Toxicological assessment of estuary water using the microalgae *Thalassiosira weissflogii*

Mario r. Alvear-Alayon
Process design and biomass utilization research group
University of Cartagena, Cartagena, Colombia
Email- malvearaamail.com

Alonso A. Doria-Manzur
Process design and biomass utilization research group & Biomedical, toxicological and Environmental Sciences Research group
University of Cartagena, Cartagena, Colombia
Email- adoriam@unicartagena.edu.co

Lesly P. Tejeda-Benitez
Process design and biomass utilization research group & Biomedical, toxicological and Environmental Sciences Research group
University of Cartagena, Cartagena, Colombia
Email- ltejedab@unicartagena.edu.co

Abstract- Cartagena Bay is localized in the northwestern region of the Colombian Caribbean coast, in the city of Cartagena. The industrial zone located in the western part of the bay, is not only between the most important ones in the country but also the source of numerous contaminants for the ecosystem, with few researches made regarding its water toxicity. This research used the microalgae *Thalassiosira weissflogii* as a biological model to evaluate the toxicological profile of Cartagena Bay. Ten water samples were taken across the bay and analyzed for phenols, hydrocarbons and heavy metals such as mercury, lead, and chrome. The *T. weissflogii* was exposed to different concentrations of the same water samples measuring cell density, growing rate and chlorophyll-A production as endpoints for the toxicity evaluation. The samples close to the industrial zone and to the connection with the Magdalena River were the most toxic ones, exhibiting cell density inhibition of 90%, reduction in the growth rate of 40 % and a diminution in the chlorophyll-a production up to an 87%. Additionally, these effects were correlated to the presence of lead in the water concluding that the microalgae *T. weissflogii* is a sensible and appropriate organism to evaluate the toxicity of estuaries.

Keywords – Estuary water; Microalgae; Heavy Metal; Toxicological Assessment.

I. INTRODUCTION

Cartagena Bay is geolocated in 10°16'25" N 75°30'35" O, with a surface of 82 km2 and a depth of 16 m. Due to its proximity to the Mamonal industrial zone the bay has been receptor of treated and untreated industrial wastewater as well as being the receptor of domestic wastewater for many years, before the construction of the submarine emissary. Additionally, it is known that the bay receives water from the Magdalena river through the artificial arm known as Dique Channel, coming with high contaminant and sediment load from the river course [1]. The wastewater dumping in the bay has been estimated to be around the 126388 m3/d, with considerable loads of hydrocarbons, pesticides, organic residues, phenols, ammonium and heavy metals such as mercury, cadmium, chrome, copper, nickel and lead [2], [3] being the coast close to the industrial zone, the most affected part, as a result of all the industrial activities held in the area, such as oil refining, agrochemicals, food processing, leather manufacturing and cement industry [4]. Some of these contaminants due to their properties can persist in the waters and thus affect the ecosystem, being the case of oysters from the Cartagena Bay which have been found to hold concentrations of cadmium in them [5].

Physical-chemical methods that analyze the presence of diverse contaminants and biological methods that determine the effects of contaminants on a subject can be used in order to evaluate the quality of surface water bodies such as Cartagena Bay. Biological essays are becoming more common due to their inexpensiveness and versatility, besides they provide data about environmental quality and effects of contaminants in living organism that let identify affected area as a result of human intervention [6], [7]. Many organisms can be used as environmental

bio-markers, as happens with nematodes [8] [9], plants [10][11], microalgae [12][13], among others. Microalgae are important organisms in aquatic ecosystems, reason why they are being used for environmental processes such as contaminants removal from wastewaters [14]-[16] or ecotoxicological essays [17]-[18]. The microalgae *Thalassiosira weissflogii*, is known for having both chlorophyll A and C pigments, and for accumulation of carotenoids [19]. It has been used in diverse researches as a biological model, such as the toxicological evaluation of the industrial and marine complex of Suape in Pernambuco, Brazil where it was found to be an appropriate model for the toxicological essays[20], in other studies it has also been used as biomarker, for herbicides glyphosate, cadmium, mixtures of herbicides and metals, and non-protein amino acids [21]-[25].

Estuary waters such as bays are transition zones where critical phytochemical and/or biological processes [26]. The bay of Cartagena lacks studies regarding its water quality, making it a necessity after the importance of the estuary in the economic development of Colombia. Besides studies covering entire estuaries are rare and normally not updated. There is limited understanding of such water bodies, as well as to how the biochemical cycle of the ecosystem is currently being affected by industrial contamination [27]. On another hand, few studies on T. weissflogii have investigated its behavior on direct exposure to estuary water of industrial zones, making it relevant to understand the behavior of its main endpoints, and how these results can affect and translate to other environments. In this work the microalgae *T. weissflogii* was used as a bio-marker to evaluate the toxicity of the Cartagena Bay water, by studying cell density, chlorophyll content, specific growth rate and LC50-72h, and transforming the acquired data to indicators that show the quality of Cartagena Bay water, identify the critical point along the estuary and the sensitivity of end-points against its direct exposure to waters in proximity of industrial zones.

II. MATERIALS AND METHODLOGY

2.1 Sampling –

Ten water samples were taken in Cartagena Bay, near the Mamonal industrial zone, 200 m away from the industrial wastewater discharge. The map with all the sampling points is shown in figure 1. The samples were taken in bottles with a capacity of 1 gal each (previously sterilized) at a depth of 2 m. physicochemical parameters as pH, dissolved oxygen, conductivity and salinity were measured in situ. Sterilized, ultrafiltered and dechlorinated sea water was used as the solution basis and as the negative control for the toxicity essays.

Figure 1. Sampling points over Cartagena's Bay (Mamonal area)

2.2. Chemical analysis –

Total hydrocarbons were determined according to the Standard Methods S.M. 5529 F. Phenols follow the ASTM D1783 protocol, consisting in a distillation, followed by a chloroform extraction and a mass spectrophotometry for quantification. Heavy metals as mercury, arsenic, chrome and lead were analyzed through spectroscopy of atomic absorption according to the 3111 methodology described in the Standard Methods.

2.3. Microalgae & Biological essays –

The T. weissflogii strain used was the CCMP 1051 supplied by the Bigelow laboratory of Ocean Sciences from the National Center for Marine Algae and Microbiota (NCMA) in East Boothbay, Maine (USA). Water samples were sterilized and then diluted to concentrations of 0 %, 25 %, 50 % and 100 % in 500 mL of filtered and sterilized seawater, being the concentration of 0 % the negative control. The microalgae were inoculated to a concentration of 1x104 cells/mL in each solution (ASTM, 2007) once they reached the exponential growth phase and the 99% were healthy and alive. Every culture was done twice and samples of 6 mL were taken each 24 h (starting at the moment of the inoculation) until 96 h.

2.5. Cell density –

Cell density was determined each 24 h in 20 mL of the inoculated solution using the haemocytometer counting method with a Neubauer chamber, which has been found to be an excellent method in counting cultures and high concentrations of small cells due to its affordability as well as its high precision [28][29].

2.6. Cell growth rate –

Measurements of growth rate, μ were conducted for each following the first order rate equation 1 using the number of cells in cultures before and after exposition. and the duplication time td using equation 2.

$$M = (\ln(N_2/N_1))/(t_2-t_1) \quad (1)$$

$$t_d = (\ln t_2)/ \mu \quad (2)$$

Where
μ: Growth rate (Number of divisions per day)
N_2: Number of cells per culture
N_1: Number of initial cells per culture
t_d: Duplication time
t_1: Initial time (days)
t_2: Final time (days)

The maximum growth rate was the highest value from each essay.

2.7. Chlorophyll production –

The chlorophyll content was measured every 24 h with a Turner Designs 10-005-R Fluorimeter, by taking 5 mL of the culture and introducing it in the cell equipment to make the quantification of the chlorophyll content in the solution expressed in μg/m3 [30].

2.8. Statistical analysis –

The treatments with P values inferior to 0.05 were considered statistically significant. The Dunnett test was used to determine the differences between the samples and the control.

III. EXPERIMENT AND RESULT

3.1. Chemical analysis

The results of the physicochemical parameters from the recollected water samples are shown in Table 1. Salinity, dissolved oxygen and pH values were found between the required ranges and protocols. The pH varied between 7.98 and 8.58, meaning there was no significant variation. On the other hand, salinity on the other hand was adjusted whenever it was necessary, and the dissolved oxygen oscillated between 5.25 and 7.7 mg/L.

Table 1. Physicochemical parameters from Cartagena's Bay water samples

PARAMETER	**Sampling points**									
	1	2	3	4	5	6	7	8	9	10
pH	8.50	8.46	8.49	8.58	8.58	8.59	8.58	7.98	8.33	8.21
Conductivity (mS/cm)	40.7	40.2	42	36.6	39.7	40	39.5	40.5	43.9	44.9
Salinity (%)	26.7	25.8	27.8	23.5	25.6	25.6	25.3	25.8	28.5	29.3
Dissolved Oxygen (mg/L)	6.60	7.70	5.98	5.25	7.40	7.49	7.10	6.82	5.79	6.60
Total Hydrocarbon (mg/L)	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
Aromatic Compounds (mg/L)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
DBO_5 (mg/L)	1.42	1.45	1.30	2.20	4.20	5.00	4.30	2.35	1.89	4.10
Kjeldahl Total Nitrogen (mg/L)	0.021	0.03	0.035	0.032	0.052	0.036	0.04	0.037	0.032	0.046
Nitrites (mg/L)	< 0.005	0.012	< 0.005	0.021	0.04	0.06	0.055	0.046	0.021	0.013
Nitrates (mg/L)	0.21	0.29	0.27	0.25	0.41	0.29	0.27	0.32	0.26	0.119
Phosphates (mg/L)	0.05	0.042	0.04	0.031	0.04	0.04	0.032	0.02	0.019	0.25
Phenols (mg/L)	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Mercury (µg/L)	0.24	0.3	0.27	0.25	0.42	0.24	0.29	0.32	0.49	0.41
Arsenic (µg/L)	0.27	0.22	0.25	0.24	0.19	0.26	0.25	0.24	0.31	0.39
Chrome (µg/L)	0.71	0.77	0.85	0.72	0.5	0.17	0.21	2.41	1.8	2.13
Lead (µg/L)	0.65	0.74	0.71	0.68	1.98	1.99	1.34	0.9	1.72	2.41
Total suspended solids (mg/L)	15.7	25.6	23.5	25.2	39.3	31.4	26.3	37.2	23.1	28.9

According to the chemical analysis, hydrocarbons, aromatic components or phenols were not detected in the different water samples as a result of their hydrophobic nature, which can only coexist in aquatic bodies due to their affinity with other components in response of extreme deterioration of the ecosystem [31]. Heavy metals in general were found in all samples, highlighting points 5, 9 and 10 with the highest concentration of mercury; points 5, 6, and 10 with high concentrations of lead and samples from point 8 with the highest concentrations of chrome. These concentrations are linked to the surroundings of the different points, such as 5 and 6 whose presence of heavy metals is influenced by its proximity to the main cement manufacturer of the zone, industry known for the liberation of heavy metals as mercury, cadmium, lead and others [32]. Point 8 is influenced by the wastewaters of the zone oil refinery and points 9 and 10 by agrochemical industries as well as the Dique Channel known for holding high concentrations of contaminants [1]. Arsenic concentrations were low in every analyzed sample. Heavy metal values were under the limit concentrations recommended by the World Health Organization (WHO).

3.2. Cell density

Fig 2 abc, represent the cell density taken each 24 h since the inoculation until the 96 h, for 25, 50, and 100 % solutions respectively. All essays reached their maximum density at 72 h of culture. A diminution in the growth curves occurred after that time meaning that all treatments had a toxicological effect in the microalgae, inhibiting the formation of new cells and thus decreasing the cell density proportionally to the samples concentrations, being this a common prove of the toxicological effects of contaminants as heavy metals [33] Additionally, the cultures done in the control treatment had a bigger population of cells. On the other hand, the exposure to samples from points 1 to 4, had a higher cell density compared to point 9 and 10 which had the lowest values of cell density, provoking an inhibition of the variable of 80 % and 97 % at 72 h of culture.

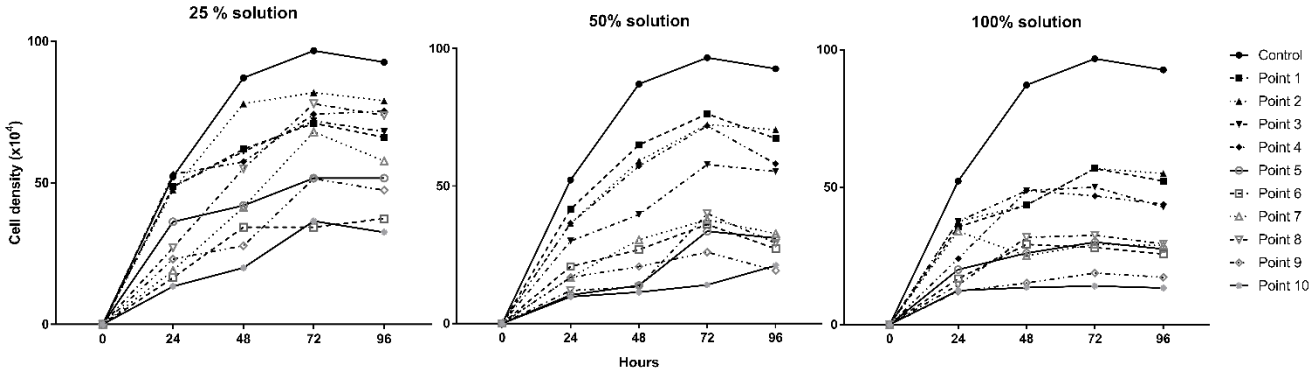

Figure 2. Cell density of *Thalassiosira weissflogii* after exposure to 25 % (a), 50 % (b) and 100 % (c) solutions of Cartagena's Bay water

The diminution on the cell density values is mainly related to the water content results from the chemical analysis, e.g. points 9 and 10, as well as being the points that affected the most the cell density of the microalgae, were also the points that presented some of the highest concentrations of heavy metals analyzed (mercury, arsenic, chrome and lead) mainly influenced by the agrochemical industries surrounding the area. Similar studies have found that heavy metals such as mercury and lead can decrease the cell production in microalgae, up to an 80% of inhibition [34], besides studies in the same microalgae (*T. weissflogii*) shows that the exposure to mercury , produces inhibitions in the division rate of algal cells [35], as a result of the high reactivity of heavy metals, which in their ionic forms can bond in the algal cell surface, affect the intracellular heavy metal ions and their chemical nature [36] as well as affecting the chloroplast of microalgae at high concentrations, influencing then the photosynthetic activities and thus the cell growth of the plant (a proportional variable with the cell density). Lead and arsenic, have as well been found to affect the cell density of microalgae directly, though the formation of starch and lipid reserves which form when growth is limited by environmental stresses [37][38], being some of the causes of the inhibition in the current microalgae.

3.3. Growth kinetics

The values of μmax, and td for each sample and concentrations are shown in Table 2. It can be seen that the growth rate trends to decrease from point 1 to 10 in every concentration evaluated from the Bay samples. The increase in the concentration had as well a negative effect on the growth rate of the microalgae showing both its dependence on the variable and the toxic effects that the bay water had on the microalgae, diminishing maximum specific growth rate

from the control value of 1.53 d-1 to values of 0.83 d-1 implying an inhibition percentage of almost 50%. Points with the lowest maximum specific growth rate were 9 and 10, for bay water concentrations of 50 and 100 %, similar to the results found with the cell density. The μ_{max} values have been found in different researches with different marine microalgae species to be influenced by the presence of heavy metals, such is the case of Chlorella vulgaris which after being exposed to them had a diminution on the specific growth rate to values of 0.02 – 0.34 d-1, being lower values to the normal values reported in the literature [39]. On the other side, a common consequence in microalgae after the exposition to wastewater is the inhibition of the μ_{max}, Lam et al. (2017) found that with the increment of the wastewater concentration of exposure, existed an increase in the variable inhibition, up to a 30%, indicating that the bay water samples used in this research contain high concentrations of contaminants in the latter sampled points, affecting the ecosystem. A similar situation happened with the exposure of *Botryococcus sp*. to industrial wastewater solutions of 25, 50 and 100 %, where the 50% concentration treatments caused a remarkable diminution of the specific growth rate, which according to the composition analysis is related to the remediating capability of the microalgae which suffered toxicological effects as a result of the bio removal of heavy metals that were in the wastewater [40].

Table 2. Bioessays Kinetic parameters

Concentration Points	***25%***		***50%***		***100%***	
	μ_{max} **(div/day)**	t_d **(days)**	μ_{max} **(div/day)**	t_d **(days)**	μ_{max} **(div/day)**	t_d **(days)**
Control					1.523	0.455
1	1.422	0.488	1.444	0.480	1.345	0.515
2	1.469	0.472	1.426	0.486	1.347	0.515
3	1.426	0.486	1.352	0.513	1.305	0.531
4	1.437	0.482	1.424	0.487	1.278	0.542
5	1.315	0.527	1.170	0.593	1.112	0.623
6	1.177	0.589	1.195	0.580	1.111	0.624
7	1.408	0.492	1.209	0.573	1.122	0.618
8	1.452	0.477	1.228	0.564	1.159	0.598
9	1.306	0.531	1.084	0.640	0.975	0.711
10	1.196	0.580	0.880	0.788	0.879	0.788

According to the cell density and growth rate results, after 72 h, the response of the cell density is more efficient, being consequent with Araujo & Souza-Santos [20], who used the T. weissflogii to evaluate this water quality of a bay in Brazil. The growth pattern of the microalgae is extremely important in the evaluation of water quality. The toxic substances can either inhibit or stimulate the growth, reason why the growth rate in the exponential phase and the final population density are adequate parameters to detect toxicity. These criteria are important for the detection of substances that inhibit or stimulate the growth and to characterize eutrophicated water bodies.

3.4. Chlorophyll production

Chlorophyll production analysis allows the quantification of the phototrophic organism development as well as being a correlated variable with both cell density and water toxicity [41]. Fig 3 abc, represents the cell density taken each 24 h since the inoculation until the 96 h, for 25, 50, and 100 % solutions, respectively. The maximum chlorophyll concentration in the essays was found at 72 h. All treatments had significant differences with the control. The Dunnet test indicates the response value of the control (0 % concentration) had statistically significant differences for each concentration dissolved and in every sample point. Samples 2 and 8 were the ones with the highest chlorophyll content in the 25 % treatment, however treatments of 100 % concentration evidenced that points 5, 7, 8, 9 and 10 were the most affected ones, consequent with the results obtained in the cell density results. Concentration increments of the water samples decreased the chlorophyll production in a direct dependence of the pollutant's concentration.

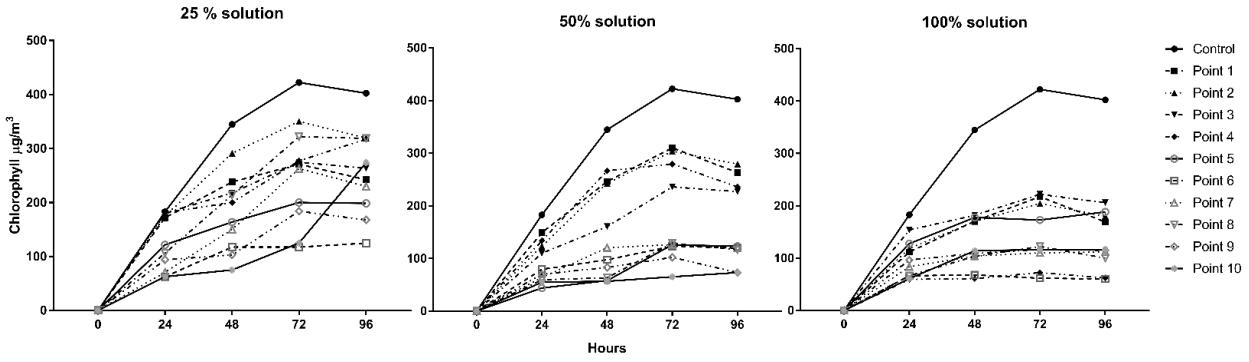

Figure 3. Chlorophyll concentration of *Thalassiosira weissflogii* after exposure to 25 % (a), 50 % (b) and 100 % (c) solutions of Cartagena's Bay water

Chlorophyll content has been widely studied as a marker for heavy metals toxicity essays in microalgae due to its importance in the photosynthetic process of microalgae as well as its affectation as a result to its exposure [42][43]. In other research it has been found that plants and unicellular organisms are heavily affected by the presence and formation of reactive oxygen species (ROS), as is the case of the chloroplasts, whose organization and structure is affected by the presence of heavy metals in the tomato plant [44]. In the case of microalgae, several studies have been done regarding the effects of contaminants in their photosynthetic system, being the situation of *Chlorella sorokiniana*, which after exposure to cadmium and lead presented a considerable diminution on the photosynthetic rate, mainly due to a diminution in the total content of chlorophyll [37], in the same way a research done in *Chlorococcum* showed that an increment in copper and cadmium concentrations led to a diminution of the chlorophyll content [45]. For marine microalgae, lead and cadmium cause diminution in the chlorophyll content at concentrations higher than 1 ppm, after 24 hours of exposure and even leading to the decease of the algae after 72 hours of exposure [46], besides other studies in *T. weissflogii*, shows that cadmium affects chlorophyll fluorescence through its decay, causing impacts on algal cells [47]. In our results, the lowest values of chlorophyll were found in the points closer to the industrial zone of Cartagena, which were as well the ones with the highest values of heavy metal content according to table 1, implying that a correlation between the low values of chlorophyll content and heavy metal presence is highly possible.

3.5. Multivariable correlation analysis

The values of the correlation coefficient R and their respective P value are shown in table 4 along with the most relevant physicochemical parameters and toxicological responses. The strongest association was found between lead concentration and cell density (R = -0.82; P = 0.010), maximum growth rate (R= -0.30; P= 0.030) and the duplication time at 72 h (R = 0.75; P = 0.010).

Table 3. Correlations between physicochemical parameters and toxic responses

R^2/(P)	**Hg**	**As**	**Cr**	**Pb**
Cell Density at 72 h	-0.49 (0.150)	-0.53 (0.110)	-0.07 (0.840)	**-0.82 (0.010)**
Chlorophyll content at 72 h	-0.57 (0.090)	0.07 (0.840)	-0.49 (0.140)	-0.30 (0.370)
μ_{max}	-0.58 (0.080)	-0.45 (0.200)	-0.35 (0.300)	**-0.30 (0.030)**
t_d at 72 h	0.59 (0.070)	0.46 (0.180)	0.36 (0.310)	**0.75 (0.010)**

The correlations found between lead and the studied parameters are a sign of the heavy metal toxicity on the microalgae, implying that the diminution on growth kinetics as well as cell density were mainly caused by the presence of Pb on the taken samples. Agreeing with this, table 1 shows high concentrations of Pb in the most affected points of sampling, being those the closest to the petrochemical industries and the Dique Channel. In concordance with these findings other studies have been reported that Pb alters chlorophyll-a content while also inhibiting growth and enzyme activity of algal cells in *Nitzschia closterium* [42], besides on *Euglena gracilis* increment in concentrations of lead showed a progressive diminution of the growth kinetics of the algae [49]. Marine microalgae *Porphyridium purpureum* and *Heterosigma akashiwo*, exhibited signs of oxidative stress which led to cell density diminution as well as growth inhibitions as a result of its exposure to heavy metal rich particle matter (from vehicular and industrial sources) present in aquatic mediums [12], similar to reports in *Scenedesmus obliquus*, which suffered inhibitions in both growth and lipid production after exposure to mixtures of heavy metals (essential and non-essential) [50], and more specifically, the exposure of the same microalgae directly to Pb produced signs of oxidative stress [51][52].

IV.CONCLUSION

In this article the microalgae *Thalassiosira weissflogii* was used in order to assess the toxicity of Cartagena's bay, which so far has not been studied from the toxicological point of view. Cartagena Bay was found to be affected by the proximity of industries. Physiochemical analysis showed imbalance on the ecosystem in those critical zones. Spatial gradients of cell density, specific growth rate and chlorophyll, showed lower values for points near petrochemical and cement industries and the Dique Channel, showing that main affectation of the water body comes from the accumulated sediments circulating through the Magdalena River, and thus implying a detrimental effect on the quality of water within time. This study helped to update the water state of the estuary, as well as helping the understanding of the ecosystem under stress conditions. Through the research it was found that the used microalgae showed good sensibility towards the evaluated contaminants in the water sample from Cartagena Bay, and assure its usage pertinence and convenience for direct use in estuary waters control essays. Cell density was the most remarkable end point (over the chlorophyll content and the growth kinetics) due to its higher variations from the control treatment.

REFERENCES

[1] L. Tejeda-Benitez, R. Flegal, K. Odigie, and J. Olivero-Verbel, "Pollution by metals and toxicity assessment using Caenorhabditis elegans in sediments from the Magdalena River, Colombia," Environ. Pollut., vol. 212, pp. 238–250, 2016, doi: 10.1016/j.envpol.2016.01.057.

[2] Invemar, "Informe del Estado de los Ambientes y Recursos Marinos y Costeros de Colombia," Ser. Publicaciones Period., no. 3, p. 200, 2017.

[3] M. Tosic, "La Bahía de Cartagena: Un destino final de la contaminación en Colombia," Arrastrando la montaña hacia el mar La Degrad. Ambient. en las cuencas hidrográficas y la Transf. impactos a los ecosistemas Mar., no. March, pp. 56–65, 2017, doi: 10.1016/j.

[4] J. Olivero-Verbel, K. Caballero-Gallardo, and N. Torres-Fuentes, "Assessment of mercury in muscle of fish from Cartagena Bay, a tropical estuary at the north of Colombia," Int. J. Environ. Health Res., vol. 19, no. 5, pp. 343–355, 2009, doi: 10.1080/09603120902749090.

[5] G. Manjarrez, I. Castro, and L. Utria, "Bioacumulación de cadmio en ostras de la bahía de Cartagena," Rev. Ing. Univ. Medellín, vol. 7, no. 13, pp. 11–20, 2008.

[6] L. Liu, H. Zhang, J. He, and S. Dong, "Investigation on the stress response of microbes in acute toxicity assay," Anal. Chim. Acta, vol. 1099, no. xxxx, pp. 46–51, 2020, doi: 10.1016/j.aca.2019.11.036.

[7] J. Xu, D. Wei, F. Wang, C. Bai, and Y. Du, "Bioassay: A useful tool for evaluating reclaimed water safety," J. Environ. Sci. (China), vol. 88, pp. 165–176, 2020, doi: 10.1016/j.jes.2019.08.014.

[8] H. Chen et al., "A review of toxicity induced by persistent organic pollutants (POPs) and endocrine-disrupting chemicals (EDCs) in the nematode Caenorhabditis elegans," J. Environ. Manage., vol. 237, no. September 2018, pp. 519–525, 2019, doi: 10.1016/j.jenvman.2019.02.102.

[9] Y. Nagar, R. Singh, and T. Parveen, "Chemosphere Toxicity assessment of parabens in Caenorhabditis elegans," Chemosphere, vol. 246, p. 125730, 2020, doi: 10.1016/j.chemosphere.2019.125730.

[10] H. Sharifan, J. Moore, and X. Ma, "Zinc oxide (ZnO) nanoparticles elevated iron and copper contents and mitigated the bioavailability of lead and cadmium in different leafy greens," Ecotoxicol. Environ. Saf., vol. 191, no. January, p. 110177, 2020, doi: 10.1016/j.ecoenv.2020.110177.

[11] X. Wang, W. Sun, and X. Ma, "Differential impacts of copper oxide nanoparticles and Copper(II) ions on the uptake and accumulation of arsenic in rice (Oryza sativa)," Environ. Pollut., vol. 252, pp. 967–973, 2019, doi: 10.1016/j.envpol.2019.06.052.

[12] K. S. Pikula et al., "Toxicity assessment of particulate matter emitted from different types of vehicles on marine microalgae," Environ. Res., vol. 179, p. 108785, 2019, doi: 10.1016/j.envres.2019.108785.
[13] W. Yang et al., "The combined toxicity influence of microplastics and nonylphenol on microalgae Chlorella pyrenoidosa," Ecotoxicol. Environ. Saf., vol. 195, no. March, p. 110484, 2020, doi: 10.1016/j.ecoenv.2020.110484.
[14] H. Chang et al., "Highly efficient reverse osmosis concentrate remediation by microalgae for biolipid production assisted with electrooxidation," Water Res., vol. 174, p. 115642, 2020, doi: 10.1016/j.watres.2020.115642.
[15] H. Nhat et al., "Micropollutants cometabolism of microalgae for wastewater remediation: Effect of carbon sources to cometabolism and degradation products," Water Res., vol. 183, 2020, doi: 10.1016/j.watres.2020.115974.
[16] J. Nie et al., "Bioremediation of water containing pesticides by microalgae: Mechanisms, methods, and prospects for future research," Sci. Total Environ., vol. 707, p. 136080, 2020, doi: 10.1016/j.scitotenv.2019.136080.
[17] K. S. Pikula, A. M. Zakharenko, V. Aruoja, K. S. Golokhvast, and A. M. Tsatsakis, "Oxidative stress and its biomarkers in microalgal ecotoxicology," Curr. Opin. Toxicol., vol. 13, pp. 8–15, 2019, doi: 10.1016/j.cotox.2018.12.006.
[18] M. S. Podder and C. B. Majumder, "Toxicity and bioremediation of As(III) and As(V) in the green microalgae Botryococcus braunii: A laboratory study," Int. J. Phytoremediation, vol. 19, no. 2, pp. 157–173, 2017, doi: 10.1080/15226514.2016.1207601.
[19] M. Latasa, "Pigment composition of Heterocapsa sp. and Thalassiosira weissflogii growing in batch cultures under different irradiances," Sci. Mar., vol. 59, no. 1, pp. 25–37, 1995.
[20] C. F. C. Araújo and L. P. Souza-Santos, "Use of the microalgae Thalassiosira weissflogii to assess water toxicity in the Suape industrial-port complex of Pernambuco, Brazil," Ecotoxicol. Environ. Saf., vol. 89, pp. 212–221, 2013, doi: 10.1016/j.ecoenv.2012.11.032.
[21] V. Filimonova et al., "Ecotoxicological and biochemical mixture effects of an herbicide and a metal at the marine primary producer diatom Thalassiosira weissflogii and the primary consumer copepod Acartia tonsa," Environ. Sci. Pollut. Res., vol. 25, pp. 22180–22195, 2018.
[22] V. Filimonova et al., "Effects of a herbicide and copper mixture on the quality of marine plankton," Ecotoxicol. Environ. Saf., vol. 156, no. 2018, pp. 9–17, 2018, doi: 10.1016/j.ecoenv.2018.02.038.
[23] S. Lage, L. Ström, A. Godhe, and S. Rydberg, "The effect of exogenous β-N-methylamino-L-alanine (BMAA) on the diatoms Phaeodactylum tricornutum and Thalassiosira weissflogii," Harmful Algae, vol. 58, pp. 85–92, 2016, doi: 10.1016/j.hal.2016.08.005.
[24] C. Wang, X. Lin, L. Li, and S. Lin, "Differential Growth Responses of Marine Phytoplankton to Herbicide Glyphosate," PLoS One, vol. 11, no. 3, pp. 1–20, 2016, doi: 10.1371/journal.pone.0151633.
[25] Y. Wu, Y. Yuan, H. Yuan, W. Zhang, and L. Zhang, "Predicting cadmium toxicity with the kinetics of phytochelatin induction in a marine diatom," Aquat. Toxicol., vol. 207, no. 2019, pp. 101–109, 2019, doi: 10.1016/j.aquatox.2018.12.008.
[26] S. Marcinek et al., "Dissolved organic matter dynamics in the pristine Krka River estuary (Croatia)," Mar. Chem., vol. 225, p. 103848, 2020, doi: 10.1016/j.marchem.2020.103848.
[27] M. Rodrigues, A. Cravo, P. Freire, A. Rosa, and D. Santos, "Temporal assessment of the water quality along an urban estuary (Tagus estuary, Portugal)," Mar. Chem., vol. 223, no. December 2019, p. 103824, 2020, doi: 10.1016/j.marchem.2020.103824.
[28] C. Camacho-Fernández, D. Hervás, A. Rivas-Sendra, M. P. Marín, and J. M. Seguí-Simarro, "Comparison of six different methods to calculate cell densities," Plant Methods, vol. 14, no. 1, pp. 1–15, 2018, doi: 10.1186/s13007-018-0297-4.
[29] M. LeGresley and G. McDermont, "Counting chamber methods for quantitative phytoplankton analysis - haemocytometer, Palmer-Maloney cell and Sedgewick-Rafter cell," in Microscopic and molecular methods for quantitative phytoplankton analysis, B. Karlston, C. Cusack, and E. Bresnan, Eds. 2010, p. 114.
[30] M. Preuss and G. C. Zuccarello, "Comparative studies of photosynthetic capacity in three pigmented red algal parasites: Chlorophyll a concentrations and PAM fluorometry measurements," Phycol. Res., vol. 67, no. 2, pp. 89–93, 2019, doi: 10.1111/pre.12356.
[31] Y. Liu and E. B. Kujawinski, "Chemical composition and potential environmental impacts of water-soluble polar crude oil components inferred from esi FT-ICR MS," PLoS One, vol. 10, no. 9, pp. 1–18, 2015, doi: 10.1371/journal.pone.0136376.
[32] Y. Arfala, J. Douch, A. Assabbane, K. Kaaouachi, H. Tian, and M. Hamdani, "Assessment of heavy metals released into the air from the cement kilns co-burning waste: Case of Oujda cement manufacturing (Northeast Morocco)," Sustain. Environ. Res., vol. 28, no. 6, pp. 363–373, 2018, doi: 10.1016/j.serj.2018.07.005.

[33] M. C. Naorbe and A. E. Serrano, "Effects of heavy metals on cell density, size, specific growth rate and chlorophyll a of tetraselmis tetrathele under controlled laboratory conditions," AACL Bioflux, vol. 11, no. 3, pp. 589–597, 2018.
[34] R. Zamani-Ahmadmahmoodi, M. B. Malekabadi, R. Rahimi, and S. A. Johari, "Aquatic pollution caused by mercury, lead, and cadmium affects cell growth and pigment content of marine microalga, Nannochloropsis oculata," Environ. Monit. Assess., vol. 192, no. 6, 2020, doi: 10.1007/s10661-020-8222-5.
[35] Y. Wu, Y. Zeng, J. Y. Qu, and W. Wang, "Mercury effects on Thalassiosira weissflogii : Applications of two-photon excitation chlorophyll fluorescence lifetime imaging and flow cytometry," Aquat. Toxicol., vol. 110–111, pp. 133–140, 2012, doi: 10.1016/j.aquatox.2012.01.003.
[36] K. K. I. U. Arunakumara and X. Zhang, "Heavy metal bioaccumulation and toxicity with special reference to microalgae," J. Ocean Univ. China, vol. 7, no. 1, pp. 60–64, 2007, doi: 10.1007/s11802-008-0060-y.
[37] S. Carfagna, N. Lanza, G. Salbitani, A. Basile, S. Sorbo, and V. Vona, "Physiological and morphological responses of Lead or Cadmium exposed Chlorella sorokiniana 211-8K (Chlorophyceae)," Springerplus, vol. 2, no. 1, pp. 1–7, 2013, doi: 10.1186/2193-1801-2-147.
[38] S. Díaz, P. de Francisco, S. Olsson, Á. Aguilera, E. González-Toril, and A. Martín-González, "Toxicity, physiological, and ultrastructural effects of arsenic and cadmium on the extremophilic microalga Chlamydomonas acidophila," Int. J. Environ. Res. Public Health, vol. 17, no. 5, 2020, doi: 10.3390/ijerph17051650.
[39] F. M. Santos, L. P. Mazur, D. A. Mayer, V. J. P. Vilar, and J. C. M. Pires, "Inhibition effect of zinc, cadmium, and nickel ions in microalgal growth and nutrient uptake from water: An experimental approach," Chem. Eng. J., vol. 366, no. February, pp. 358–367, 2019, doi: 10.1016/j.cej.2019.02.080.
[40] J. I. Onalo, H. M. Matias-peralta, and N. M. Sunar, "Growth of Freshwater Microalga , Botryococcus sp . in Heavy Metal Contaminated Industrial Wastewater," Joirnal Sci. Technol., vol. 6, no. 2, 2014.
[41] R. F. Serpa Ibáñez and A. Calderón Rodríguez, "EFECTO DE DIFERENTES FUENTES DE NITRÓGENO EN EL CONTENIDO DE CAROTENOIDES Y CLOROFILA DE CUATRO CEPAS PERUANAS DE Dunaliella salina Teod.," Ecol. Apl., vol. 5, no. 1–2, p. 93, 2006, doi: 10.21704/rea.v5i1-2.895.
[42] T. Gan et al., "Optimal chlorophyll fluorescence parameter selection for rapid and sensitive detection of lead toxicity to marine microalgae Nitzschia closterium based on chlorophyll fluorescence technology," J. Photochem. Photobiol. B Biol., vol. 197, no. July, p. 111551, 2019, doi: 10.1016/j.jphotobiol.2019.111551.
[43] Y. K. Leong and J. S. Chang, "Bioremediation of heavy metals using microalgae: Recent advances and mechanisms," Bioresour. Technol., p. 122886, 2020, doi: 10.1016/j.biortech.2020.122886.
[44] P. L. Gratão et al., "Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium," Environ. Exp. Bot., vol. 67, no. 2, pp. 387–394, 2009, doi: 10.1016/j.envexpbot.2009.06.017.
[45] C. E. Qiu, Q. J. Kuang, Y. H. Bi, G. X. Liu, and Z. Y. Hu, "Response of Chlorococcum sp. AZHB to copper and cadmius stress," Bull. Environ. Contam. Toxicol., vol. 77, no. 5, pp. 772–778, 2006, doi: 10.1007/s00128-006-1130-8.
[46] W. Mu, Y. Chen, Y. Liu, X. Pan, and Y. Fan, "Toxicological effects of cadmium and lead on two freshwater diatoms," Environ. Toxicol. Pharmacol., vol. 59, no. 2010, pp. 152–162, 2018, doi: 10.1016/j.etap.2018.03.013.
[47] Y. Zeng, Y. Wu, D. Li, and W. Zheng, "Two-photon excitation chlorophyll fluorescence lifetime imaging : a rapid and noninvasive method for in vivo assessment of cadmium toxicity in a marine diatom Thalassiosira weissflogii," pp. 1653–1663, 2012, doi: 10.1007/s00425-012-1703-1.
[48] F. Yadegarnia, N. Id, H. Azimzadeh, A. M. Arani, A. S. Id, and B. K. Id, "Ecological risk assessment of heavy metals from cement factory dust," Environ. Heatlh Eng. Manag. J., vol. 6, no. 2, pp. 129–137, 2019, doi: 10.15171/EHEM.2019.15.
[49] B. Khatiwada et al., "Probing the role of the chloroplasts in heavy metal tolerance and accumulation in Euglena gracilis," Microorganisms, vol. 8, no. 1, 2020, doi: 10.3390/microorganisms8010115.
[50] K. Napan, L. Teng, J. C. Quinn, and B. D. Wood, "Impact of heavy metals from flue gas integration with microalgae production," Algal Res., vol. 8, pp. 83–88, 2015, doi: 10.1016/j.algal.2015.01.003.
[51] M. Danouche, N. El Ghachtouli, A. El Baouchi, and H. El Arroussi, "Heavy metals phycoremediation using tolerant green microalgae: Enzymatic and non-enzymatic antioxidant systems for the management of oxidative stress," J. Environ. Chem. Eng., p. 104460, 2020, doi: https://doi.org/10.1016/j.jece.2020.104460.
[52] B. Debelius, J. M. Forja, L. M. Lubia, Á. DelValls, and L. M. Lubián, "Toxicity and bioaccumulation of copper and lead in five marine microalgae," Ecotoxicol. Environ. Saf., vol. 72, no. 5, pp. 1503–1513, 2009, doi: 10.1016/j.ecoenv.2009.04.006.