Phosphorylation of MAP Kinases crucially controls the response to environmental stress in *Dunaliella viridis*

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Abstract

The green unicellular microalga *Dunaliella viridis* has the ability to cope with a wide variety of environmental stressful conditions, such as thermal and osmotic shocks, high PAR, UV radiation and nitrogen deficiency. The lack of a rigid cell wall makes *D. viridis* an excellent model organism to study stress signaling in eukaryotic unicellular organisms. Mitogen-activated protein kinases (MAPKs) are highly conserved serine/threonine kinases that convert extracellular stimuli into a wide range of responses at both cellular and nuclear levels. In eukaryotic cells, MAPKs are involved in both cell proliferation and differentiation (ERK pathway) and stress responses (JNK and p38 pathways), through protein kinase cascades. Significantly lesser phosphorylation levels of ERK-like protein were observed in *D. viridis* cultures acclimated to high salinity (3-4M NaCl). In contrast, JNK-like and p38-like proteins phosphorylation levels increased in stressed cells. Likewise, the efficacy of specific commercial inhibitors of the phosphorylation of ERK (PD98059), JNK (SP600125) and p38 (SB203580) revealed the importance of JNK-like proteins in the maintenance of cell viability, the highlighted participation of p38-like proteins and the non-direct implication of the ERK-like proteins in the acclimatization process. In summary, specific blockade of JNK- and p38-like cascades in stressed cells led to rapid cell death. The behavior of MAPK-like proteins in algae is not known in depth, so the analysis of their mechanism of action, as well as their function in this model microalga, will allow to estimate the fate of unicellular eukaryotic organisms in aquatic ecosystems subjected to environmental stress derived from the conditions prevailing within a framework of global climate change.

Keywords: *Dunaliella*, MAP Kinases, Environmental stress, Hypersalinity, UV radiation, High irradiance, Acclimatization, Climate change

Introduction

All organisms have to acclimate to their environment in order to survive. The response to a changing environment requires that organisms must be able to perceive the stimuli and external cellular signals and, consequently, to develop appropriate cellular responses that enable them to survive against environmental changes. Microalgae, as well as vascular plants, are permanently exposed to changes in environmental conditions. To survive, they have developed a complex network of biochemical signals that allow them to perceive environmental changes and respond to them. This process usually leads to a response within the cell that allows it either to acclimate to the new conditions of the environment or to start a process that will cause cell death. In general, external information is transmitted to the cell nucleus through a series of phosphorylation and de-phosphorylation reactions of certain proteins (kinases), which induce the activation or deactivation of specific genes (Kyriakis & Avruch, 2001).

Increasing evidences show that mitogen-activated protein kinases (MAPKs) occupy the central core of the network of phosphorylations and de-phosphorylations that takes place when plants have to deal with a situation of stress. MAPKs are a group of highly conserved serine/threonine kinases, ubiquitous in all eukaryotic cells. They have been widely studied in organisms from yeast to humans as transducers of extracellular signals into a variety of cytoplasmic and nuclear responses (Widman et al., 1999). The MAPKs, together with their activators (the so-called MAPK kinases - MAPKK and MAPK kinases kinases - MAPKKK) form a cascade.

To date, six MAPKs cascades have been identified in mammalian cells, but only for some of them have all the components been completely identified: extracellular signal-regulated kinase (ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38. When phosphorylated, both p38 and JNK trigger the response to stress, while ERK controls cell proliferation and differentiation (Ligterink, 2000). The presence of MAPK-like pathway components in algae has been described previously by our group (Jiménez et al., 2004, 2007). We reported the presence of both JNK- and p38-like MAPKs in the unicellular chlorophyte *Dunaliella viridis* and their involvement in survival of cells under stress, also showing the presence of ERK1/2 in this microalga and its participation in cell division. In addition, the phosphorylation of MAPK-like proteins in response to environmental stress has been shown in macroalgae by Parages

et al., 2012, 2013, 2014. These results, together with others (García-Gómez et al., 2012; Gasulla et al., 2016), indicate that algae possess MAPK-like signaling components that are essential to sense and respond to different stress, allowing cell acclimation and survival.

Exposure to low levels of stress usually generates an acclimatization process in the organisms, resulting in a transient resistance at higher levels of the same stress. This acclimatization to stress can also induce a greater tolerance (cross-protection) to other stresses (Chen et al., 2003, Lurie et al., 1994), which is usually short, requiring *de novo* protein synthesis. Thus, the phenomenon of cross-protection suggests a general response mechanism to stress that may confer a basic level of protection against the action of any factor responsible for the stress. Responses against different environmental stress situations must be given to maintain cell survival and proliferation, and so the characterization of these responses, the mechanisms involved in the detection of stress, the transmitting signals of this information to the cell and the nucleus, and compensatory changes resulting in physiology and gene expression, are essential to understand how cells respond and survive under non-ideal conditions (Chen et al., 2003).

The present study was performed in a model microalga of the genus *Dunaliella,* that thrives in hypersaline aquatic environments and which needs to cope with rapid changes in salinity, radiation and temperature, among other stresses. Halotolerant algae of the genus *Dunaliella* are the most ubiquitous eukaryotic microorganisms in hypersaline environments, and can survive even in saturated salt solutions (≈5.5 M NaCl) (Ben-Amotz & Avron, 1983, 1990). These organisms have to face sudden changes in salinity, irradiance and nutrient availability that usually occur in their natural habitat. The lack of a rigid cell wall permits rapid changes in cell shape and volume in response to osmotic shocks. After a change in the external salinity, *Dunaliella* osmoregulates by varying the intracellular concentration of glycerol (Ben-Amotz & Avron, 1973). Transition to the new osmotic conditions lasts 1–3 h, and during most of this period cell division does not occur (Avron, 1992).

The experiments were designed to explore the possibility that these lower organism respond to sudden changes of the environmental conditions (hypersalinity, high irradiance and UV radiation, all stressful but non-lethal exposures) by activating MAP kinase signaling pathways such as p38 and JNK, while deactivation of ERK would

reduce cell proliferation. Inhibiting these pathways would compromise the ability of this microalga to acclimate to the new prevailing conditions, thus leading to cell death.

Material and methods

Microalgae culture

D. viridis Teodoresco was isolated from the athalassic lake of Fuente de Piedra (Málaga, Spain) and grown in batch culture as previously described (Jiménez et al., 2007), in a basal medium that contained 1 M NaCl, 5 mM KNO_3, 0.25 mM KH_2PO_4, 50 mM $NaHCO_3^-$. Cells were cultivated under continuous orbital shaking and illumination (30 μmol $m^{-2}s^{-1}$), being this illumination provided by commercial cool white fluorescent lamps, at a temperature of 25º C. Cell density was determined by means of counting viable cells using a hemocytometer and a flow cytometer (BD Biosciences, BD Accuri C6 Flow Cytometer, San José, CA, USA).

Experimental conditions

The ability of *D. viridis* to survive at increasing salinities was estimated by growing the cells at 1, 2, 3 and 4 M NaCl for a period of several weeks. The time that each culture took to reach the desired cell density, depending on the salinity in the medium, can be observed in Figure 1.

For short-time experiments, cells in their mid-exponential phase of growth, at a density around $4x10^6$ cell ml^{-1}, were used. To study the acclimatization process under different stress situations, cells were exposed to three types of stress, including: (i) hyperosmotic stress, (ii) high irradiance, and (iii) UV radiation. All the three stressful conditions described below are non-lethal for *D. viridis* under the detailed conditions. Hyperosmotic stress consisted in a sudden increase of the osmotic pressure in the medium by the addition of NaCl, from an initial concentration of 1 M NaCl to a final of 2.5 M NaCl. For high irradiance stress, cultures were exposed to an incident irradiance of 3000 μmol $m^{-2}s^{-1}$ of white light for 35 minutes, using a LED Explosion proof lamp (120 W), and were then kept under the same conditions as described above (30 μmol $m^{-2}s^{-1}$ under orbital shaking). The emission spectrum of the lamp is shown in Figure 2a. After exposure, cells were kept under continuous orbital shaking at an irradiance of 30 μmol $m^{-2}s^{-1}$ of PAR. Samples were withdrawn at the described times in the figures. The

148 influence of UV radiation was studied through the exposure of the cultures of *D. viridis*
149 to a total dose of 480 mJ cm^{-2} (20 W m^{-2}) of UV light in the range 200-400 nm, using
150 two UV lamps (Philips TL 36W/12, Philips Co. Holland, Philips Co. Holland) as
151 previously reported (Jiménez et al., 2004). Spectral irradiance of the UV lamps is shown
152 in Figure 2b. 50 ml of 1M NaCl cultures were placed in Petri dishes of 14 cm in
153 diameter (in triplicate), and after UV exposure they were kept under continuous orbital
154 shaking at an irradiance of 30 μmol $m^{-2}s^{-1}$ of PAR during the next 24 h for recovery.
155 Samples were withdrawn at the described times in the figures.

156

157 *Treatment with inhibitors*

158 Before exposure to stress, aliquots of the microalgae culture were transferred to 50
159 ml conical tubes (in triplicate). Appropriate volumes of very specific inhibitor solutions
160 in dimethilsulphoxide (DMSO) (1000× stock) were added to make the culture medium
161 1 μM in SP600125 (JNK inhibitor), 20 μM in PD98059 (ERK inhibitor) (Calbiochem,
162 La Jolla, CA, USA) or 20 μM in SB203580 (p38 inhibitor) (Caymanchem, Ann Arbor,
163 MI, USA). At these concentrations, these molecules have been largely proved to be very
164 selective inhibitors of the JNK (Bennet et al., 2001), ERK (Jiménez et al., 2007) and
165 p38 (Capasso et al., 2001) pathways, respectively, being selective and cell wall
166 permeable that act by inhibiting the phosphorylation of the MAPKs and the subsequent
167 phosphorylation of downstream substrates. No significant inhibition of phosphorylation
168 of other MAPKs is expected. After 2 h of incubation in darkness with the inhibitor, cells
169 were exposed to the different environmental stress conditions as described above.

170

171 *Western blot analysis*

172 At fixed times, an aliquot of culture was removed and centrifuged at 1500x*g* for 10
173 min. Pellets were then resuspended in 1 ml of MAPK lysis buffer (Capasso et al., 2001)
174 and placed in ice for 1 h, with vortexing every 10 min. Samples were frozen in liquid N_2
175 and kept at -80º C or processed immediately. After thawing, samples were centrifuged
176 at 15,000 *g* for 60 min in an Eppendorf ® Centrifuge 5804/5804R. Supernatants were
177 frozen at -80º C until further analysis. Protein concentration of each supernatant was
178 determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA). Equal
179 amounts of protein were loaded per lane (40 μg/lane) for SDS-PAGE. A certain number

of samples were run in duplicate gels, with one of them stained with Coomassie Blue to confirm uniform protein loading, and the second one used for MAPK immunodetection. SDS-PAGE, electroblot to poly(vinylidene difluoride) membrane (Amersham Hybond-P PVDF membrane; GE Healthcare, Buckinghamshire, UK), and immunodetection were performed as previously described (Capasso et al., 2001; Jiménez et al., 2004). Immunodetection was carried out using antibodies against the phosphorylated forms of mammalian p38, ERK and JNK proteins (Ph-p38 #9211, Ph-JNK #9251, Ph-p44/p42 #9106; Cell Signaling Technology, Beverly, MA, USA). Phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibody detects endogenous levels of p44 and p42 MAP kinase (ERK1 and ERK2), but only when catalytically activated by phosphorylation at Thr202 and Tyr204 of human ERK, or Thr183 and Tyr185 of rat ERK2. This antibody does not cross-react with the corresponding phosphorylated residues of either JNK/SAPK or p38 MAPK. The phospho-p38 antibody detects only the phosphorylated form of this kinase and is specific for the antigen sequence T*GY*, not showing cross-reaction with similar sequences such as TPY (JNK) or TEY (ERK). This phospho-p38 does not react with either the non-phosphorylated or single phosphorylated form of the protein. The phospho-JNK antibody used is specific for the T*PY* sequence and only when double-phosphorylated, not recognizing either the non-phosphorylated or single phosphorylated form of this protein and not cross-reacting with p38 or ERK. This antibody recognizes all isoforms of the JNK protein. Secondary antibody used against the primary antibodies was Anti-rabbit IgG, HRP linked (#7074, Cell Signaling Technology, Beverly, MA, USA). The antibody concentration was 1/1000 (primary antibody) and 1/2000 (secondary antibody), diluted in 10 ml of bovine serum albumin (BSA) with 3% TTBS. Membranes were analysed using a Kodak Gel Logic 1500 Image System, using Amersham ECL Advance (GE Healthcare, Buckinghamshire, UK) as a chemiluminiscence agent following the recommended conditions by the manufacturer. Further band intensity analysis was performed using the ImageJ 1.440 free software (National Institute of Health, USA).

PAM fluorometry

The physiological fitness of *D. viridis* was followed by measuring the photosynthetic efficiency using PAM-fluorometry, with a WATER-PAM (Heinz Walz GmbH, Effeltrich, Germany). Maximum quantum yield of Photosystem II (PSII) fluorescence

(F_v/F_m) was measured in parallel with sampling for the subsequent Western Blot analysis, with the WinControl-3 software, as previously described (Schreiber et al., 1986). F_v/F_m was considered as $(F_m - F_o)/F_m$, where F_v is the maximal variable fluorescence of a dark-adapted sample, F_m is the maximal fluorescence intensity with all PSII reaction centers closed, and F_0 is the basal fluorescence, thus obtaining the parameter F_v/F_m, which represents the maximum quantum yield of the photosystem II when all reaction centers are open after a period of darkness adaptation (Baker and Oxborough, 2004). High F_v/F_m values indicate the good condition of the cells, whereas low values indicate stress and photoinhibition.

Maximum relative electron transport rate ($rETR_{max}$, measured in μmol electrons $m^{-2}s^{-1}$) through PSII was directly given by the Water PAM, and no transformation was carried out.

Statistical data analysis

All experiments were run in triplicate. Data presented here are means ± SD. Results were analysed by using the SigmaPlot 11.0 statistical package (SPSS). A value of $p < 0.05$ was considered significant and is represented in the graphs with a single asterisk "*", while a value of $p \leq 0.001$ was considered very significant and is represented by two asterisks "**".

Results

Long-term acclimation to hypersaline conditions

Before performing the above-detailed experiments for the identification of the role of the three MAPKs in the response to acute stress, photosynthetic performance of *D. viridis* long-term acclimatized to four increasing salinities (1, 2, 3 and 4M NaCl) was assessed by determination of F_v/F_m, and the level of phosphorylation of the three MAPK-like proteins was studied. As already mentioned in Material and Methods, *D. viridis* was cultured for several weeks at 1, 2, 3 and 4M NaCl, and a significant decrease of F_v/F_m values at high salinity was detected; the lowest performance was found at 3 and 4M, increasing at 1 and 2M NaCl. In addition, no significant differences were found between the cultures subjected to 1 and 2M NaCl (Fig. 3a). Even though F_v/F_m

244 was significantly lower at the highest salinity, absolute values of this photosynthetic
245 parameter at 4M NaCl (>0.55) support the very well known capacity of *D. viridis* to
246 thrive at salinities near saturation. This conclusion was supported also by the high
247 values of the maximum relative electron transport rate ($rETR_{max}$) found in all conditions
248 (Fig. 3b). These results indicate that even at salinity as high as 234 g NaCl/l *D. viridis*
249 maintains a high capacity to develop a very efficient photosynthesis. Since F_v/F_m values
250 provided detailed information on the physiological state of the microalga, this parameter
251 was chosen preferably during the rest of the work to estimate its physiological condition
252 when exposed to sudden stresses both in the presence and absence of specific inhibitors
253 of the MAPKs.

254 MAPKs phosphorylation was also studied in cultures that were long-term
255 acclimatized to the salinity range 1-4M NaCl (Fig. 4). No significant differences were
256 found in the phosphorylation levels of either JNK-like or p38-like proteins in cultures
257 exposed to increasing salinities. A basal and similar level of phosphorylation of both
258 MAPKs was always detected in these long-term acclimated conditions, independently
259 of the salinity in which cells were cultured. However, a significant difference in the
260 intensity level of phosphorylation of ERK-like proteins could be observed. The level of
261 phosphorylation of ERK was significantly lower at the highest salinities ($p \leq 0.001$),
262 which is consistent with previously reported data of growth rate of *D. viridis* under
263 different salinities.

264

265 *MAPK-like phosphorylation after hypersaline stress*

266 The role played by the three MAPKs (ERK, JNK and p38) in *D. viridis* cultures
267 exposed to different environmental stress situations has been assessed using very
268 specific commercial inhibitors of each protein, largely proved to specifically block
269 phosphorylation of the detailed MAPK. Thus, the comparison between the experiments
270 performed either in the presence or the absence of inhibitors, permits to estimate the
271 different role played by these MAPKs in the maintenance of cell viability after an
272 environmental stress situation.

273 The response of *D. viridis* photosynthetic performance after hypersaline stress
274 (mediated by the non-lethal addition of NaCl from 1M to 2.5) is shown in Figure 5. As
275 it can be seen, the mean F_v/F_m value obtained in basal conditions, without stress or
276 inhibitor (control treatment), was 0.687. This value decreased when the cultures

277 underwent osmotic shock, reaching its lowest value at t=4 h (F_v/F_m=0.509). From that
278 minimum, F_v/F_m steadily recovered during the next hours attaining values at 24 h
279 similar to those expected at the new osmotic pressure. However, in the presence of the
280 specific inhibitors of the MAPKs cascades, differential responses of F_v/F_m were found.
281 The inhibition of the ERK cascade (see Materials and Methods for details) followed by
282 the addition of NaCl to make 2.5M NaCl did not have any effect on the photosynthetic
283 performance of *D. viridis*, remaining F_v/F_m values very similar to those from the
284 control. These results suggest that ERK-like proteins are not involved in the response to
285 stress. When the cascade of p38-like proteins was inhibited, significant differences
286 could be found when compared to the control. In the first 4 hours after subjecting *D.*
287 *viridis* to hyperosmotic stress in the presence of the p38 cascade inhibitor, the drop of
288 F_v/F_m was not significantly different when compared to the control ($p = 0.073$).
289 However, after this time differences with the control were observed, and F_v/F_m never
290 recovered to values similar to control ($p \leq 0.001$ at 24 h). *D. viridis* seemed to be unable
291 in the mid term to fully recover its cell performance when p38 was inhibited.
292 In addition, pre-incubation with the JNK cascade inhibitor produced the most drastic
293 effect. When exposed to hyperosmotic stress (after incubation with the JNK inhibitor)
294 F_v/F_m values dropped rapidly, from 0.687 to 0.189 within 30 minutes. One hour after
295 stress, F_v/F_m values were around 0.08, dropping to 0 in the successive hours.
296 Microscopic analysis of the cells (not shown) indicated that 100% of the cells were dead
297 since the cell membrane was broken. These results show the crucial dependence of
298 response to hyperosmotic stress in *D. viridis* and phosphorylation of the JNK.

299 Additionally, the phosphorylation level of the three MAPKs was studied by means of
300 immunodetection. Figure 6 shows the time-course of the phosphorylated forms of ERK,
301 p38 and JNK when the cultures of *D. viridis* were exposed to hypersalinity both in the
302 presence and absence of their specific inhibitors. A band of 42 kDa of estimated
303 molecular weight was identified by the specific antibody against the phosphorylated
304 form of the ERK (Fig. 6a), while the specific antibody against the JNK/SAPK cross-
305 reacted with a 45 kDa protein (Fig. 6b). Finally, a phosphorylated protein of 57 kDa
306 was detected by the antibody against the phospho-p38-MAPK (Fig. 6c).

307 Two noticeable facts seem to occur after the increase of the salinity: first, while the
308 phosphorylation level of both p38 and JNK immediately increased after the onset of the
309 stress, phosphorylation of the ERK rapidly decreased; second, the phosphorylation of

all three MAPKs decreased in the presence of the inhibitor. In the case of ERK, the extent of the decrease was more pronounced than in control cells (absence of inhibitor). In all cases, the phosphorylation of the MAPKs remained lowest in the presence of the inhibitor 24 h after the increase of the salinity.

In the controls, an increase on the phosphorylation levels of JNK-like proteins could be seen in the short and medium term after hyperosmotic stress, whereas in the presence of the specific JNK cascade inhibitor, this MAPK was progressively dephosphorylated. An initial presence of phosphorylated JNK-like proteins, even after being incubated with the inhibitor, seems to correspond to an existing basal reserve of JNK, despite the arrest on the phosphorylation of these proteins.

There was also a phosphorylation of p38-like proteins after hyperosmotic shock in control cells, experiencing a slight dephosphorylation within 2 hours after the shock, but maintaining high phosphorylation levels in the medium term. On the other hand, by inhibiting the p38 cascade, this protein remained dephosphorylated, presenting the aforementioned effects on cell viability.

ERK phosphorylation, as expected, highly decreased after the shock, indicating a sudden arrest of cell division. In addition, de-phosphorylation of this MAPK-like protein was more pronounced after stress in the presence of the inhibitor.

MAPK-like phosphorylation after high irradiance stress

The effect of high irradiance was studied exposing *D. viridis* cultures to 3000 µmol $m^{-2}s^{-1}$ of PAR for 35 minutes. By using MAPKs specific inhibitors, as in the previous case, it was possible to estimate the different role played by these three MAPKs in maintaining cell viability after an environmental stress situation such as a high irradiance event.

After the shock, an immediate drop of F_v/F_m occurred in control cells to figures around 0.38, which steadily increased to initial values during the next 24 h (Fig. 7). A similar response was found in the cultures incubated with the ERK inhibitor before the stress. In contrast, cells treated with either the p38 or the JNK inhibitor before the stress, showed a clear decrease of photosynthetic efficiency ($p \leq 0.001$) after the transient exposure to high irradiance. The loss of efficiency was much more drastic and lasting in the presence of the JNK inhibitor. In this case, F_v/F_m values 24 h after exposure to high

342 irradiance were below 0.1, indicating that *D. viridis* was not capable of recovering (not
343 being able to trigger its acclimatization responses) after the high irradiance shock.

344 Protein analysis by means of immunodetection revealed that phosphorylation of the
345 42 kDa band (ERK-like MAPK) was immediately reduced after the stress, and that the
346 extent of this inhibition was much more patent in the presence of the ERK specific
347 inhibitor (Fig. 8a), like in the previous experiment. The response of JNK was, as
348 expected, somehow different. In the control, an initial and fast phosphorylation of JNK-
349 like proteins occurred, remaining at high levels 24 h after the shock, while when
350 inhibiting this MAPK, its phosphorylation levels were significantly lower. Under this
351 stress, two JNK-like proteins of 45 and 43 kDa were identified (Fig. 8b). Finally, p38-
352 like proteins showed a similar behavior to that presented in the hyperosmotic shock. A
353 rapid phosphorylation of p38-like proteins occurred in the control, which remained 24 h
354 after stress (Fig. 8c). In the presence of the inhibitor of p38 phosphorylation, no
355 response to high irradiance stress was detected, and a fast de-phosphorylation of the 57
356 kDa phosphoprotein occurred in the first minutes.

357

358 *MAPK-like phosphorylation after UV radiation stress*

359 *D. viridis* response to stress induced by non-lethal doses of UV radiation was
360 estimated by exposing the cultures to 480 mJ cm^{-2} (20 W m^{-2}) of UV in the range 200-
361 400 nm. Photosynthetic efficiency of this microalga dropped to values around 0.57 a
362 few minutes after exposure, both in control cultures and in the presence of ERK
363 inhibitor (Fig. 9). However, inhibition of either p38 or JNK caused a very significant
364 decrease of F_v/F_m values (in accordance with their roles in environmental stress
365 acclimation), being this decrease far more drastic when inhibiting JNK (0.42 in the
366 presence of p38 inhibitor and 0.15 in the presence of the JNK one).

367 When exposed to a non-lethal UVR dose, phosphorylation of ERK-like proteins did
368 not show significant differences along the experiment (Fig. 10a). However, incubating
369 the cultures with the ERK inhibitor produced a very significant decrease of the
370 phosphorylation of this MAPK-like protein. In the case of the JNK, treatment with non-
371 lethal UVR caused its immediate phosphorylation, which levels increased during the
372 following 24 h after stress (Fig. 10b). By employing the inhibitor of the JNK,
373 phosphorylation of this MAPK was prevented, and only residual phosphorylation

374 remained after 24 h. Finally, the p38-like MAPK (57 kDa) did not show significant
375 changes along time when exposed to the above-mentioned levels of UVR (Fig. 10c).
376 Furthermore, when incubating with the p38 inhibitor, the phosphorylation level of p38-
377 like MAPK decreased significantly, reaching its lowest level after 30 minutes, and
378 remaining low for the rest of the experiments.

379

380 **Discussion**

381 Animals have several well-characterized MAPK cascades that participate in cellular
382 responses to a wide variety of stress and in the control of cell division and
383 differentiation. These cascades consist of a series of protein kinases that are
384 phosphorylated and activated in a sequential way, associated with the downstream
385 protein kinase. Both the p38 and the JNK cascades are responsible for stress adaptation,
386 whereas the ERK cascade is involved in mitogenic stimuli and differentiation
387 (Widmann et al., 1999). MAPKs may also be involved in the signal transduction of
388 several environmental factors in plants (Hirt, 1997; Tena & Renaudin, 1998; Sinha et al
389 2011; Danquah et al., 2014; Yanagawa et al., 2016). These proteins participate in the
390 response to a broad range of extracellular stimuli, such as growth factors, drought, UV
391 radiation, osmotic changes, temperature stress, etc., and are involved in the operation of
392 several cell programs, such as cell proliferation, movement and cell death. MAPKs are
393 ubiquitous (Jonak et al., 1999), and their sequences are very conservative, with
394 serine/threonine residues in the active core (Kyriakis & Avruch, 2001). Several authors
395 have shown that all plants possess MAPK homologs, which play a critical role in the
396 cell signaling network (for a review, see Sinha et al. 2011). Despite the fact that signal
397 transduction in algae has only recently become a research focus, different studies with
398 microalgae (Jiménez et al., 2004, Jiménez et al., 2007, García- Gómez et al., 2012,
399 Gasulla et al., 2016) have demonstrated that p38, JNK and ERK components are present
400 in algae, and that they have a crucial role in acclimation to stress and in cell division.

401 Already in 1931, Baas-Becking proved that *D. viridis* presented a great ability to
402 survive in high salinity conditions. Accordingly, Borowitzka et al. (1977), Brown &
403 Borowitzka (1979), and Jiménez & Niell (1991) showed that *D. viridis* grows optimally
404 in 5.8-11.6 % (w/v) NaCl (1-2M NaCl), and tolerates up to 23.2% (4M NaCl). At lower
405 concentrations (e.g. 0.5M NaCl), *D. viridis* was unable to grow (Jiménez & Niell,
406 1991). Jiménez & Niell (2003) also reported that after a sudden increase of the salinity

of the medium, cell carbon and nitrogen content of *D. viridis* rapidly increased, and a new stationary state was reached in a period of time that ranged between 8 and 24 h. According to Berner et al. (1989), 24 h is also the time needed for a complete rearrangement of the thylakoid membranes following a light intensity transition in *D. tertiolecta*. In addition, Jiménez & Niell (2003) concluded that different kind of stress induces similar responses in *Dunaliella*. Among others, there was a significant reduction of the growth rate and of the package of the pigments, and an increase of the cell volume and of the C/N ratio.

In the present work it is demonstrated that MAPKs have a crucial role in the response of *D. viridis* to environmental stress, and that acclimation to the new conditions occurs through phosphorylation of both p38-like and JNK-like MAPKs. The estimated time for acclimation (measured as recovery of the photosynthetic efficiency) to the new conditions ranged between 8-24 h. The inhibition of either p38 or JNK phosphorylation in *D. viridis* highly impaired acclimation under stress conditions, eventually leading to cell death, in agreement with previous reports (Jiménez et al., 2004). A decrease in the capacity to acclimate to new environmental conditions, compromising eventually cell survival in the presence of specific inhibitors of the signaling pathways, suggests the existence of signaling mechanisms in algae similar to those found in mammalian cells, and that algae survive under stress conditions by activating several cell programs, among them p38 and JNK MAPK pathways.

This study has allowed the identification of the mechanisms underlying in stress acclimatization responses in the unicellular chlorophyte *D. viridis*. It has been determined the phosphorylation/de-phosphorylation levels of JNK-like, p38-like and ERK-like proteins in *D. viridis* cultures at their exponential growth phase long-time acclimated to different salinities (1M to 4M NaCl), with significantly lower phosphorylation levels of ERK-like proteins in the cultures exposed to the maximum NaCl concentration (4M), and no significant differences in the phosphorylation levels of JNK-like and p38-like proteins between cultures, independently of the salinity, which indicates that a certain level of phosphorylation is necessary to keep homeostasis of this species. It was also assessed, in the short and medium term (up to 24 h), the evolution of the phosphorylation levels of JNK-like, p38-like and ERK-like proteins after the cells being challenged with hyperosmotic shock, high irradiance shock and UV radiation shock (all non-lethal treatments). In general, there was an immediate increase of

phosphorylation levels of JNK-like and p38-like proteins after stress (that coincided with a recovery of photosynthetic capacity); on the contrary, phosphorylation level of ERK-like MAPKs suddenly dropped, in correlation with their role in the control of cell division (Jiménez et al., 2007).

The use of specific inhibitors of the three MAPKs studied demonstrates a direct and strong correlation between phosphorylation of the MAPKs and response to stress. A very significant decrease of photosynthetic efficiency occurs when either JNK or p38 inhibitors were applied to the cultures prior to a non-lethal stress (as determined by the *in vivo* fluorescence of photosystem II), being always more drastic in the case of the JNK inhibitor. This coincides with previous results of Jiménez et al. (2004) who measured a dramatic drop of cell viability when specific inhibitors were applied. Thus, it could be determined the crucial role played by JNK in the maintenance of cell viability after an environmental stress situation, and the involvement of p38 in the acclimatization process, while ERKs appear to control the cell division process, not being directly involved in the acclimatization process in the short and medium term after stress. Both p38 and JNK are essential for acclimatization (Jiménez et al., 2004) and their basal phosphorylation allows starting the response when a situation of environmental stress occurs. Blocking the phosphorylation of either MAPK significantly impaired the capacity to respond to stress in; in parallel to a decrease of the phosphorylation of these MAPKs a rapid and very significant drop of cell viability occurs, leading, in the case of total blocking of JNK, to cell death, even at NaCl concentrations, irradiance or UV levels compatible with a normal development of this species. Homeostasis of *D. viridis* is crucially dependent of the phosphorylation of p38 and JNK MAPKs.

ERK plays an essential role in cell division (Jiménez et al., 2007; Gasulla et al., 2016) and its phosphorylation is an indicative of this division. It is well known that cell division in *Dunaliella* is hampered or completely arrested in stressful growth conditions (Avron, 1992). De-phosphorylation of p42/44 ERK MAPKs in *D. viridis* coincides with the arrest of cell division, but does not imply loss of cell viability (Jiménez et al., 2007).

In conclusion, phosphorylation of MAPK-like proteins of the type JNK and p38 is crucial for response to environmental stress in the microalga *D. viridis*. Activation of both kinases occurs immediately after stress, being needed between 8 and 24 h for a complete acclimation to new environmental conditions. ERK proteins are not involved

in the response to stress, and they are rapidly deactivated after stress, leading to a transient inhibition of cell division.

Acknowledgements

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Figure captions

Fig. 1. Growth of *D. viridis* in function of the salinity (1 to 4M NaCl). Data are the mean of three replicates ± standard deviation.

Fig. 2. Spectral irradiance emitted by the LED Explosion Proof Lamp (a), for the high irradiance experiments, and by the UV lamps (b), for the UVR experiments.

Fig. 3. a) F_V/F_m values of long-term acclimated *D. viridis* cultures during the exponential phase of growth at increasing salinities. Cultures at 2M, 3M and 4M NaCl were compared against 1M NaCl treatment (control). A p-value < 0.05 was considered significant and is represented with a single asterisk "*", while a p-value ≤ 0.001 was considered very significant and is represented by two asterisks "**". Data are the mean of three replicates ± standard deviation. b) Relative maximum electron transport rate ($rETR_{max}$) of *D. viridis* long-term acclimated to 1, 2, 3 and 4M NaCl salinity in function of increasing irradiance. No significant differences between treatments were found.

Fig. 4. Band intensity (in relative units) of the three MAPK-like proteins (ERK, JNK and p38) during exponential growth at increasing salinity (1-4M NaCl) of long-term acclimated cultures, with examples of the bands obtained by means of immunedetection with specific antibodies of the phosphorylated forms of the MAPKs. Data are the mean of three replicates ± standard deviation. A p-value < 0.05 was considered significantly different than the 1M NaCl control, and is represented with a single asterisk "*", while a p-value ≤ 0.001 was considered very significant and is represented by two asterisks "**".

Fig. 5. Evolution of the maximum quantum yield of *D. viridis* after hypersaline shock (from 1 to 2.5M NaCl) in the presence or absence of the specific inhibitors of the ERK, JNK and p38 cascades. Data presented correspond to time 0 before stress (0,BS), time 0 after 2h of incubation with designated inhibitor and before the detailed stress (0,I), time 0 after stress (0,AS), and 0.5, 1, 2, 4, 8, 24 hours after stress. Data are the mean of five replicates ± standard deviation. A p-value < 0.05 was considered significantly different than the time 0 before stress (0,BS) and is represented with a single asterisk "*", while a p-value ≤ 0.001 was considered very significant and is represented by two asterisks "**".

Fig. 6. Time course (24 h) of the phosphorylation level of ERK-like (a), JNK-like (b) and p38-like (c) proteins after hypersaline stress, both in control cells (stress without inhibitor) and in the cells stressed after being incubated for 2 h with the inhibitors of the corresponding phosphorylation cascade. Relative band intensity values are shown, with examples of the bands obtained. Samples labelled 0h-BS correspond in the controls to the moment previous to the stress situation, while in the inhibition experiments they refer to the moment previous to the stress situation but 2 hours after incubation with the corresponding inhibitor. Samples labelled as 0h-AS correspond in both cases to the first moments (less than 1 min) after the shock was applied. Data are the mean of three replicates ± standard deviation. A p-value < 0.05 was considered significantly different than the time 0 before stress (0h-BS) and is represented with a single asterisk "*", while a p-value ≤ 0.001 was considered very significant and is represented with two asterisks "**". Open symbols – treatments with specific inhibitors; closed symbols – control treatments.

Fig. 7. Evolution of the maximum quantum yield of *D. viridis* after high irradiance shock (3000 μmol m^{-2} s^{-1} for 35 minutes) in the presence or absence of the specific inhibitors of the ERK, JNK and p38 cascade. Details of the data points as in Figure 5.

Fig. 8. Time course (24 h) of the phosphorylation level of ERK-like (a), JNK-like (b) and p38-like (c) proteins after high irradiance stress, both in control cells (stress without inhibitor) and in the cells stressed after being incubated for 2 h with the inhibitors of the corresponding phosphorylation cascade. Relative band intensity values are shown, with examples of the bands obtained. Samples labelled 0h-BS correspond in the controls to the moment previous to the stress situation, while in the inhibition experiments they refer to the moment previous to the stress situation but 2 hours after incubation with the corresponding inhibitor. Samples labelled as 0h-AS correspond in both cases to the first moments (less than 1 min) after the shock was applied. Data are the mean of three replicates ± standard deviation. A p-value < 0.05 was considered significantly different than the time 0 before stress (0h-BS) and is represented with a single asterisk "*", while a p-value ≤ 0.001 was considered very significant and is represented with two asterisks "**". Open symbols – treatments with specific inhibitors; closed symbols – control treatments.

Fig. 9. Evolution of the maximum quantum yield of *D. viridis* after non-lethal UV shock (480 mJ cm^{-2} of UV light in the range of 200-400 nm) in the presence or absence of the specific inhibitors of the ERK, JNK and p38 cascade. Details of the data points as in Figure 5.

Fig. 10. Time course (24 h) of the phosphorylation level of ERK-like (a), JNK-like (b) and p38-like (c) proteins after exposure to UVR, both in control cells (stress without inhibitor) and in the cells stressed after being incubated for 2 h with the inhibitors of the corresponding phosphorylation cascade. Relative band intensity values are shown, with examples of the bands obtained Samples labelled 0h-BS correspond in the controls to the moment previous to the stress situation, while in the inhibition experiments they refer to the moment previous to the stress situation but 2 hours after incubation with the corresponding inhibitor. Samples labelled as 0h-AS correspond in both cases to the first moments (less than 1 min) after the shock was applied. Data are the mean of three replicates ± standard deviation. A p-value < 0.05 was considered significantly different than the time 0 before stress (0h-BS) and is represented with a single asterisk "*", while a p-value ≤ 0.001 was considered very significant and is represented with two asterisks "**". Open symbols – treatments with specific inhibitors; closed symbols – control treatments.

Figure 1

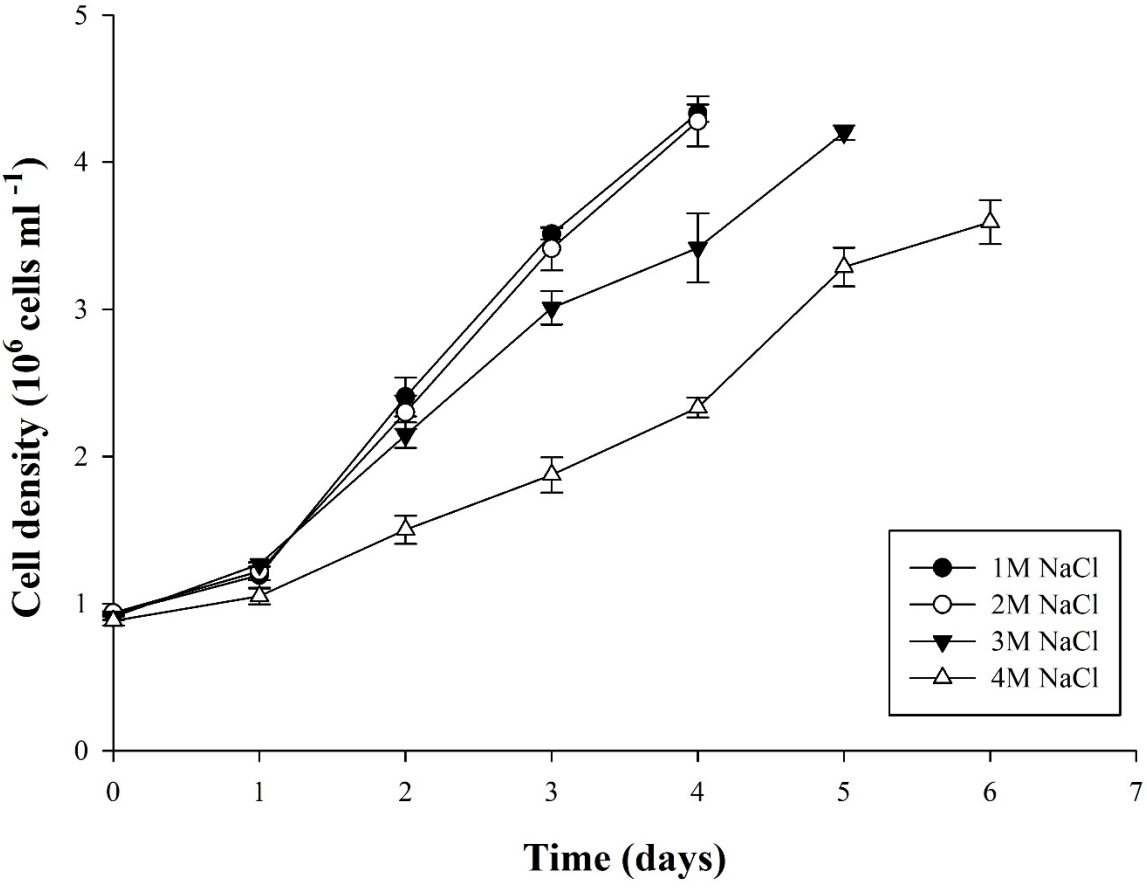

Cell density (10^6 cells ml^{-1})
Time (days)
0
1
2
3
4
5
6
7
1M NaCl
2M NaCl
3M NaCl
4M NaCl

Figure 2

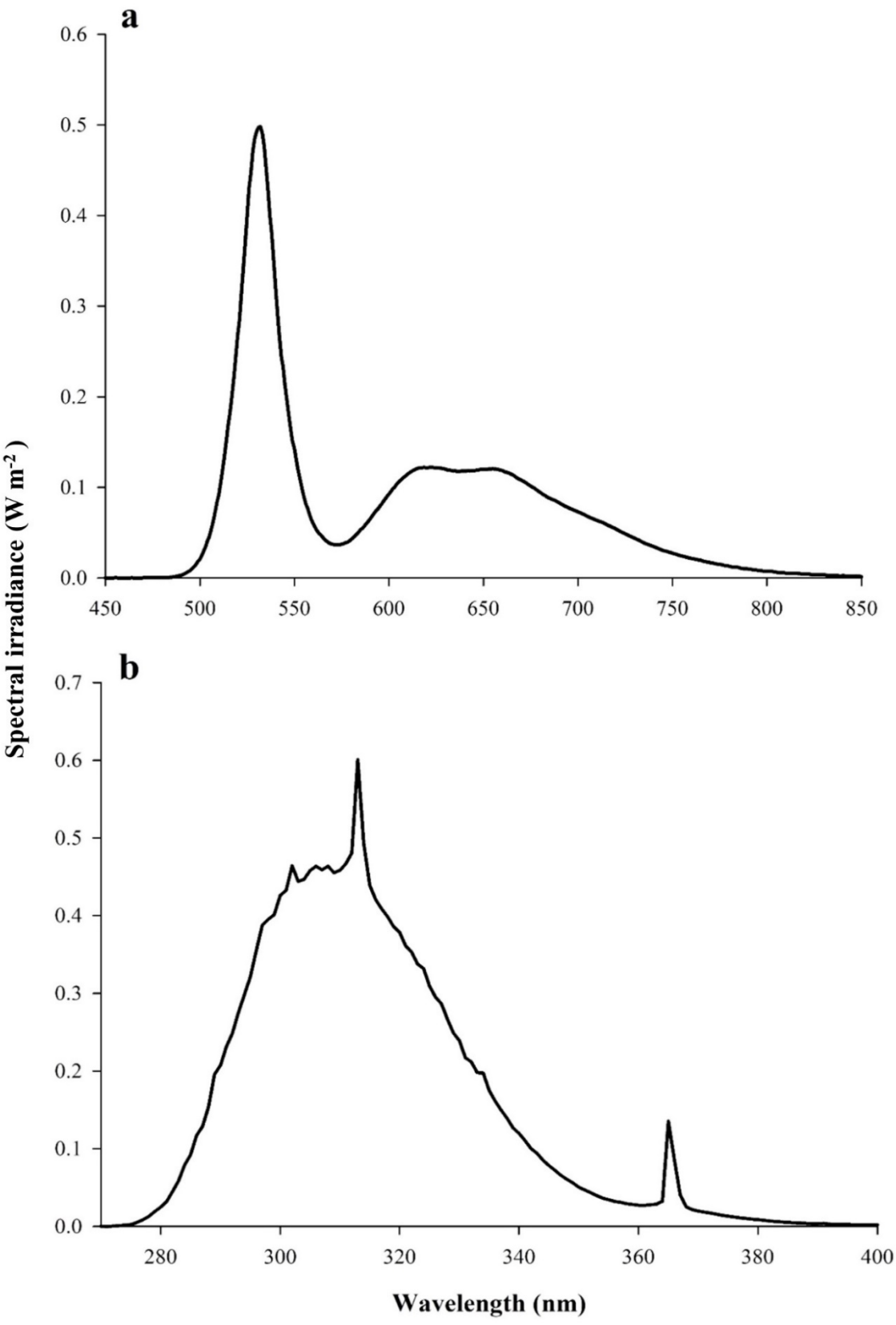

a
b
Spectral irradiance (W m-2)
Wavelength (nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
450
500
550
600
650
700
750
800
850
280
300
320
340
360
380
400

Figure 3

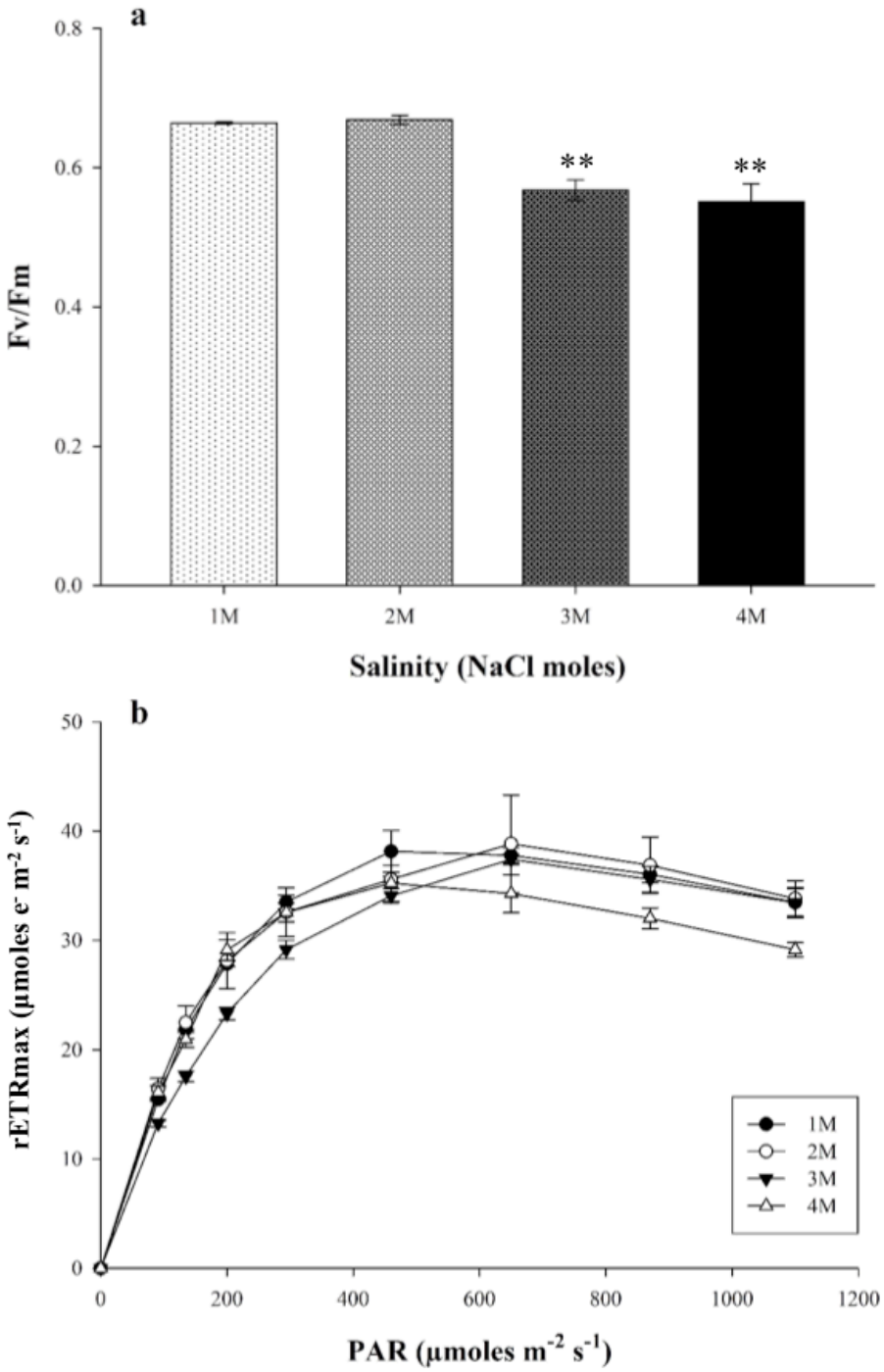

a
0.8
0.6
0.4
0.2
0.0
Fv/Fm
**
**
1M
2M
3M
4M
Salinity (NaCl moles)
b
50
40
30
20
10
0
rETRmax (µmoles e⁻ m⁻² s⁻¹)
0
200
400
600
800
1000
1200
PAR (µmoles m⁻² s⁻¹)
1M
2M
3M
4M

Figure 4

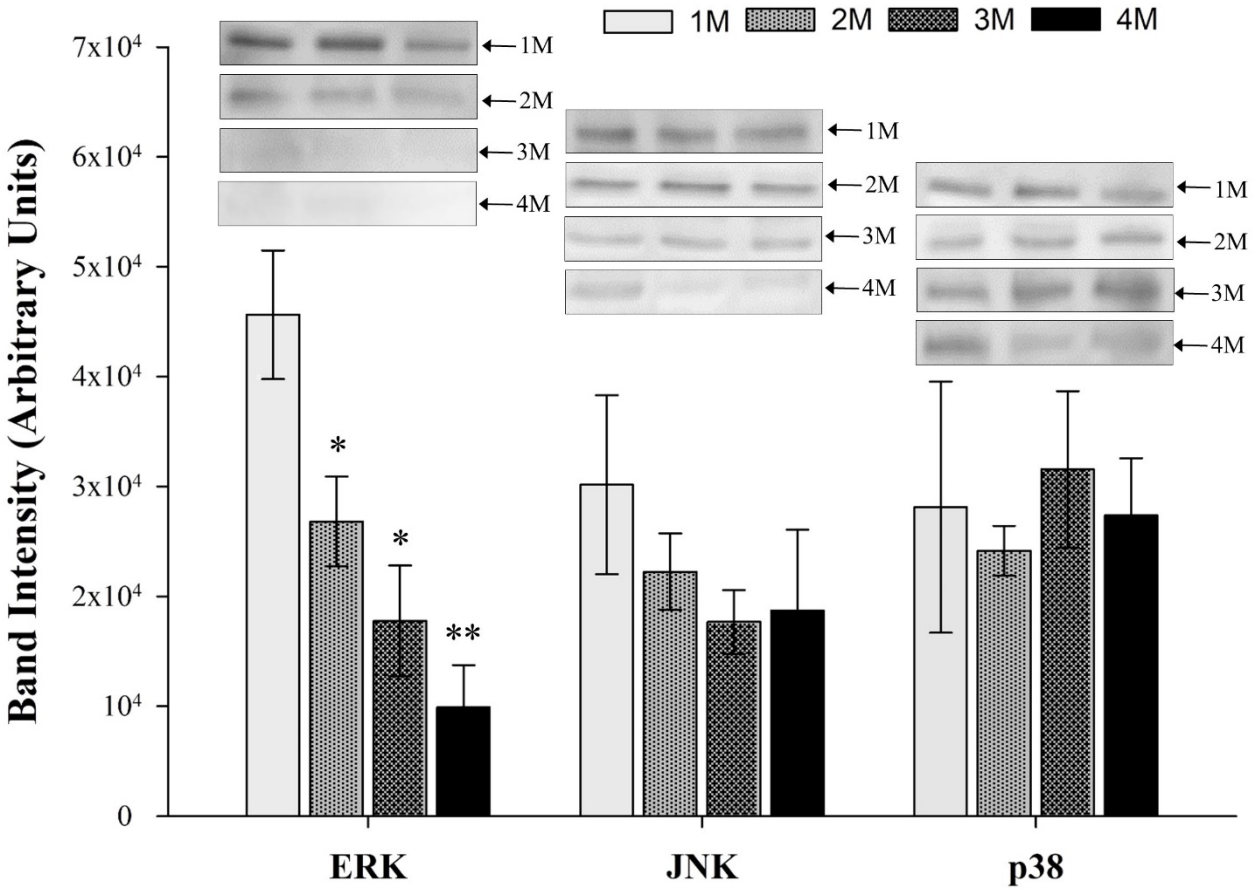

1M
2M
3M
4M
Band Intensity (Arbitrary Units)
7x10⁴
6x10⁴
5x10⁴
4x10⁴
3x10⁴
2x10⁴
10⁴
0
*
*
**
ERK
JNK
p38

Figure 5

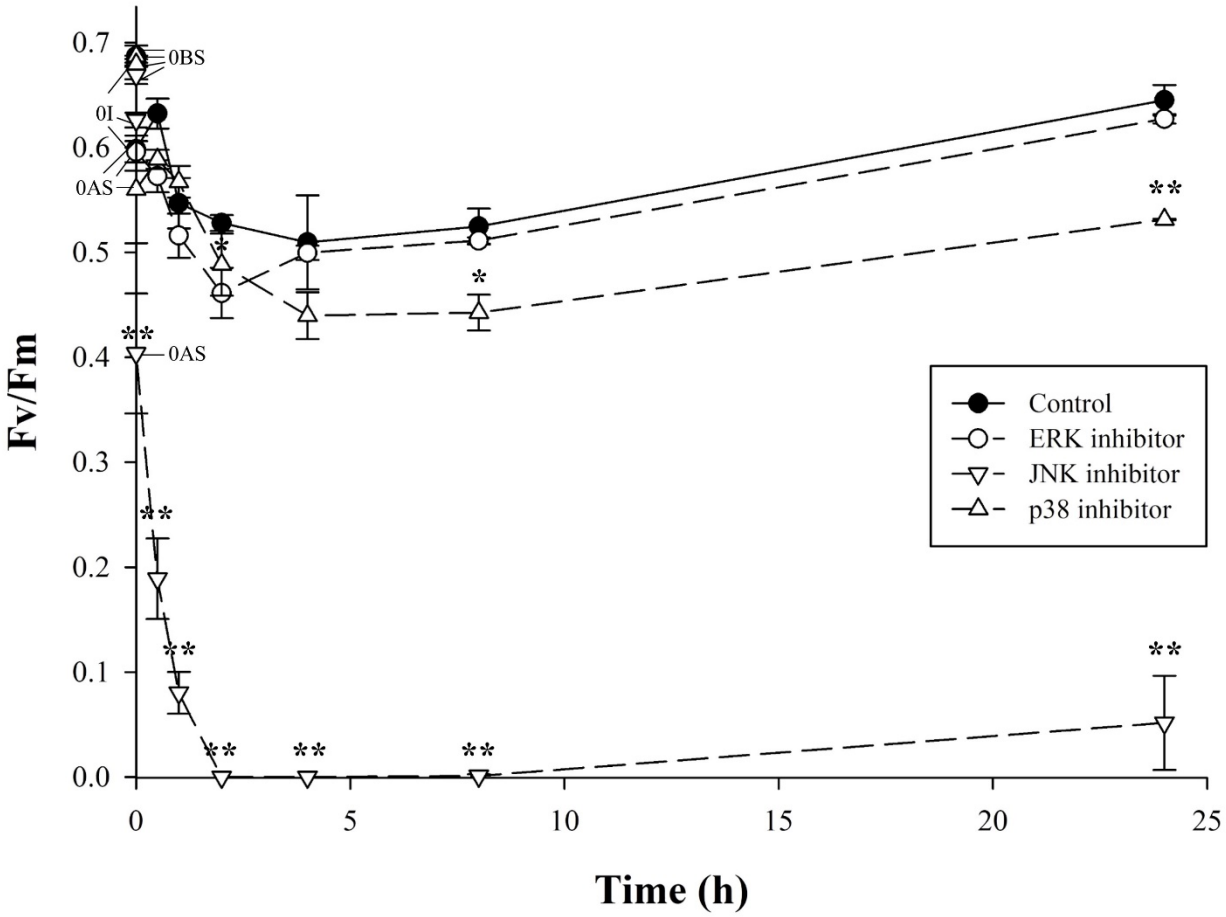

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Fv/Fm
0
5
10
15
20
25
Time (h)
0BS
0I
0AS
0AS
Control
ERK inhibitor
JNK inhibitor
p38 inhibitor
**
*

Figure 6

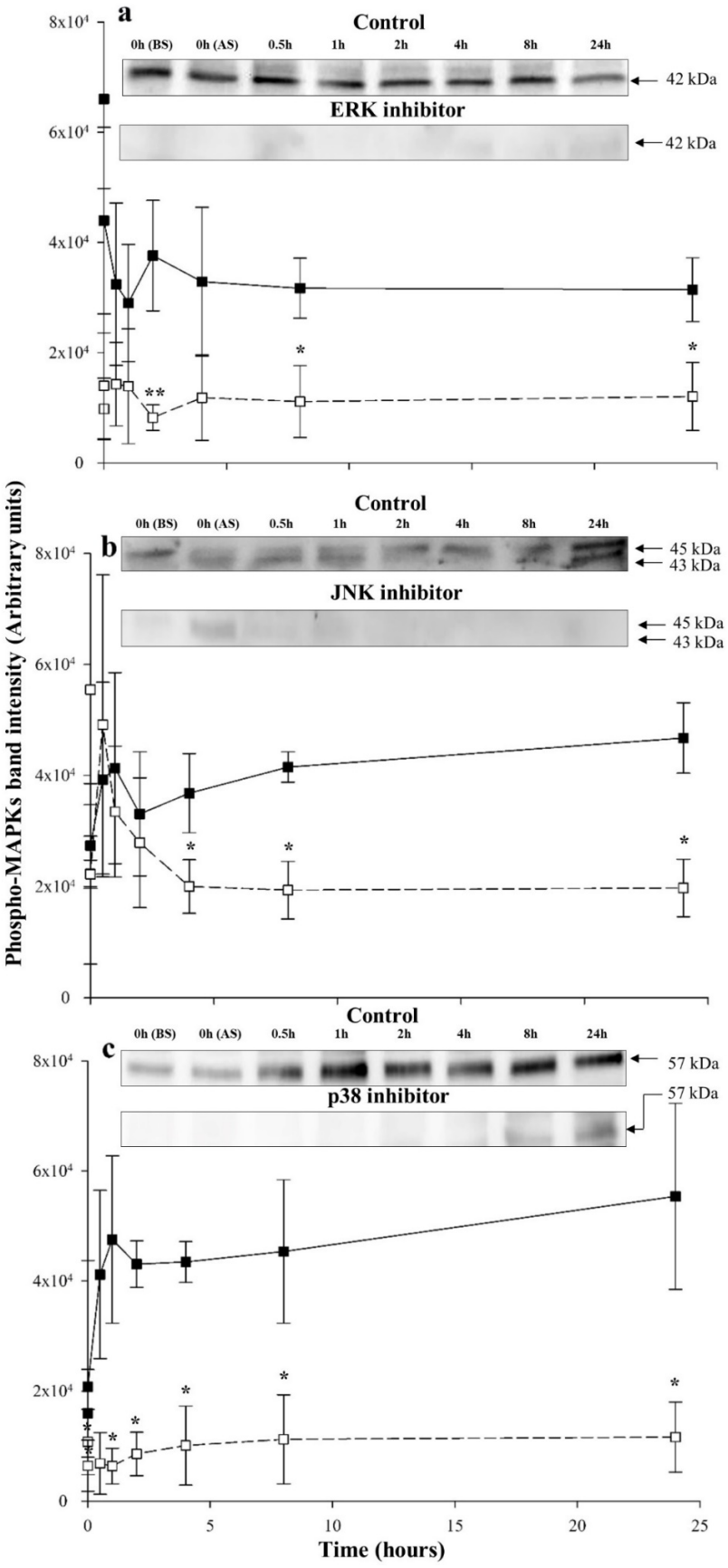

a
Control
0h (BS)
0h (AS)
0.5h
1h
2h
4h
8h
24h
42 kDa
ERK inhibitor
42 kDa
b
Control
45 kDa
43 kDa
JNK inhibitor
45 kDa
43 kDa
c
Control
57 kDa
p38 inhibitor
57 kDa
Phospho-MAPKs band intensity (Arbitrary units)
8x10⁴
6x10⁴
4x10⁴
2x10⁴
0
0
5
10
15
20
25
Time (hours)

Figure 7

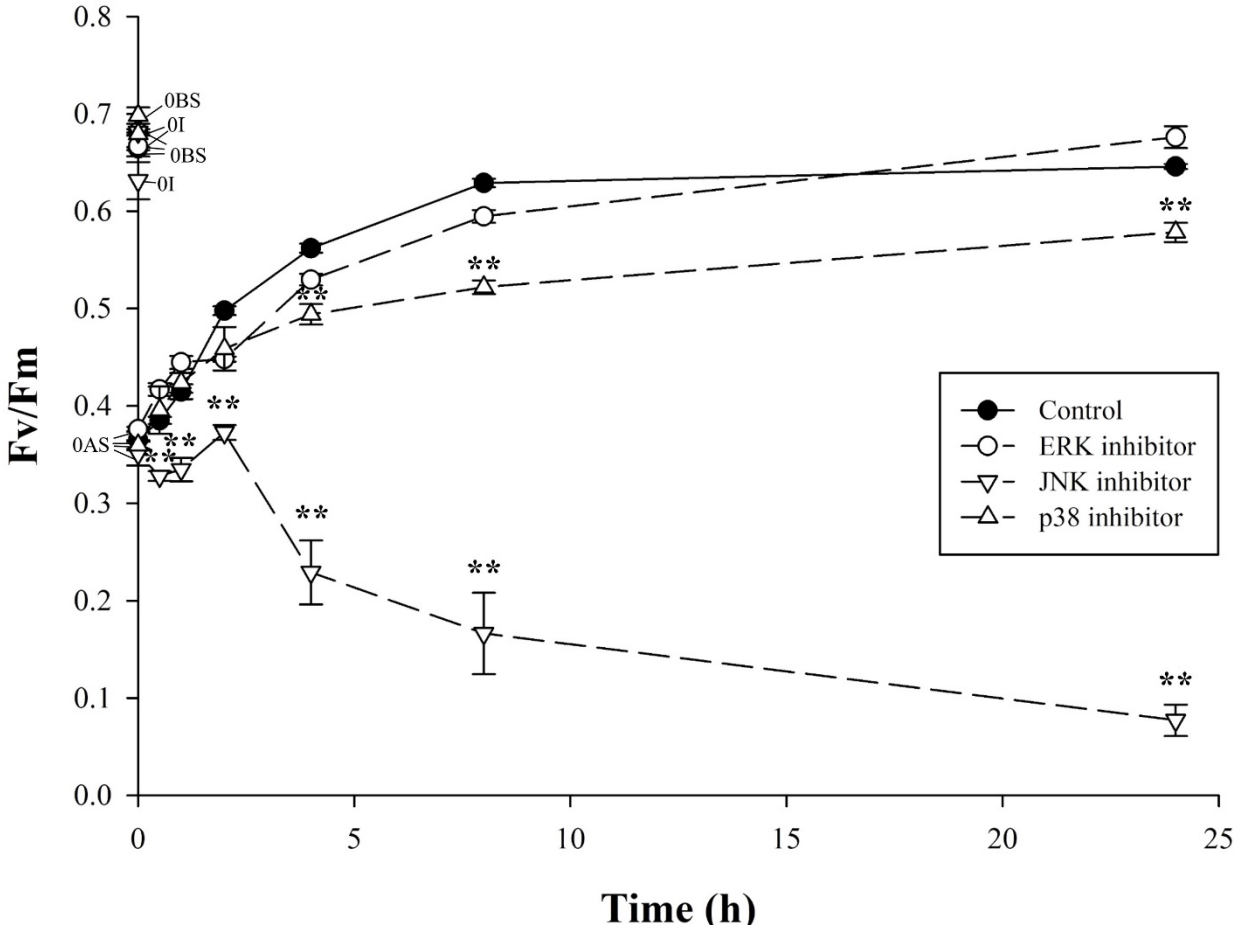

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Fv/Fm
0
5
10
15
20
25
Time (h)
0BS
0I
0BS
0I
0AS
**
Control
ERK inhibitor
JNK inhibitor
p38 inhibitor

Figure 8

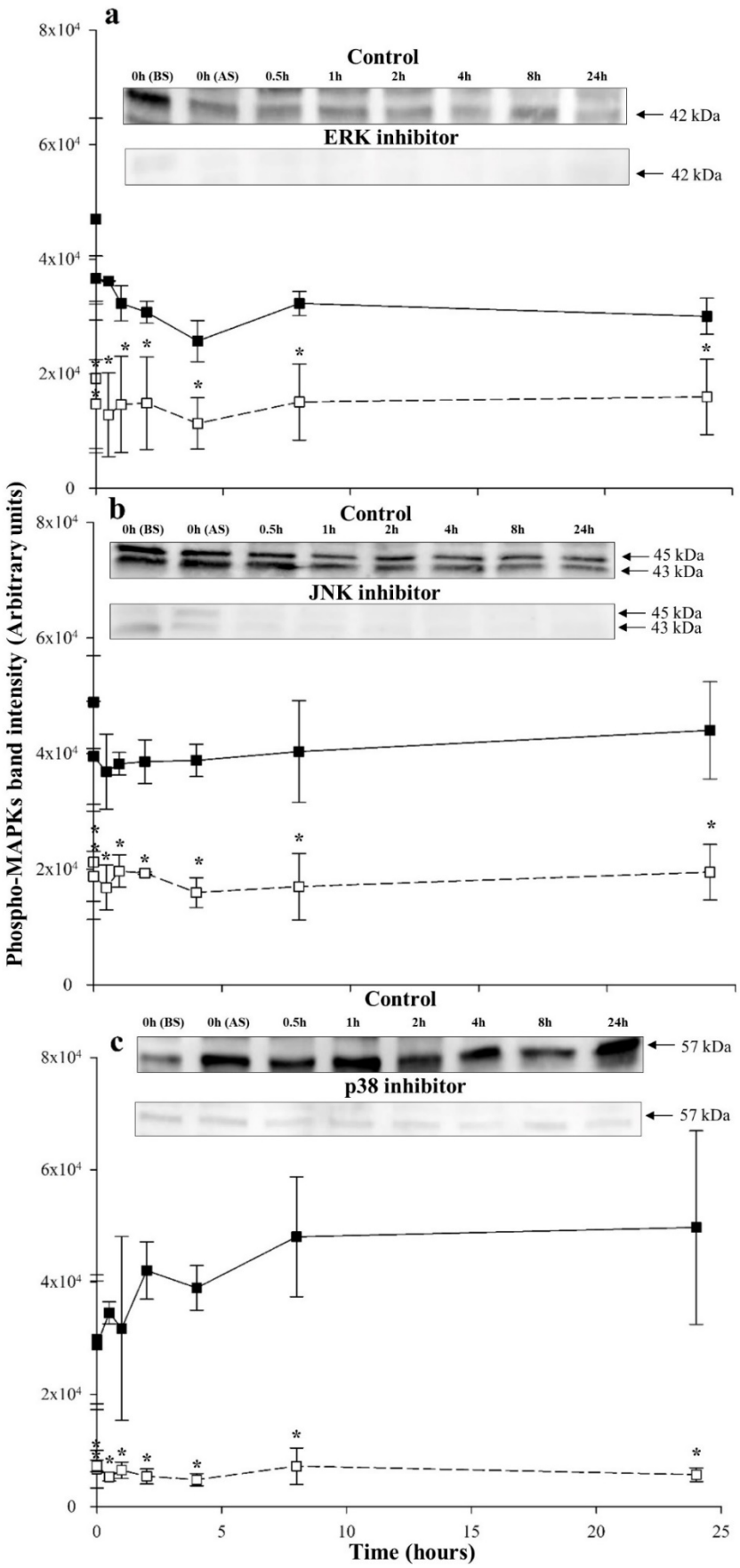

a
Control
0h (BS)
0h (AS)
0.5h
1h
2h
4h
8h
24h
42 kDa
ERK inhibitor
42 kDa
b
Control
45 kDa
43 kDa
JNK inhibitor
45 kDa
43 kDa
c
Control
57 kDa
p38 inhibitor
57 kDa
Phospho-MAPKs band intensity (Arbitrary units)
8x10⁴
6x10⁴
4x10⁴
2x10⁴
0
0
5
10
15
20
25
Time (hours)

Figure 9

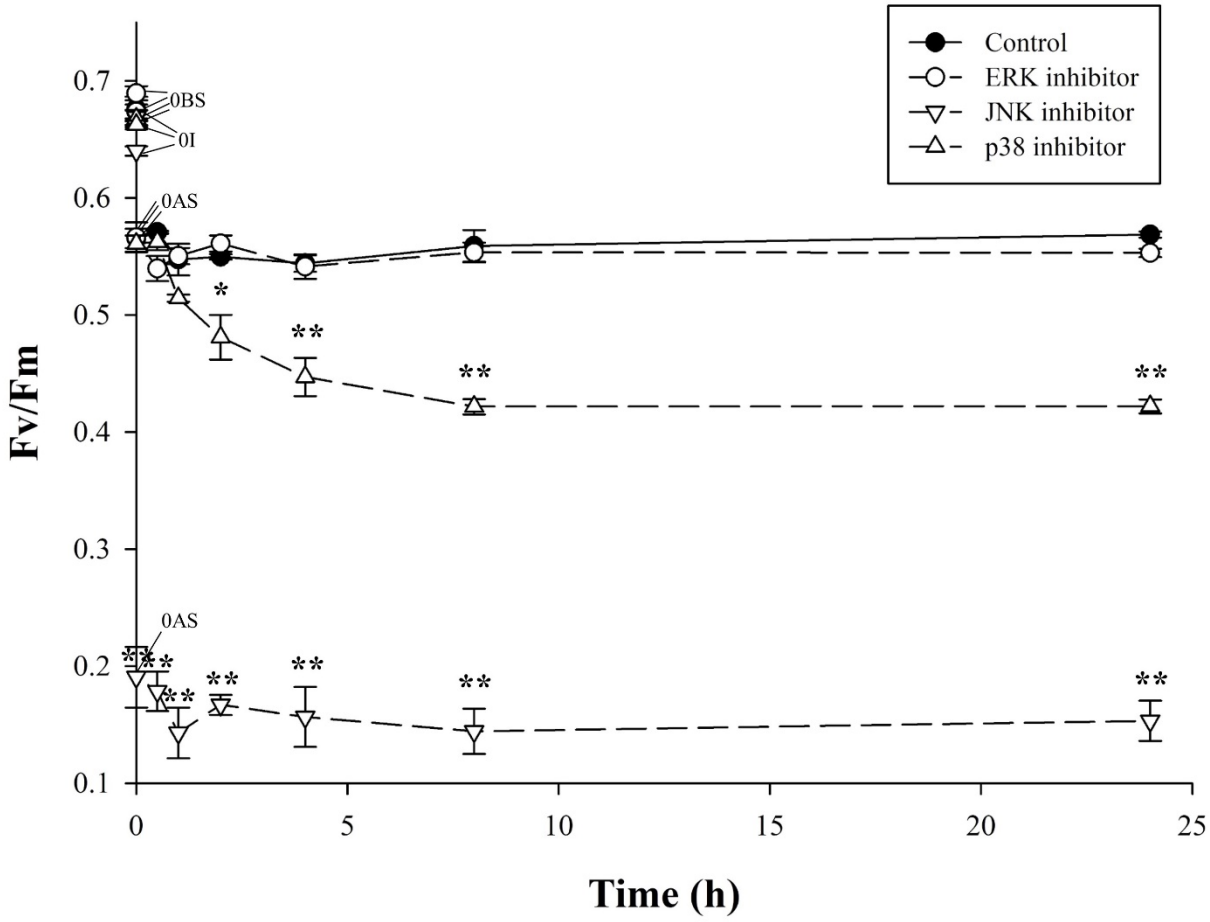
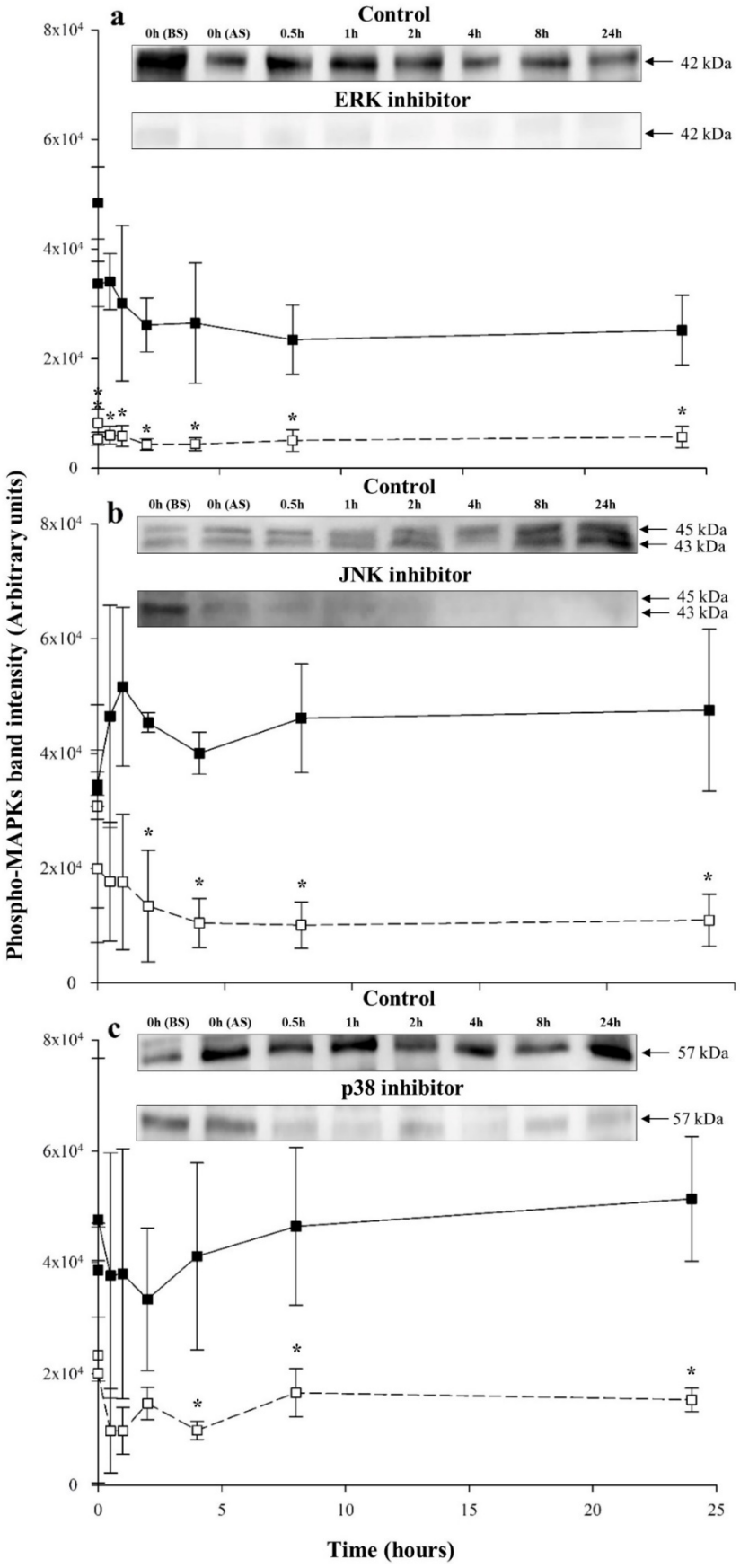

Control
ERK inhibitor
JNK inhibitor
p38 inhibitor
0BS
0I
0AS
0AS
Fv/Fm
Time (h)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
*
**

Figure 10


a
Control
0h (BS)
0h (AS)
0.5h
1h
2h
4h
8h
24h
42 kDa
ERK inhibitor
42 kDa
b
Control
45 kDa
43 kDa
JNK inhibitor
45 kDa
43 kDa
c
Control
57 kDa
p38 inhibitor
57 kDa
Phospho-MAPKs band intensity (Arbitrary units)
8x10⁴
6x10⁴
4x10⁴
2x10⁴
0
0
5
10
15
20
25
Time (hours)