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Scientific Research

The Effect of UV Light and CO_2 in the Production of Polyunsaturated Aldehydes in *Skeletonema costatum* (Bacillariophycea)

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Abstract

***Skeletonema costatum* was submitted to two experiments using UV lights and $CO_2$with the purpose of observing changes in the lipids profile and the synthesis of polyunsaturated aldehydes (PUA) after cell disruption. When cells receive CO_2 supply, it was noticed that the production of PUA was significantly lower. The same was observed when the culture was treated with a dose of 45.9 kJ·m^{-2} of ultra-violet A/B ray. The premise to all experiments was the production of 2,4-heptadienal compared to the supply of EPA as substrate. As a result, the same synthesis rate was observed both when the CO_2 treatment was applied and in the experiment control. On the other hand, the culture subjected to ultraviolet radiation showed a 68% greater demand with the utilization of the substrate. These observations suggested that EPA was consumed before cell disruption and was probably exuded to the surrounding environment as a sign of stress. Changes in cell morphology could be observed by the migration of the chloroplast nearby the cell wall, where PUA was produced, indicating a defense strategy.**

Keywords

Diatom, Physiology, Oxilipins, *Skeletonema costatum*, Polyunsaturated Aldehyde (PUA)

1. Introduction

The ocean covers 70% of the planet's surface and presents an extraordinary microbial diversity, constituting the

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largest ecosystem in the world [1]. Diatoms are an outstanding group of phytoplankton microalgae playing a crucial role in the ocean's ecological and biogeochemical systems, cycling important nutrients such as carbon, nitrogen and silica [2]. Planktonic organisms drifting on ocean currents are usually challenged with unfavorable conditions [3] that can generate great changes in the metabolic functioning of some diatoms, e.g. increasing their level of toxicity through the synthesis of polyunsaturated aldehydes [4] [5].

The rising of CO_2 level in the atmosphere is causing ocean's acidification [6] [7] and the continuous stratospheric ozone depletion will increase UVB radiation in 5% to 10% during de next decades [8] [9]. Ocean acidification can cause an increase in carbon fixation rates in some photosynthetic organisms [10] [11] and the capability of marine organisms to adapt to this rise, as well as its implications for ocean ecosystems, is not well known. Trying to understand if the UVB-stressed-algae can indirectly affect primary and secondary consumers in the marine environment lead to the deepening of the present research. Wichard *et al*. found that about 30% of the marine diatoms produce polyunsaturated aldehydes (PUA) as secondary metabolites derived from fatty acid metabolism [12]. It has been demonstrated that PUA have negative effects on copepod development and reproduction, as well as on other marine invertebrates [13]-[16], since their first identification in marine diatoms [17]. The exudation of aldehydes during algae development represents a chemical sign for the ending of the bloom at specific PUA concentrations [18]. Species belonging to different taxonomical groups of phytoplankton, are also inhibited by PUA acting as allelochemicals [19] [20]. It is found that stress-surveillance system triggers the rise of an intracellular messenger (Ca^{+2}), as an increase of gene activity, producing nitric oxide (NO), that induces cell death [21] [22]. In a recent report, Vardi [23] demonstrates how cells employ a complex mechanism to sense changes in environment and activate chemical-based defense strategies, elucidating how chemical signals (e.g., infochemicals), derived from biotic interactions, have the potential to shape complex community structures in aquatic systems. On the other hand, healthy diatoms that are growing in favorable conditions are capable of overcoming the risk of self-poisoning even tough producing toxic compounds [24].

This report compares how cells respond to the influence of UV light and the increase of CO_2, by changing concentrations of polyunsaturated fatty acids (PUFAs) and producing oxylipins, under the influence of the environmental conditions in the intra-specific response.

2. Material and Methods

2.1. Collection and Screening

Phytoplancton samples were collected in a Coquimbo Bay (29˚55'S and 71˚19'W) in a campaign between September to December 2009 using a network mesh of 23 µm. Cells of *Skeletonema costatum* was isolated and cultivated in a f/2 + Si [25] for a quick analysis of PUA content through a semi-quantitative solid phase microextraction (SPME) by the concentrated biomass [12]. The paste of microalgae was obtained by centrifugation of 1600 ml of the culture in 50 ml tubes at 4˚C for 15 min at 1500 rpm. Of this, 500 µL of biomass was introduced in a vial of 4 ml whit a silicon septum cap. A SPME fiber of polydimetilsiloxan (PDMAS, 100 µm) was used to adsorb aldehydes from a head space of the vial, after the cell disruption by sonication from 10 min at 40˚C. The sample recovered in SPME fiber was injected into the CG-MS for desorption of the analytes and further characterization. After this analysis, the richer strain in aldehydes was selected to achieve the next experiments.

2.2. Experimental Design

The cultures were performed in triplicate in a controlled environment at 20˚C, light intensity of 50 µmol/m^2/s and constant aeration in Erlenmeyer of 2 L. For the treatment carried out with addition of CO_2 cultivation was supplied with a flow of 0.5 mm^3/s of carbon dioxide with a purity of 99%, for 24 h in parallel aeration. For the test with ultraviolet cultures received photosynthetic active radiation PAR (700 - 400 nm) while were subjected to UV A/B, of wavelength 400 - 280 nm. A filter Schott WG 305 was used to prevent radiation at frequencies lower than 280 nm. Equation (1) calculates the doses of the UV light the irradiated [26].

$$J \cdot m^{-2} = W \cdot m^{-2} \cdot s \quad (1)$$

The final dose was calculates in 45.9 KJ·m^{-2}, concerning the radiation of 15.3 KJ·m^{-2} found at 1 m depth accumulated over three days, as described [27].

2.3. Experimental Design

To descript the change profile of fatty acids during the curve growth, atransesterification methodology was applied [28]. A solution of methanol, hydrochloridric acid and chloroform was prepared (10:1:1 v/v/v), and was added 3 mL to a sample of 50 mg of lyophilized microalgae. The sample was homogenated and maintained during 60 min at 90˚C in a water bath. Before, the sample was cooled at room temperature. The sample was homogenized again and 1 mL pure water and 2 mL of a solution of hexane and chloroform (4:1 v/v) was added and then homogenate. Finally, the sample was centrifuged for 15 min at 6800 rpm and the supernatant was used for the GC-MS analysis.

2.4. Chemical Analysis of Derivatized Aldehydes and Fatty Acids

A standard sample of 50 mL with 7.4×10^4 cells/mL obtained from each culture was concentrated in a glass fiber filter (What man GB) under a reduced pressure of 700 mbar. The concentrated biomass was washed with 1 mL of the derivatized reagent (25 mM PFBHA. HCl in 100 mM Tris/HCl, pH 7.0). The sample was transferred to a 4 mL vial that was exposed to ultrasound for 1 min at 4˚C. As internal standard 5 µL of benzaldehyde solution (1 mM) was added, the sample remained at room temperature for 30 min to complete the enzymatic reaction and after then it was stored at −20˚C. For the extraction of the derivatized compounds 0.5 mL of methanol and 1 mL of hexane were added and homogenate in vortex, 2 drops of sulfuric acid (5 mM) was added and homogenate again. The upper layer of the sample was removed pipetting and sodium sulfate was added to remove water. The sample was filtrated with a Millipore® membrane of 0.2 µm and transferred to a 1 mL conical vial and dried by a nitrogen gas flow. At the end, 50 µL of hexane was added and further analyzed in a GC-MS [29].

2.5. Coupled Gas Chromatography-Mass Spectrometry (GC-MS)

A capillary GC column (50 m × 0.32 mm i.d. HP-1) fitted with a cold on-column injector was directly coupled to a mass spectrometer (Thermo Finnigan). Ionization was by electron impact at 70 V, 250˚C. The GC oven temperature was maintained at 30˚C for 5 min and then increased at $5^{\circ}C \cdot min^{-1}$ to 250˚C. Tentative identifications were made by comparison with mass spectra databases (NIST 2002).

2.6. Statistic Analysis of the Metabolism of PUA

To compare concentrations of PUA and fatty acids between strains and treatments a one way ANOVA was performed and the a posteriori Tukey test was applied when necessary to indicate the significant differences ($p < 0.05$).

3. Results

3.1. SPME Analysis

The three pre-selected strains subjected to a semiquantitative analysis by SPME-PDMS presented a pronounced difference of PUA synthesis **Figure 1**. Strains B and C present a production of 130 fold higher of 2,4-heptadenal and 255 fold higher of 2,4-octadienal than the strain A. In the strain A just trace of toxic aldehydes was detected as shows in **Figure 1**.

3.2. Fatty Acids Pofile

Fatty acids presented a tendency of change the profile during de growing curve as describe in **Table 1**. The saturated fatty acid shoed a reduction in their concentration over the time, down from 55% in the 3rd day to 37% on the 7th day. Instead monounsaturated fatty acids remained stable, increase between the 3rd and 5th day, from 25% to 30%, decreasing again to 25% in the last day. On the other hand, polyunsaturated fatty acids (PUFA) increased in the lipid profile from 19% to 36% between days 3 to 7. These fatty acids cumulated in *Skeletonema costatum* could be a potential for the PUA production. Polyunsaturated fatty acids with 18 carbons (C18: x) increased from 8% to 18% in the lipid profile. At the same time the eicosapentaenoic acid (EPA) accumulated from 10% to 17% in the algae cells. Docosahexaenoic acid (DHA) also was detected in low concentrations and it did not change significantly during the growth as show in **Figure 2**.

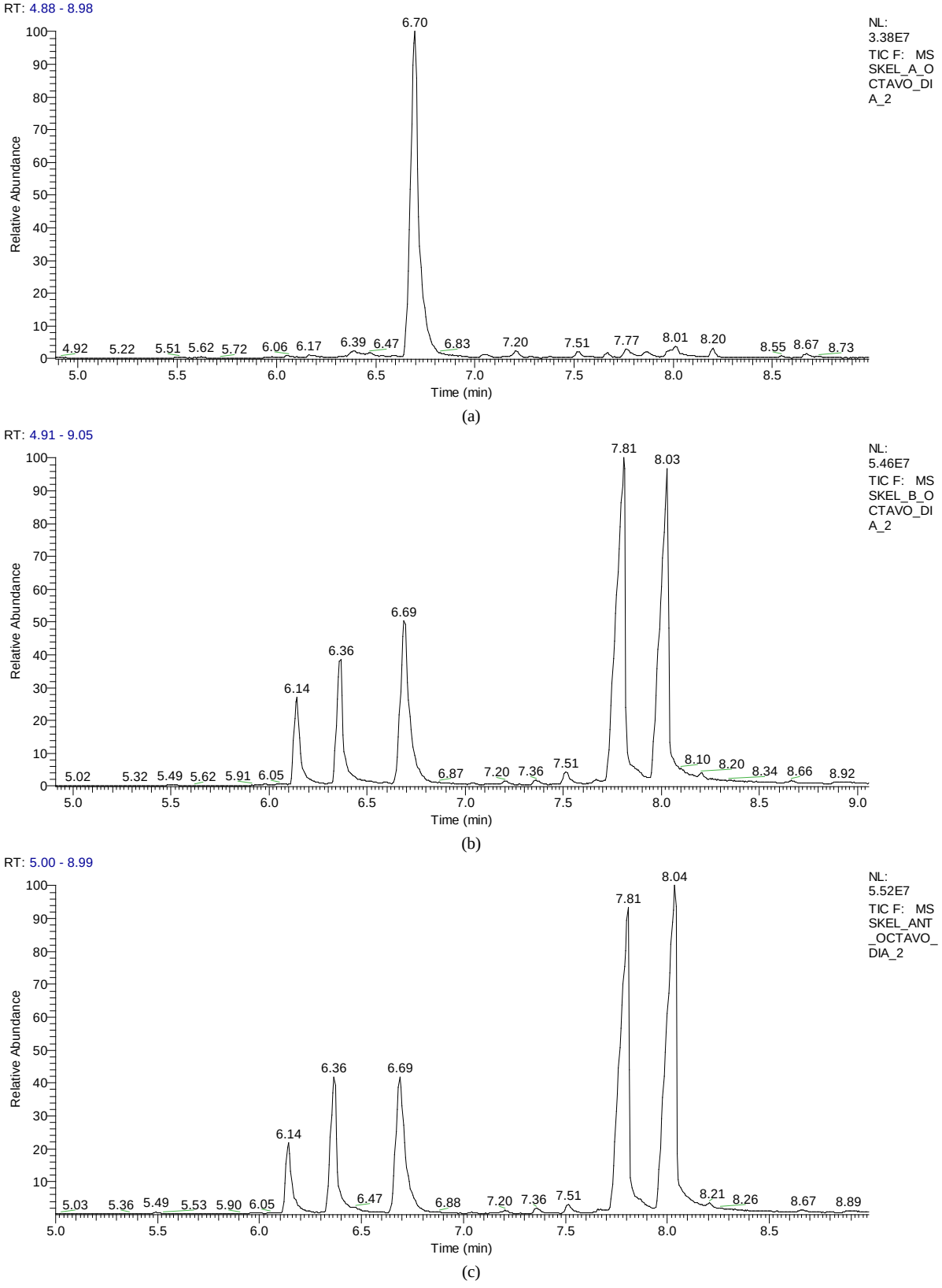

Figure 1. Aldehydes profile of the three selected strains in chromatography by SPME technique. Strain (a) with no toxic aldehydes. Strain (b) and (c) showed isomers of 2,4-heptadienal (RT: 6.14 and 6.36) and 2,4-octadienal (RT: 7.81 and 8.03).

Table 1. Relative abundance of fatty acids of *Skeletonema costatum* strain in the cellular life cycle.

	Abundance of fattyacids					
	Day 3		Day 5		Day 7	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
C14:0	38.36%	3.44%	34.31%	5.92%	22.98%	18.99%
C16:0	11.31%	2.63%	7.15%	6.95%	14.40%	12.26%
C18:0	5.35%	7.69%	0.00%	0.00%	0.00%	0.00%
C16:1	25.39%	2.51%	30.07%	4.85%	25.97%	20.72%
C18:2	0.52%	0.45%	0.17%	0.30%	1.71%	0.94%
C18:3	7.57%	2.59%	11.99%	1.55%	15.94%	3.45%
C20:5	10.11%	2.44%	15.43%	5.16%	17.01%	3.08%
C22:6	1.39%	0.28%	0.87%	0.10%	2.00%	0.64%

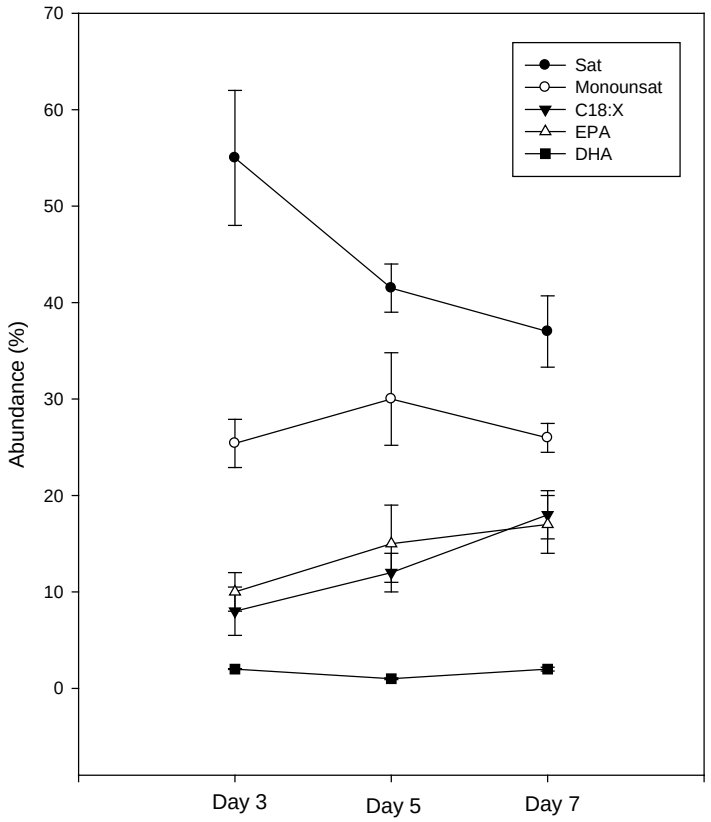

Figure 2. Fatty acids profile during the growth curve.

Through the methodology of derivatization was possible to trap and identify some fatty acids used in the metabolic pathway of oxylipins. In cells culture grown with CO_2 and UV light was observed a decrease in the total fatty acid concentration. The abundance of EPA was significantly lower ($p < 0.05$) in the treatment whit CO_2, especially with cells challenged with UV light ($p < 0.01$) as presented in **Figure 3**. The tendency to accumulate higher amounts of saturated fatty acids was observed in crops that receive a supply of carbon dioxide increasing from 35% for 42% under the experimental conditions. At the same time a decrease of PUFA was observer with concentrations lowering from 16% to 9% in compare to the control.

3.3. Aldehydes Productions

The total production of aldehydes was accessed and in both treatments. The amount of PUA produced was significant lower ($p < 0.05$) in compeer to control (**Figure 4**). For 2,4-octadienal the tendency was the same, and the concentrations observed was significantly lowers ($p < 0,05$) in cells treated. In 2,4-heptadienal this difference was just observed in crops challenged with UV light, showing a significantly lower concentration ($p < 0.05$). The 2,4,7-octatrienal was detected in trace amounts, in this analysis no significant difference was observed between treatments as shows in **Figure 4**. Taking the EPA as the precursor of the 2,4-heptadienal and comparing the concentrations of this substratum and the final product, was observed that was basely no difference between control and CO_2 crops (4%). On the other hand, those cultures irradiated with UV light consumed 68% fold more EPA than the control.

4. Discussion

4.1. Screening and Selection

In the present work, a screening was conducted to select strains that produce the larger amount of aldehydes. From these three microalgae analyzed the strain A did not produce the molecules of interest. However, these algae (*Skeletonema costatum*) could be a good source of food for plankton consumption, because the low toxicityeven after cell disruption. About the two other strains of algae tested, it was observed a very similar profile of aldehydes which leads us to believe that it was the same species. Moreover the microalgae that not produce aldehydes could be another species of *Skeletonema* sp. and more accurate analysis should be conducted for the identification that isolated strain.

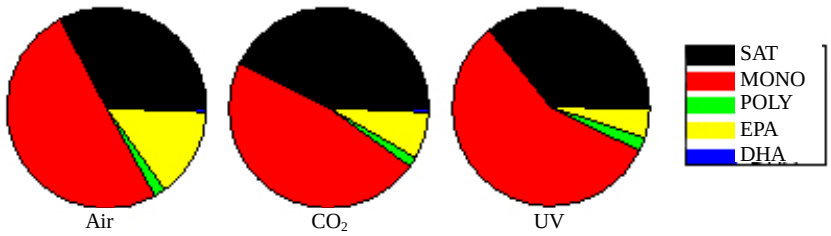

Figure 3. Distribution of fatty acids abundance trapped after de cell disruption by the derivatization methodology in microalgae challenged by three different experiments.

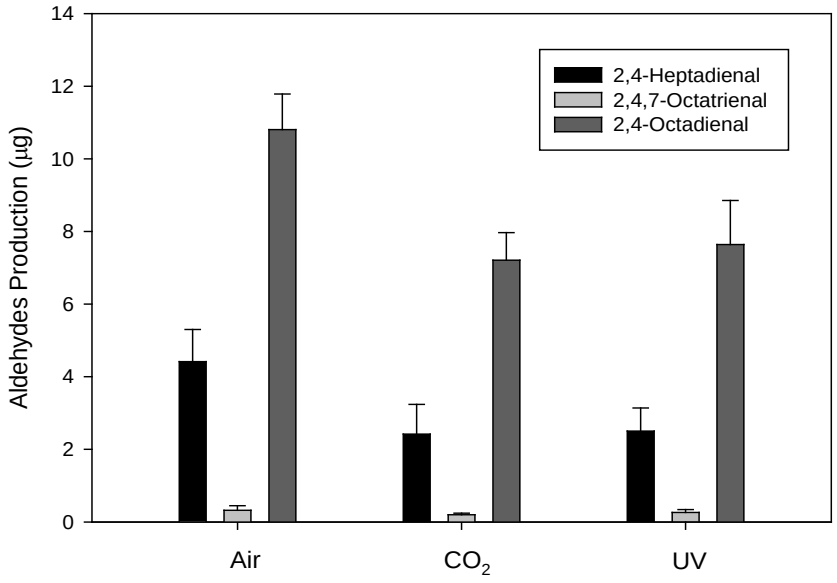

Figure 4. Comparative data between concentrations of aldehydes in the sample of *Skeletonema costatum* collected from the experiencedcultures.

4.2. The Effect of Increase of CO_2

In cultures that grow with supply of carbon dioxide, the cells density does not surpass the control culture. In a work made by Blanchemain and Grize [30], a crop of *Skeletonema costatum* that received CO_2 with the controlling pH in 8 do not register significant difference between treatments, corroborating the results of this work and conclude that diatom is insensitive to the increased supply of carbon dioxide. The Rubisco activity and the ability to capture CO_2 decrease with gradual increase in available carbon dioxide [31]. However, carbon fixation by diatoms still remains considerable debate about how diatoms process the CO_2 by the enzyme Rubisco, with sets carbon [32]. In a study by Monsalve [33], it was observed with increasing CO_2 concentrations. The diatom *Thalassiosirapseudonana* increased the averaged concentration of saturated fatty acids and decreased production of PUFA including EPA. The same was observed in this work with *Skeletonema costatum*.

The effect of CO_2 on the lipid composition was also studied in the others microalgae. In strains of *Chlamidomonasreinhardtii* was observed that increasing concentrations of PUFA occurred when CO_2 concentrations was reduced from 2% to 0.03% [33]. Probably the strain of *Skeletonema costatum* under high CO_2 concentrations showed a similar inhibition of the activity of the desaturase and elongase enzymes, responsible for the synthesisof PUFA. In this experiment, *Skeletonema costatum* showed low productivity of aldehydes that corroborate on the lipid profile that was generated by the CO_2 saturation. Their profiles show an increase in saturated fatty acid synthesis and decreased from 46.7% in the production of EPA. These polyunsaturated fatty acid, used as substrate for synthesis of 2,4-heptadienal decreased proportionally with aldehyde observed, as compared to control. Through this treatment was obtained less toxic microalgae.

4.3. The Action of Ultra-Violet Rays

With regard to the physiology of the defense and the production of oxylipins after the cell wall damage was possible to observe the same proportions between the aldehydes produced. We can assume that the path of enzymes involved in the synthesis of PUA remained active with no direct effect by UV light in their functions. Kouwenber and Lantoine [27] suggested that the effect of ultra-violet in *Skeletonema costatum* cells, could potentiate their toxicity through the synthesis of secondary metabolites, subjected to UV irradiation, mainly for the production of reactive oxygen species (ROS). In this study we found that the ultra-violet has a direct effect on the physiology of microalgae promoting similar conditions of radiation in temperate zones. In this experiment the dose of irradiation of 45.9 KJ/m^2 was sufficient to generate changes in cell morphology. It was observed a migration chloroplast to the periphery, nearby de cell wall and the increase of the cell volume, also described by Kouwenber and Lantoine [27]. At the same time, these results cannot be extrapolated to what happens in oceanic conditions or in the sea of Chile. Gieskes and Buma [34] and Neale *et al*. [35] mentioned that the effects of UV radiation on phytoplankton can only be faithfully reproduced when is used a model that realistically consider the attenuation of UV and PAR light, the vertical mixing dynamics, the seasons and cloud cover.

The result of this experiment with indirect evidence suggests that microalgae synthesize and release oxylipins before cell disruption respond to a stress signal, as described by Vidoudez and Pohnert [18] in bloom termination of *Skeletonemamarinoi*. The microalgae under the influence of UV irradiation may exude aldehydes as a form of chemical signals between the same specie [36] like many other examples of cell-to-cell communication in the marine environmental [37]. The release of 2,4-heptadienal and 2,4-octadienal directly affects the metabolism of surrounding cells preventing the cells to continue dividing [35]-[39]. Brownlee [36] proposes that aldehydes are released in response to abiotic stress, such as reduced nutrient supply and irradiance acting intracellularly as an info-chemical that can lead to a mechanism for regulating the growth and to promote programmed cell death. Kouwenberg and Lantione [27] propose the transformation of these cells in a resting spore. Vardi *et al*. [23] observed an increase in PtNOA1 gene expression synthesizing a group of proteins called GTP associated to stress for aldehyde 2,4-decadienal (DD). These authors were able to determine the activation of the pathway that regulates the production of NO in diatoms in response to the DD present in the environmental. PtNOA1 gene, located in the chloroplast has upregulation when in the presence of DD, increases the synthesis of superoxide and metacaspases, responsible for triggering apoptosis. In a new study, it was also observed that aldehydes, 2,4-heptadienal and 2,4-octadienal produce the same effect on the cells of *Skeletonema marinoi*, functioning as infochemicals [22]. It is also the reason why, aldehydes trapped after cell rupture were lower in microalgae subjected to this treatment, as part of the production of these compound have been released as chemical signals [23] from the microalgae. This explains why, fatty acid concentrations decreased under this stress, as

enzyme activity and the production of oxylipins remained normal, while the cells are changing their morphological characteristics, lowering its nutritional value and its digestibility [27] [39].

5. Conclusion

Despite cultures were not axenic, the PUA that was produced by and identified in *Skeletonema costatum* corroborated with the literature [11], confirming that any picoplanktoncontamination was insignificant to the experiment's outcome. In this work, the production of 2,4-heptadienal using EPA as substrate was addressed as a model for the synthesis of PUA. Among the treatments of aeration (control) and CO_2-enriched cultures, the results of the enzymatic utilization and consumption of substrate for the synthesis of aldehydes were virtually the same (difference of 4%). However, the culture subjected to UV radiation showed a 68% greater demand for EPA compared to the control and the CO_2 treatment, although the final production of PUA itself was not greater. This leads us to conclude that EPA consumption may have been made before cell disruption. In this way, the 2,4-heptadienal could be exuded during the stress period, confirming the positioning of chloroplasts near the cell wall, where EPA prevails. For future studies and bioassays, extracellular amounts should be quantified to clarify this behavior and the influence on the environment under the influence of CO_2 concentration and UV irradiation, using marine plankton communities enclosed in mesocosms systems.

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