 2008). The figure correspond to *B. moritziana*.

In this study a very simple method for protein extraction was performed, the details are shown below.

1. Freeze seaweed material at -80°C before pulverization.
2. Homogenize seaweed material using a mortar-driven homogenizer in sample lysis solution composed of 7 M urea, 2 M thiourea, containing 4% w/v CHAPS, 1% w/v DTT, 2% v/v ampholytes and 1 mM benzamidine.
3. Perform freezing and thawing steps for five times for 1 day*.
4. Extract proteins for 1 h at room temperature with vortexing.
5. Centrifuge mixture at 15,000 *g* for 1 h at 18°C.
6. Retain soluble fraction and discard insoluble material.
7. Determine protein concentration of the sample (e.g., with a Bradford assay or a BCA assay) and keep at –80°C until used for isoelectric focusing.

* If cell lysing is found to be difficult, used a bead beater in order to facilitate the process.

Saccharina japonica and *Ecklonia cava* **(Chromista, Ochrophyta)** (Yotsukura et al., 2010; 2012). The figures correspond to the species mentioned.

1. Remove the salt excess in Milli Q water.
2. Freeze seaweed material at -80°C before pulverization. Homogenize the tissue in 99.5% cold ethanol and centrifuged.
3. The protein pellet is rinsed in 99.5% ethanol and 100% acetone and resuspended in 0.1 M Tris-HCl buffer pH 8.0, 30% sucrose, 2% SDS, 5% β-mercaptoethanol and phenol.

4. The solution is vortexed and centrifuged at room temperature and the upper phase collected. Agitate solution in 0.1 M ammonium acetate and kept at -20°C.
5. The protein pellet obtained by centrifugation is rinsed in 0.1 M ammonium acetate in methanol and 80% acetone, subsequently dried in evaporator and preserved at –80°C until protein quantification.

Porphyra columbina **(Plantae, Rhodophyta).** The figure correspond to the species mentioned.

This method is an adaptation of the method performed by Contreras et al. 2008, where the details of it are shown below*.

1. Pulverize 3-5 g of frozen seaweeds at -80°C to a fine powder with a mortar and pestle in liquid nitrogen.

2. Resuspend pulverized tissue in 5-10 mL of buffer lysis containing 0.5 M Tris-HCl pH 7.5, 0.7 M sucrose, 0.5 M KCl, 250 mM EDTA, 1.5% w/v PVP, 0.5% w/v CHAPS and 2% v/v β-mercaptoethanol and homogenize for 15 min at 4°C.
3. Add an equal volume of Tris–HCl pH 7.5-saturated phenol and homogenize for 15 min at 4° C. Centrifuge the homogenate at 2,000 *g* for 30 min.
4. Retain only upper phenol phase containing proteins being careful not to remove the interphase.
5. Add ½ volumes of Tris–HCl pH 7.5-saturated phenol and mix well by inversion.
6. Centrifuge the homogenate at 2,000 *g* for 20 min.
7. Retain newly only upper phenol phase containing proteins being careful not to remove the interphase and mix with the previously retained upper phase.
8. Add 5 volumes of 0.1 M ammonium acetate on methanol ice-cold.
9. Shake vigorously to mix the solution and leave to precipitate for 4 h at -20°C.
10. Centrifuge at 2,000 *g* for 40 min. Discard supernatant and wash pellet with 8 volumes of 0.1 M ammonium acetate on methanol ice-cold.
11. Shake vigorously to mix the solution and leave to precipitate for 30 min at -20°C.
12. Centrifuge at 2,000 *g* for 30 min.
13. The proteins pellet is washed in 80% ice-cold acetone and cold acetone containing 20 mM DTT.
14. The protein pellet is then washed in ice-cold acetone 80% and ice-acetone 60% in methanol to remove the majority of contaminants.
15. Determine protein concentration of the sample and keep at -20°C until used for isoelectric focusing.

* Method not yet published.

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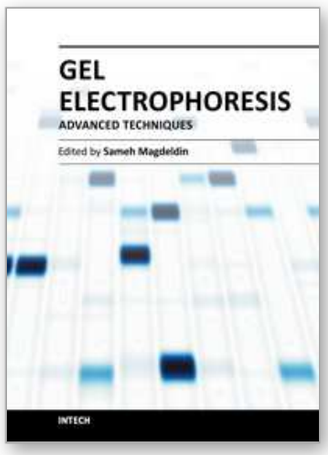

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As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

How to reference

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