Changes on foliar nutrients, proteins and photosynthetic pigments due to controlled exposures to sulfur dioxide in *Rhizophora mangle, Laguncularia racemosa* and *Conocapus erectus* individuals

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Abstract*—Rhizophora mangle, Laguncularia racemosa*, and *Conocarpus erectus* three months old individuals were exposed during 8 weeks, one hour daily at sulfur dioxide at three different concentrations (50, 100 and 150 ppb) using charcoal filtered air within an open-top chamber from August 8 to October 15 in 2010. Visible damages were identified, and changes on foliar nutrients concentrations, photosynthetic pigment content (chlorophyll *a*, chlorophyll *b*, total chlorophyll and total charotenoids) and soluble proteins concentrations were determined. All mangrove species studied showed sensitiveness to sulfur dioxide exposure levels, being red mangrove and buttonwood mangrove the most sensitive species to sulfur dioxide.

***Keywords*—**Campeche (México), Foliar visual symptoms, Mangroves, Open Top Chambers, Sulfur Dioxide.

I. Introduction

SULFUR dioxide still remains as an atmospheric pollutant due to many countries in development use fuels with a considerable content of sulfur resulting in an increase in sulfur dioxide concentrations (SO_2), in the lower atmosphere [1]. In Mexico for instance, the content of sulfur in combustibles is important yet and there is a standard which establishes that SO_2 concentrations in air ambient must not exceed 0.13 ppm (341 $\mu g\ m^{-3}$) 24-h average once a year and 0.03 ppm (79 $\mu g\ m^{-3}$) arithmetic annual average to protect susceptible population [2]. However there is not a secondary standard to control the SO_2 concentrations in order to protect vegetation and agricultural crops due to there are not enough studies about the effects of air pollutants in plants. Most of them are focused to forestry species located in the surroundings of big urban areas like Mexico City [3-6]. Plants show a special sensitiveness to most of air pollutants and suffer significant damages at concentrations lower than those necessary to cause harmful effects on human health. It is difficult to establish permissible maximum values to protect plants because of the negative effects manifestation depends on the plant constitution, foliar tissue kind and the specific specie [7, 33]. There are a great variety of factors influencing the plant responses when it is exposed to atmospheric pollutants, for example: duration of exposures (in a short time/ in a long time), plant age, and morphological characteristics as size of leaves, foliar area index, foliar cover, and so on [8, 9, 34, 35]. Tolerance of plants to SO_2 is related to its capacity to defend from the toxicity of active oxygen [10]. At low concentrations, SO_2 it can be considered as a nutrient, however, at high doses it behaves as a toxic. This pollutant is deposited by dry via on the cuticle or to spread toward the inside of the leaves through the stomas. Inside the leaves, SO_2 entries in contact with water to be converted in HSO_3^* (bisulfite, a free radical) or/and SO_3- (sulfite), which are more toxic to plants than the former compound [11]. These radicals destroy chlorophyll, cause lipids per oxidation and damages in the chloroplast, causing affections in the photosynthetic activity with a decrease in the uptake rate of CO_2. Finally, SO_4^{2-} is accumulated in the vacuoles of the cells. Acute damages are produced due to high concentrations of SO_2 and produce the death of cell tissue [12]. At lower concentrations, the effects are chronicle showing adverse damages without death of tissue; however this condition can occur in a long term with injuries externally visible. In wide leaf plants, acute

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symptoms can be presented as necrosis in the edges of the leaves, with an ivory color in some species, whereas other species presented brown coloration tending towards red or black. In narrow leaf plants, symptoms are observed as necrotic spots with an ivory color or bleached yellow on the apexes and edges of the leaf. Typical chronicle damages of this pollutant are chlorosis, it means, loss of chlorophyll [4]. When SO_2 is accumulated in the leaf, sulfur content has a trend to be increased, then analyzing the content of sulfur in foliar tissue it is possible to detect the observed damages attributable to SO_2 [13]. In active photosynthetically tissues of higher plants are found the photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and total charotenoids). These pigments are associated to specific proteins constituting systems binded to the chloroplast membranes structures. The main pigment is chlorophyll *a*, whereas, chlorophyll *b* and charotenoids are auxiliary in the light absorption, acting as photo-protectors pigments against photo oxidation and degradation toward pheophytines [13, 14]. Therefore, photosynthetic pigments quantification is an useful method to evaluate visible damages of vegetation affected by air pollutants [14]. Other damage indexes have been proposed as pheophytinization index (A_{430}/A_{665}), when the absorbances relations at these wave length decreases, it means that a degradation process has occurred from chlorophyll a to pheophytines. Charotenoids and chlorophyll degradation products has an absorbance relation A_{430}/A_{665} greater than chlorophyll a. So, A_{430}/A_{665} index determines the proportion of total pigments in regard to chlorophyll a. Likewise, proteins concentrations can be used as an indicator of damages due to atmospheric pollution on vegetation [15].

Atmospheric sulfur dioxide can induce changes in foliar nutrients balance. Many nutrients compete each other in order to be absorbed by the plant. For this reason, it is important to maintain the right proportions to avoid deficiencies in any nutrient. A potassium excess can compete with the absorption of calcium and manganese and a high iron/manganese ratio can result in a manganese deficiency. A high concentration of sulfur can diminish nitrates absorption [25]. Deficiencies in some nutrients as potassium can be showed severe interveinal chlorosis in *E.globulus ssp. Maidenii* [26]. Manganese deficiencies are common in soils with high pH values and tropical soils highly lixiviated. Common symptoms of manganese deficiencies appear first in younger leaves. Symptoms vary among the different species; some of them appear as grey dots, yellow spots or chlorotic lines. In oak individuals, symptoms are showed as grey dots [27]. Therefore, the effects of sulfur dioxide on foliar nutrients can be analyzed determining the levels of these nutrients before and after the SO_2 controlled exposures.

This study had as objective to use these biochemical responses (changes in foliar micronutrients, photosynthetic pigments and soluble proteins) as indicators of damages due to controlled SO_2 exposures in *Rhizophora mangle, Laguncularia racemosa*, and *Conocarpus erectus* three months old individuals and to determine the sensitiveness of each mangrove species. These species were chosen due to in Mexico, mangrove is a very important ecosystem because of its natural productivity and biodiversity [16]. One of them with a great cover is located in the surroundings of the Terminos Lagoon (more than 200,000 ha of shore) in the coastal zone of Mexican Gulf. The most important mangrove species in Terminos Lagoon mangrove are: *Rhizophora mangle* (red mangrove), *Laguncularia racemosa* (white mangrove) and *Conocarpus erectus* (known as buttonwood mangrove). Red mangrove is commonly located between the terrestrial and marine limits, in greater inmersion conditions and low salinity (from 0 to 37 ups). White mangrove it is located in severe soil immersion conditions with salinities ranged from 0 to 42 ups. Buttonwood mangrove occasionally is found under soil immersion conditions, it is located at high salinity concentrations (from 0 a 90 ups) [28].

II. Materials and Methods

A. Propagation and Fumigation

The study was conducted in a site located within the Botanical Garden of the Autonomous University of Carmen Island (Lat. 18º 38' 36''N, Long. 91º 49' 51'' W, elev. 2 m asl) on the southeast edge of Carmen City in Campeche, Mexico (Fig. 1). To carried out the sulfur dioxide exposures, Open-top chambers (OTC) of 3 m diameter x 3 m height were constructed according the scheme described by Heagle and Johnston [8]. These OTC were operated from August 8 to October 15 in 2010 during the day-time from 08:00 to 09:00 h. Three different SO_2 exposure concentrations were used: 50, 100 and 150 ppbv. Exposures were carried out everyday using charcoal filtered air (CF) at the three different concentrations for each studied species. A total of 28 individuals for each species were exposed for each concentration level (control samples were not exposed) and 7 replicates were analyzed for each treatment.

All seedlings were selected under homogeneous conditions of size, foliage and age (three months old). All plants received daily irrigation during the experiment, to keep the soil moisture close to field capacity. SO_2 was generated by a dynamic calibration system (Teledyne Advanced Pollution Instrumentation Model 700 and using a calibration standard with EPA protocol 0.483% Vol. Sulfur in Nitrogen making dilutions with filtered air using two mass flow controllers. SO_2 produced by this way was introduced into the Open-top-chamber and concentrations generated were verified by an SO_2 automatic analyzer (API Teledyne Series 100 A).

B. Visual assessment and harvest of plants

A first sampling was carried out before exposures and a second sampling was done after eight weeks at the end of the exposures. Two foliar tissue samples were collected, one of them was split in two parts, the first one was processed in fresh to determine photosynthetic pigments and the second one was dried at 80ºC for 48 h and used to determine soluble proteins

content. The second foliar tissue sample was used to determine total sulfur concentration and foliar micronutrients (Mn^{2+} and K^{+}). Once a week, visual assessments were carried out on plants, counting number the leaves and identifying possible visible injuries.

Fig. 1. Sampling site location.

C. Photosynthetic Pigments Content

Samples were processed and weighed immediately after collection. Pigments were extracted using a 80% acetone-20% water solution. Extracts were centrifuged at 1500 rpm during one minute and absorbances were measured in an UV-visible Hach DR201Q spectrophotometer at 663.2, 646.8, 470, 430, and 665 nm. Finally, chlorophyll total, chlorophyll *a*, chlorophyll *b* and total charotenoids contents were calculated per foliar mass unit using Lichtenthaler equations [14].

D. Soluble Proteins Concentrations

Samples were extracted with 10 ml of a buffer solution of potassium phosphate 0.1 M at pH 7.4. Proteins were precipitated adding 1 ml of trichloroacetic acid (at 10%) to 1 ml of the extract, then stirring and let it stand overnight in refrigeration. The next day, sample was centrifuged from 5 to 10 minutes at 10 000 rpm. The sediment obtained was dissolved into 1 ml of NaOH and let it stand for two hours. 50 ml of the sediment were taken and then 250 µl of distilled water and 1.7 ml of Folin reactive mixture were added and let it stand during 10 minutes [17]. Finally, absorbance was measured at 750 nm in an UV-visible Hach DR201Q spectrophotometer. The calibration curve was prepared from bovine serum, at concentrations in water at 200 µg/ml. The curve was prepared whenever required in the same way as samples.

E. Total Sulfur

Foliar tissue sample was digested in order to determine total sulfur according the technique described by Chapman and Pratt [29]. Dried samples were crushed and then digested in Teflon ® 100 ml vessels within Autoclave equipment. Then 1 ml of nitric acid was added and let it stand during 15 minutes, after, 0.5 ml of perchloric acid was added and it was digested again in the autoclave equipment for 15 minutes at 134ºC. A complete digestion is obtained when the sample shows a clear yellow color. Samples were filtered and one aliquot was diluted with distillated water, then 500 µl of sulfate conditioner solution and bario chloride crystals were added and stirred during one minute. The resulting suspension was quartz cell and absorbance was measured at 420 nm using a spectrophotometer UV-visible Hach DR201Q quantifying the turbidity.

F. Foliar Micronutrients (Mn^{2+} and K^{+}).

Foliar tissue samples were dried at 80ºC during 24 h in Fisher Scientific Drier equipment. Dried weight was registered for each sample. Then samples were crushed and then verted in Teflon® 100 ml vessels with 3 ml of nitric acid (65.2% solution) and let stand for 30 minutes. After, 1.5 ml of perchloric acid (70% solution) and 0.700 ml of sulfuric acid (98% solution) were added. Samples were digested in an autoclave equipment (Tutthauer 2340M) at 273°C for 30 minutes (volumes of used acid adjusted depending on the dried weight for each sample). A complete digestion is reached when a clear yellow liquid is obtained. Samples then were filtered and analyzed by atomic absorption spectrophotometer [29 - 31]. Calibration curves were prepared for K^{+} and Mn^{2+} from a 1000 ppm certified standard. Calibration curves for potassium ranged from 1-40 µg ml^{-1} with absorptions between 222 and 926 nm. Lecture time was of 45 s for each sample and three lectures were carried out for each sample. Calibration curves for manganese ranged from 0.5-3 µg/ml with maximum absorptions between 307 and 403 nm. Analysis were conducted by direct aspiration using an atomic absorption equipment Thermo Scientific iCE 3300 (A True Dual Atomizer AAS) using an air-acetylene flame, with combustible flow of 1.2 l min^{-1} and 10 l min^{-1} of air. PHOTRON HOLLOW CATHODE lamps for potassium and manganese were used.

G. Severity Scale

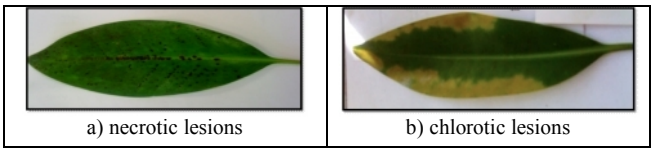
Damaged leafs were scanned and processed using Adobe Photoshop CS e Image Tool for Windows v. 1.28 (UTHSCSA1995-97). Foliar damaged percentage was used to obtain a severity scale by 2LOG v1.0 program [18]. Each class shows lower, middle and upper limits expressed as damaged area percentage. Each processed leaf was classified according to Horsfall-Barratt method [19].

III. Results

A. Visual Assesment

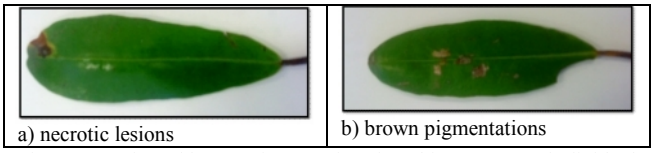
The three mangrove species showed visual damages as necrosis and chlorosis. Red mangrove showed symptoms of necrosis and chlorosis (Fig. 2 a and 2 b). In White mangrove individuals, necrosis was observed in some leaves (Fig. 3 a) and in some cases, brown pigmentations (Fig. 3 b). Buttonwood mangrove showed the most severe visual

damages, showing necrotic lesions and reddish brown pigmentations (Fig. 4 a and 4 b).



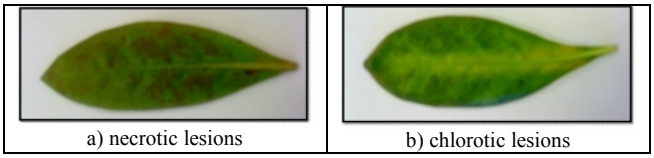
a) necrotic lesions b) chlorotic lesions

Fig 2. Necrotic and chlorotic lesions in Red mangrove individuals exposed to SO_2.



a) necrotic lesions b) brown pigmentations

Fig 3. Necrotic and brown pigmentations in White mangrove individuals exposed to SO_2.



a) necrotic lesions b) chlorotic lesions

Fig 4. Necrotic and chlorotic visual damages in Buttonwood mangrove individuals exposed to SO_2.

B. Photosynthetic Pigments Content

Percentages of change in the content of total chlorophyll, chlorophyll *a*, chlorophyll *b* and total charotenoids, before and after exposure to SO_2 at different concentrations are shown in Table I for the three mangrove species. Figure 5 (a, b, and c) shows photosynthetic pigments concentrations before (control individuals) and after exposure at three different concentrations of SO_2 (50, 100 and 150 ppb) for Rhizophora mangle (red mangrove), Laguncularia racemosa (white mangrove) and Conocarpus erectus (buttonwood mangrove), respectively.

It can be observed in Table I that all mangrove species showed a decrease in the content of photosynthetic pigments after expositions to SO_2, being greater at 150 ppb. The three mangrove species showed a considerable increase of the rate A_{430}/A_{665} absorbances indicating a possible degradation of chlorophyll by oxidation to pheophytine a, being greater at higher SO_2 concentrations (150 ppb) for red mangrove (Table II).

C. Soluble Proteins Concentrations

Concentrations of soluble proteins were determined before and eight weeks after the SO_2 exposition. In fig. 6 are shown the mean soluble proteins content for the three different treatments of SO_2 for the studied mangrove species. Red, white and buttonwood mangrove individuals showed a high correlation between SO_2 concentrations and soluble proteins content for the second sampling (0.860, 0.945, and 0.888, respectively) and it was found that 74%, 89.4% and 78.9% of the variation in the response could be due to the changes in SO_2 concentrations. These results are in agreement with those reported for other authors in OTC expositions to SO_2 concentrations, where, the soluble proteins concentrations were possibly increased as a response to the enzymatic activity induced by the oxidative stress derived from SO_2 exposition [20-25], however, it would be necessary to measure the enzymatic activity in later studies.

Table I. Decrease percentage of photosynthetic pigments for mangrove species exposed to SO_2.

Red Mangrove (DP)					White mangrove (DP)			
TR	Ca* %	Cb* %	Cx+c * %	Cl T %	Ca* %	Cb* %	Cx+c * %	Cl T %
50 ppb	24.12	32.05	35.8	24.84	4.35†	4.71	2.13	3.46
100 ppb	27.05	37.61	46.56	31.68	1.39	9.83	5.38	16.0
150 ppb	73.53	77.52	51.29	75.28	7.44	31.7	11.29	17.47

Buttonwood mangrove (DP)				
TR	Ca* %	Cb* %	Cx+c* %	Cl T %
50 ppb	13.42	17.17	13.42	15.73
100 ppb	25.62	53.93	25.62	37.07
150 ppb	55.73	75.06	55.73	61.83

Note:
TR.- Treatment Ca*.- Chlorophyll a Cb*.-Chlorophyll b
ClT.-Total Chlorophyll Cx+c*.-Total charotenoids DP%.-Decrease Percentage. † This treatment showed an increase percentage (4.35%)
Note: There were not changes in control individuals (They were not exposed).

Table II. Pheophytinization and increase percentage for the three Mangrove individuals exposed to SO_2.

RED MANGROVE			WHITE MANGROVE	
TR	Pheo-phytinization	IP %	Pheo-phytinization	IP %
50 ppb	1.752	0.809	1.716	3.392
100 ppb	1.778	2.307	1.811	9.103
150 ppb	2.980	71.468	1.862	12.183

BUTTONWOOD MANGROVE		
TR	Pheophytinization	IP %
50 ppb	1.901	0.976
100 ppb	2.113	12.194
150 ppb	2.446	29.93

Note:
TR.- SO_2 Treatment IP%.-Increase Percentage
Pheophytinization.- Absorbances Rate A_{430}/A_{465}
There were not changes in control individuals (They were not exposed).

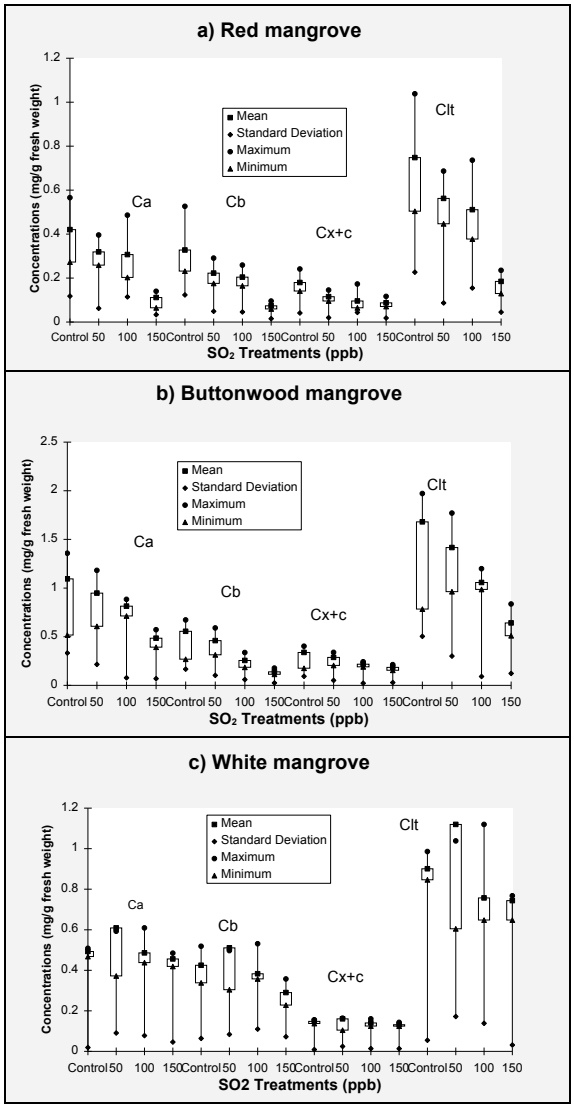


Fig 5. Photosynthetic pigments concentrations at the different SO2 treatments. Where: a: chlorophyll a., b: chlorophyll b., Cx+c :Total Charotenoids, and Cl t : Total Chlorophyll.

D. Total Sulfur

Total sulfur content increased after SO_2 exposures, being more important this increase at 50 and 100 ppb. At higher concentrations of SO_2 (150 ppb), total sulfur probably decreased due to this pollutant activated defense mechanisms closing the plant stomas.

Total sulfur concentrations increased for all exposed individuals after the treatments (Fig. 7, Fig. 8 and Fig. 9), being more evident at higher SO_2 concentrations (100 ppb), so, it can be inferred that the increase in total sulfur can be induced by an oxidative stress.

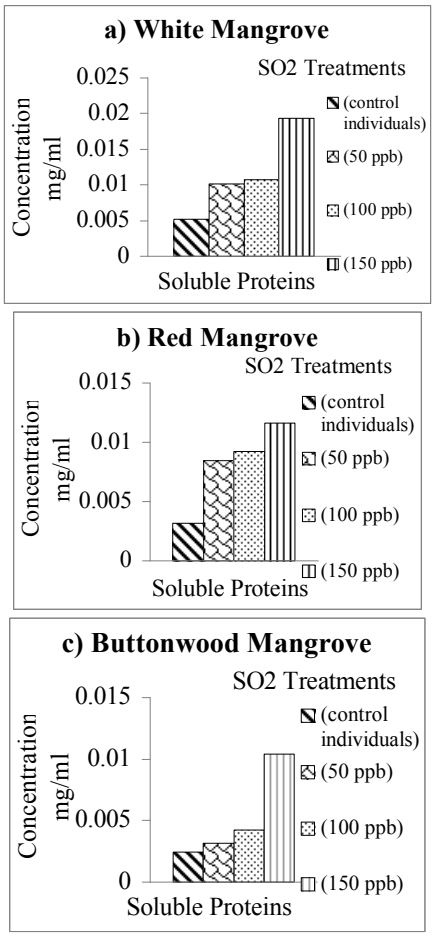


Fig 6. Soluble protein content for mangrove species at the different SO_2 treatments: a) White mangrove, b) Red mangrove and c) Buttonwood mangrove.

E. Foliar Micronutrients (K^+ and Mn^{2+})

Significant variations were found among SO_2 treatments (0 ppm, 50 ppm, 100 ppm and 150 ppm) when mean values were compared for Mn^{2+} and K^+ concentrations (Table IV).

Significant variations were observed among treatments for K^+ in White mangrove (Table IV, Figure 10). On the other hand, for this specie, Mn^{2+} showed a trend to be increased at higher SO_2 concentrations. For Red mangrove, an increase in Mn^{2+} concentrations was observed showing significant differences (Table IV, Fig. 11), whereas K^+ showed a decrease as SO_2 concentrations increased showing significant changes. In Buttonwood mangrove individuals, both foliar micronutrients (Mn^{2+} and K^+) increased at higher SO_2 concentrations (Table IV and Fig. 12).

Table III. Changes in Total sulfur foliar content at the different treatments for the three mangrove species.

TR SO_2 (ppb)	TOTAL SULFUR (IP)		
	White Mangrove	Red Mangrove	Buttonwood Mangrove
0	0.3052	0.1810(cd)	0.5096(c)
50	0.2298	0.3515(c)	0.6859(c)
100	0.3724	0.5429(ab)	0.4715(abd)
150	0.3167	0.3711(a)	0.7448(c)
ANOVA (Pr > F)	0.4932	0.0075*	0.0241*

Note:
TR.- Treatment. IP.- Increase percentage (%)
*Significant differences at 0.05. Different letters indicate that significant differences were found among treatments.

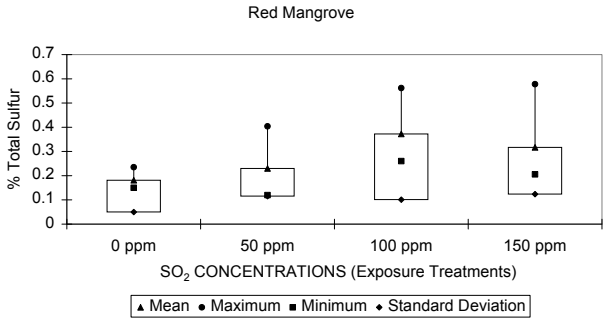


Fig. 7. Total sulfur concentrations for Red Mangrove at the different SO_2 treatments.

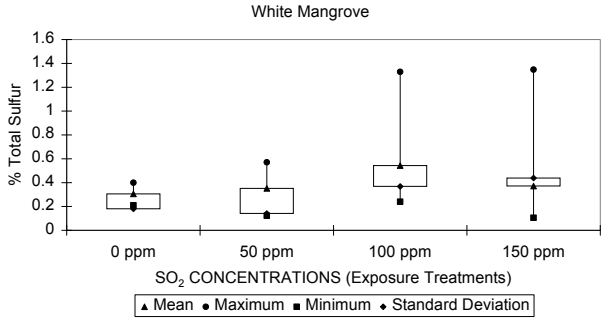


Fig. 8. Total sulfur concentrations for White Mangrove at the different SO_2 treatments.

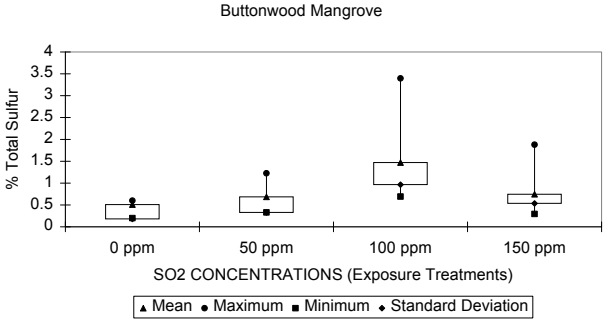


Fig. 9. Total sulfur concentrations for Buttonwood Mangrove at the different SO_2 treatments.

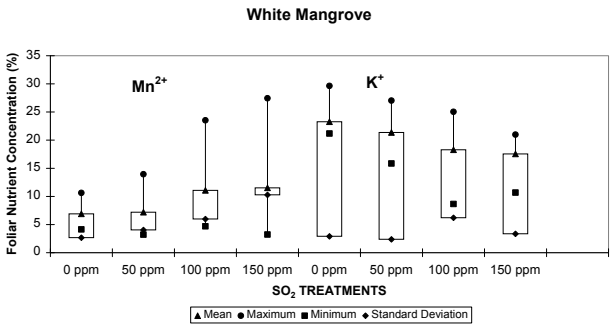


Fig. 10. Foliar nutrients content (%$Mn^{2+,}$ %K^{+}) for White Mangrove at the different SO_2 treatments.

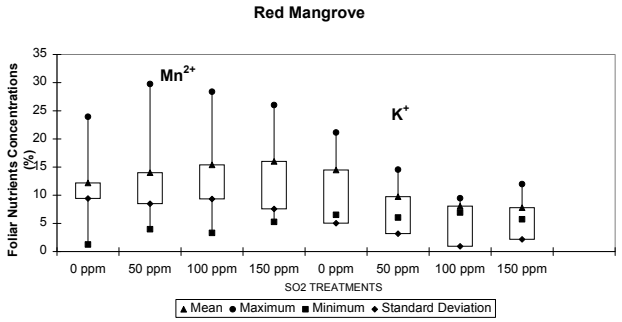


Fig. 11. Foliar nutrients content (%$Mn^{2+,}$ %K^{+}) for Red Mangrove at the different S O_2 treatments.

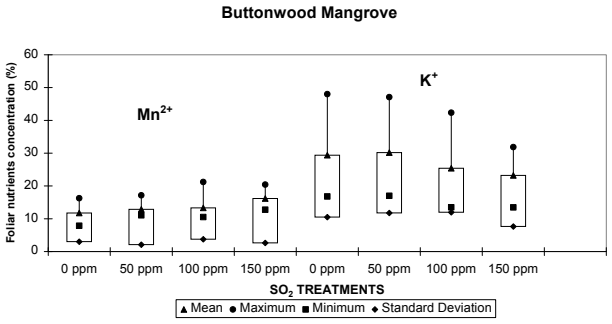


Fig. 12. Foliar nutrients content (%$Mn^{2+,}$ %K^{+}) for Buttonwood Mangrove at the different SO_2 treatments.

F. Severity Scale

A severity scale was obtained from representative images for each class. In Table V, the severity scale and class distribution for each mangrove species are shown. For Red mangrove 38% of the individuals were class 4 with the greater percentage of damaged area corresponding with the highest SO_2 concentration. Butonwood mangrove showed the highest percentage of damaged area for individuals of class 4 at 150 ppb of SO_2. White mangrove showed the same pattern .

Table IV. Mean concentrations for foliar micronutrients (%$Mn^{2+,}$ %K^{+}) for the three studied mangrove species.

SO_2 (ppb)	White Mangrove		Red Mangrove		Buttonwood Mangrove	
	%Mn^{2+}	%K^{+}	%Mn^{2+}	%K^{+}	%Mn^{2+}	%K^{+}
0	6.91	23.26	12.19	14.48	11.74	29.38
50	7.19	21.37	14.00	9.74	12.87	28.45
100	11.08	18.28	15.39	8.07	13.33	25.39
150	11.53	17.55	16.02	7.79	16.18	23.24
Anova (Pr > F)	0.10	0.006*	0.025*	0.023*	0.016*	0.17
*Significant differences at 0.05.						

Table 5. Severity scale and class distribution for each mangrove studied specie.

RED MANGROVE	Class 1	Class 2	Class 3	Class 4
Damaged Area	4% (50 ppb)	13% (100 ppb)	35% (50 ppb)	63% (150 ppb)
Class Distribution	14%	24%	24%	38%
WHITE MANGROVE	Class 1	Class 2	Class 3	Class 4
Damaged Area	4% (50 ppb)	12% (50ppb)	21% (100 ppb)	74% (100 ppb)
Class Distribution	5%	62%	19%	14%
BUTTON WOOD MANGROVE	Class 1	Class 2	Class 3	Class 4
Damaged Area	5% (50 ppb)	17% (100ppb)	44% (150ppb)	73% (150ppb)
Class Distribution	5%	38%	38%	19%

IV. CONCLUSION

According to visible damages, all the mangrove species showed symptoms when they were exposed to different concentrations of SO_2, being Red and Buttonwood mangrove the species that showed the most severe damages. Changes observed in chlorophyll *a* were related to visual damages in the three mangrove species. Total chlorophyll losses were greater at higher SO_2 concentrations being greater in Red and Buttonwood Mangrove.

All the mangrove species showed increases in soluble proteins as SO_2 concentrations were increased as sulfur source and even being benign if the plant has a deficiency of the roots [26]. This can be explained due to SO_2 can be metabolized and used SO_2 probably induced to the formation of reactive oxygen species in the plant tissue and this possibly caused the activation of antioxidant mechanisms (PR protein synthesis and other defense proteins) [27, 28]. It is possible to infer that damages and observed changes in soluble proteins and photosynthetic pigments were related to SO_2, being more evident at higher concentrations (150 ppb). Red and Buttonwood mangrove species were the most sensitive to he studied concentrations of SO_2.

It was found a relation among SO_2 concentrations, visible damages, changes in foliar total sulfur content and variations in micronutrients ($Mn^{2+,}$ K^{+}) concentrations for the three mangrove species. The increase in total sulfur could cause an imbalance of the foliar nutrients ($Mn^{2+,}$ K^{+}) [32].

In Red mangrove, there was a significant increase in foliar total sulfur. It is possible that SO_2 in high concentrations could affect biological mechanisms in the plant, activating the stomatic mechanism and resulting in deficiencies in other nutrients.

Red and buttonwood mangrove individuals showed decreases in K^{+} content for all SO_2 treatments. This nutrient showed a clear trend to be diminished for all white mangrove individuals. Mn^{2+} concentrations were higher after SO_2 exposures [31].

ACKNOWLEDGMENT

Authors thank to the Mexican Council of Science and Technology (CONACYT) for the economical support to carry out this research through the Mixed Funds of Campeche State Project 69526-2007.

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