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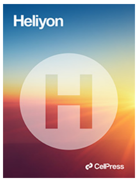

Research article

Comparative study between immobilized and suspended *Chlorella* sp in treatment of pollutant sites in Dhiba port Kingdom of Saudi Arabia

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ABSTRACT

Dhiba port has a strategic location near the Neom project. Various anthropogenic activities contributed to the discharge of metals, metalloids and oil spills in the aquatic system and caused environmental pollution. Microalgae are the best microorganisms in aquatic conditions known to be capable of eliminating contaminants. In this work the *Chlorella* sp. was isolated from seawater, the metals, metalloids were determine using ICP- OES (Inductively Coupled Plasma-Optical Emission Spectrometer) and hydrocarbons were determine using GC-MS in different five sites in Dhiba port, after and before treated with *Chlorella* sp, and immobilized *Chlorella* sp. The growth parameters (optical density and pigment contents) of *Chlorella* sp and immobilized *Chlorella* sp. were investigated during 14 days of grown. The results showed that the most contaminated site by metals and metalloids was site no 3, by Sb, As, Be, Se, and Zn with concentrations 0.07546, 0.05709, 0.09326, 0.4618, and 0.00979 mg/L respectively, and site no 1 was the most contamination by organic compounds, so the site no 1 and site no 3 were chosen to test the efficiency of *Chlorella* sp. and immobilized *Chlorella* sp. to remove hydrocarbons and both metals and metalloids. *Chlorella* sp. and immobilized *Chlorella* sp. had completely removed metals and metalloids that were present in site 3. There were only 6 compounds remained, after treatments with immobilized alga in site 1. Immobilized *Chlorella* sp. is the most effective than suspended *Chlorella* sp in reduces the number of organic compounds in contaminated area. It is an economic tool due to simplifying harvesting and then retaining for further processing.

1. Introduction

Since coastal and maritime tourism is a new vital economic activity and pioneer in advancing the economic diversity of the Kingdom of Saudi Arabia, beaches and coastal areas' quality are based on the nearby areas' environmental quality, including ports. Ports activities of tourism and transportation, including ship discharges of ballast water, loading and unloading of cargo, and accidental discharge of oil and other chemicals in the sea have various environmental impacts that affect the extent of physiochemical and biological constituents run in the port water (Bastami et al., 2015; Jahan and Strezov, 2017). These impacts are significant, ranging from heavy metal contamination, oil pollution, fecal pollution to the introduction of exotic species through ballast water uptake and discharge (Luna et al., 2019; Niimi, 2004; Sany et al., 2013; Suneel et al., 2019). Due to their high toxicity to the marine environment, several studies have been examining the bioremediation of Polycyclic aromatic hydrocarbons (PAHs) and heavy metals. The term "Bioremediation" has broadly defined as the usage of microorganisms or their products to remove or eliminate pollutants. The bioremediation of contaminants in the marine environment is carried out mainly by diverse microorganisms. Algae are low-cost sorbents for the elimination of oil and can impact the fate and vehicle of spilled oil (Mishra and Mukherji, 2012). Many studies have depicted that alga eliminate nutrients such as nitrogen and phosphorus (Kim et al., 2013; Amenorfenyo et al., 2019), heavy metals (Tam et al., 2001; 11. Romera et al., 2006; Kaplan, 2013), toxic hydrocarbon, inorganic toxins, and pesticides (Abe et al., 2003; Hultberg et al., 2016; Kottuparambil and Agusti, 2018), from enclosing water by adsorption and absorption (Kızılkaya et al., 2012; El-Naggar et al., 2018) of bioaccumulation abilities of the cells (Leong and Chang, 2020). Algae bioremediate phenolics using different mechanisms such as adsorption, bioaccumulation, biodegradation, and photodegradation (Wu et al., 2022). Several species of microalgae have shown an

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influential role in the remediation of both heavy metals, and hydrocarbons. Nweze and Aniebonam (2009) reported the probability of using naturally present algae isolated from a puddle near Nsukka Fire Service Station to remove hydrocarbon from water polluted with petroleum products. Microalga *Chlorella kessleri* could grow at different crude oil concentrations (0.5, 1, and 1.5%), mixotrophically solely and in combination with *Anabaena oryzae* (Hamouda et al., 2016a). Wang et al. (2018) argued that the acclimation process is a potential method of wastewater treatment using *Chlorella vulgaris*. *C. vulgaris* showed high efficiency of biodegradation under a low concentration of 0.5% and 1% of crude oil. The growth reached a high level even with the 2% of crude oil in an experiment of 15 days (El-Sheekh et al., 2013). *C. vulgaris* can be used for the biodegradation of crude and refined oil in contaminated aquatic environments (Samuel et al., 2020). *Ankistrodesmus braunii* and *Scenedesmus quadricauda* were able to eliminate more than 70% of phenol from olive-oil mill wastewaters within five days (Al-Dahhan et al., 2018). Hamouda et al., (2016b) reported that *Scenedesmus obliquus* was able to remove heavy metals Pb, Cd, Cu, and Mn, from wastewater under different conditions. Sharma and Khan (2013) noticed that *Chlorella minutissima* was a better efficient alga in removing heavy metals from polluted habitats than *Scendesmus* spp and *Nostoc muscorum*. *Chlorella* sp. effectively removed by 76%–96% of cadmium and 78%–94% of nickel under laboratory condition (Rehman and Shakoori, 2004). Other results showed that *C. vulgaris* was able to remove up to 70% and tolerate 200 mg/L of As^{5+} present in the growth medium (Jiang et al., 2011). Immobilization of microalgae simplifies biomass harvesting, contributes to the resistance of cultures against stresses, and simplifies the development of hardware for cultivation which leads to higher productivity of cultures and to an increase in the efficiency of wastewater treatment (Vasilieva et al., 2016). Moreover, the immobilization of marine microalgae could overcome the problems of high water volumes and very low concentrations of marine environments (Moreno-Garrido et al., 2005). The biomass produced during wastewater treatment may also be used to produce biofuels, bioplastics, and exopolysaccharides (Silva et al., 2022). de Jesus et al. (2019) tested the chemical stability of immobilised *Desmodesmus subspicatus* by counting the remaining beads over seven days of immersion in different solutions. They found that the recovery was 100% in all cases. Murujew et al. (2021) showed that recycled alginate from algae beads at a recovery rate of approximately 70% can be obtained where the recovery of alginate can bring a 60% net operational cost reduction. The disadvantages of immobilized biomass includes: added cost related to immobilization, higher mechanical diffusion resistance, and lower absorbance capacity (Blaga et al., 2021). The use of a microalgae consortium could be better than a monoculture system in terms of biomass and lipid productivity and pollutant removal (Beacham et al., 2017). The use of microalgae in many aspects could be improved by their immobilization into alginate beads. Benasla and Hausler (2021), found that the immobilized green alga *Raphidocelis subcapitata* accumulated 37.9 ± 3.8% of their dry weight in lipid with approximately 3.6 times higher than direct cultures which makes it a promising candidate for biodiesel production. The decolorization and nitrogen removal reached rates of 80% and 71%, respectively, from textile wastewater at a pH of 12, 1000 lux intensity and 150 microalgae beads (Kassim et al., 2018). Furthermore, the immobilized *C. vulgaris* were employed to capture CO_2 from the flue and exhaust gas and produced biomass yields approximating 100 g DM/dm^3 (Dębowski et al., 2021). Sarkheil et al. (2022) compared sodium alginate immobilized *Scenedesmus* spp. and *Chlorella* spp. and sodium alginate beads without microalgae in recirculating aquaculture system for water purification. They found that hat the use of sodium alginate–immobilized microalgae as a biofilter resulted in a significant reduction in water total ammonia nitrogen and total phosphorus concentrations and thus improved the survival rate and growth performance of African cichlid (*Labidochromis lividus*) fingerlings. Lee et al. (2020) investigated the optimal alginate bead size for the nutrient removal using *C. vulgaris* and suggested the cell immobilization technology as an efficient technique for the wastewater treatment systems. Algal biorefinery concept with wastewater treatment will reduce the overall residual waste component of biomass and provide efficient utilization of algae biomass for fuel generation (Chandra et al., 2019). The recent studies on microalgae biomass have revealed that there is a huge potential for co-products that can be recovered after the bioremediation processes. The microalgae were classified as potential candidates in biorefinery processes due to their capability of producing multiple products (González-Delgado and Kafarov, 2011). This microalgae biomass refining includes mechanical, chemical post-harvest, mechanical or chemical disruption, or selective extraction of microalgae products and co-products (Barsanti and Gualtieri, 2018). The combination of wastewater bioremediation with the mass of microalgae improved the conventional treatment process and environmental impacts. From a bio-economy viewpoint, biofuels and value-added product recovery are important areas of technological intervention (Ummalyma et al., 2021). The main objectives of the current study are to investigate the contamination pattern in Dhiba port marine environment, analyze water in the five sites inside the port related to contaminations of heavy metals and organic carbon, and study the potential of fresh alga *Chlorella* sp. and immobilized *Chlorella* sp for possible treatment pollutants that exist in the most contaminated two sites in port. It also aims to compare between fresh alga *Chlorella* sp and immobilized alga for possible remediation of heavy metals and organic compounds in the most contaminated two sites.

2. Materials and methods

2.1. Sampling location and collection

The study location is Dhiba port (27° 34′ N to 34° 33′ E), located at the north-western corner of the Kingdom of Saudi Arabia. It is the nearest Saudi port to the Suez Canal and the Mediterranean basin countries' ports, including Turkey 593 miles, Greece 491 miles, and 988 miles to the nearest French ports (Saudi Ports Authority, 2021). Thus, it acquires unique importance in its strategic location near the NEOM project, which is the Saudi Crown Prince Mohammed bin Salman's vision and a centerpiece of Saudi Arabia's 2030 Vision (NEOM, 2021). The registration of vessel arrivals from various ports worldwide showed that a total number of 12029 vessels had navigated the port during the period 2005–2019 (Saudi Ports Authority, 2021) (Supplementary Table S1).

Water samples were collected from the water surface on 25th January 2020 from five different Dhiba port locations (Supplementary Figure S1) in dark graduated bottles. For heavy metals and hydrocarbons determination, samples were stored in the dark at a low temperature of 4 °C until examination.

2.2. Isolation and identification of Chlorella sp.

The green microalga *Chlorella* sp was isolated from water samples collected from Thuwal beach, Red Sea, Saudi Arabia (22°16′35.0″N 39°05′22.3″E). The isolation was done through a serial dilution technique followed by plating on a modified BG-11 medium (Rippka, 1988; Stanier et al., 1971). The microalga identification was based on Algae Base (Guiry and Guiry, 2019), Stanier et al. (1971) and Bellinger and Sigee (2015).

2.3. Preparation of immobilized microalga in alginate beads

For each flask, 30 ml of algal suspension in its exponential growth phase were harvested by centrifugation at 3000 rpm for 10 min. The supernatant was then decanted, and the volume of sediment was adjusted to 2 ml with sterilized deionized water. After that, the concentrated algal suspension was mixed with 2% (w/v) sodium alginate solution and dropped into a 2% calcium chloride solution using a sterilized burette. Beads were left to harden overnight then rinsed with distilled water.

2.4. Growth assessment

2.4.1. Optical density

For microalga growth and pigments measurement, alginate beads should be dissolved in 100 ml of 0.1 M sodium citrate solution with pH 5 that was prepared by adding 10 ml of sodium citrate to a specified number of beads at 45 °C with stirring, and the beads would dissolve within one hour. Then, the solution was centrifuged at 5000 rpm for 5 min. After that, the supernatant was decanted, and the volume was adjusted to 3 ml with sterilized water. Alga's biomass was determined every three days by measuring the algal suspension's optical density at 600 nm using a SHIMADZU UV-2600 spectrophotometer, Japan.

2.4.2. Pigments determination

A known volume of culture was centrifuged at a speed of 3000 rpm for 10 min. After that, the algal pellets were treated with a known volume of methanol, kept in the water bath for 30 min at 55 °C, and then centrifuged again. The absorbance of the pooled extracts was registered by SHIMADZU UV-2600 spectrophotometer, Japan, at 666, 653, and 470 nm. Calculations were made according to the formulae devised by Costache et al. (2012) for chlorophyll a, chlorophyll b, and carotenoids.

2.5. The bioremediation experiment design

Two treatments were conducted triplicate to study the potential of *Chlorella* sp in the bioremediation of metals, metalloids, and the biodegradation of hydrocarbons. For each treatment, two Erlenmeyer flasks (250 ml) containing 150 ml of sterilized seawater were enriched with nitrogen and phosphate source (0.225 g of $NaNO_3$ and 0.006 g of K_2HPO_4). Under a laminar flow cabinet, three flasks were cultivated with the algal beads, and the other three were cultivated with the residue of 30 ml of centrifuged algal cells of each flask. The cultures were incubated under the conditions of 12:12h light: dark and at 25 °C temperature and slight aeration for two weeks (Supplementary Figure S2).

2.6. Chemical parameters analysis

2.6.1. Metals and metalloids

Laboratory analysis was carried out for metals (Aluminum, Barium, Cadmium, Chromium, Cobalt, Copper, Iron, Lead, Manganese, Nickel Silver, Titanium, Vanadium, and Zinc), and metalloids (Antimony, Arsenic, Beryllium, and Selenium) were determination before and after the experiment. Metals and metalloids were measured using ICP- OES (Inductively Coupled Plasma-Optical Emission Spectrometer). Agilent Technologies 720 ICP-OES (Agilent Technologies Inc., Santa Clara, CA, USA). Axial. Seawater samples were filtered then diluted 10 times. No digestion needed. Calibration and its range were done with 5, 2 and 1 ppm standard solution of each metal element and were prepared in 2% nitric acid.

2.6.2. Determination of petroleum hydrocarbons

Petroleum derivatives were extracted from 100 ml of seawater of each sample. The pH was adjusted with 1 M HCl to get a pH < 3. Organic compounds were extracted via liquid-liquid phase extraction thrice, using 10 ml and 5 ml of dichloromethane ($CH_2 Cl_2$). The organic lower phase was collected, and the moisture was removed by adding about 2g anhydrous sodium sulfate (Na_2SO_4). The clear extract was transferred to a test tube and evaporated with a gentle nitrogen gas stream at room temperature. The sample concentrated to about 10 μL (Suhrhoff and Scholz-Böttcher, 2016). The analysis was performed using a gas chromatograph (GCMS-QP2010 Plus, Shimadzu, Japan) equipped with a mass spectrometer with a fuse-silica capillary column (30 m × 0.25 mm ID × 0.25 μm-Rtx®-1, Restek, USA) was used. Helium was used as a carrier gas, and the temperature programming was 60–300 °C, 1/5 min. GC-MS internal library search was used to identify the organic compounds. The analysis was conducted before and after the experiment.

2.7. Statistical analysis

Experiments were conducted in triplicate and expressed as ± standard error of the mean. The data were compared by analysis of variance one-way and three-way ANOVA. Significance was determined using Duncan's multiple range tests ($p \leq 0.05$). Analysis was carried out using MS Excel (2016) and SPSS (Version 16).

3. Results and discussion

The results showed the number of vessels arrived Dhiba port from ports worldwide. 12029 vessels, through 14 years ago, denoting anthropogenic activities during these years and hence accumulation of waste products (Supplementary Table S1). Results also showed nineteen heavy metals investigated in five Dhiba port sites (Supplementary Table S2).

The World Health Organization (WHO) resulting a guideline value of 3 μg/L for antimony in drinking water (WHO, 1993). The doses of arsenic in natural waters, including open ocean seawater, generally ranges between 1 and 2 μg/L 40 (Hindmarsh et al., 1986). The safe doses of beryllium concentration of 0.1 μg/L (Lytle et al., 1992). The levels of selenium in surface water range from 0.06 μg/L to about 400 μg/L (Lindberg, 1968) so the concentrations above the previous denoted the contamination. The results demonstrated different concentrations of As, Be, and Se among nineteen investigated metals. The Be and Se were found at all five locations. Site no. 1 was contaminated by Sb, As, Be, and Se with concentrations 0.03168, 0.04126, 0.08985, and 0.199 mg/L respectively where the site no. 3 was contaminated by the previous metalloid Sb, As, Be, Se, in addition to Zn metal with concentrations 0.07546, 0.05709, 0.09326, 0.4618, and 0.00979 mg/L respectively. The concentrations of metals and metalloids in surface seawaters varied from one site to another. Zinc metal has been depicted only in the third site, which has a high total concentration of metals (Ms) compared with other sites, so it was chosen for the bioremediation experiment.

The organic compounds concentrations were estimated before the experiment (Supplementary Table S3). The level of total organic compounds ranged from 0.21 ppm to 0.55 ppm. The first site was the most highly polluted with organic compounds. It showed particular compounds that were not found in the other sites (1,1,3-Trimethylcyclopentane and Diethyl Phthalate), so it was chosen for the biodegradation experiment.

3.1. Assessment of Chlorella. sp. growth

Green microalga *Chlorella* sp. is halotolerant, proliferating, and growing in marine environments and favorably using it for bioremediation and biodegradation experiments. Luangpipat and Chisti (2017) indicated that *C. vulgaris* thrived in a full-strength seawater medium and enhanced lipid productivity by nearly 2-fold compared to freshwater. *Chlorella* sp. is a microgreen alga that is usually found in seawater (Maghfiroh et al., 2018) *C. vulgaris* was cultivated in a photobioreactor with controlled conditions of NaCl that was extracted from salt from brackish and seawater (Sahle-Demessie et al., 2019) Figure 1a-b shows the suspension of *Chlorella* sp and *Chlorella* sp beads growth that was measured by optical density at 600 nm. The growth of immobilized cells reached a high level compared to fresh cells.

The immobilized cells grown in sample one reached their highest growth level close to the eighth day of cultivation, and optical density reached 2.4 nm. In the case of site 3, the maximum growth of alga beads reached at (O.D 1.8 nm) after ten days and at (O.D 0.4 nm) within seven days in the case of fresh alga. A plausible explanation for this result is that the third site was mostly contaminated with metals and metalloids as a result of the negative effect of alga growth, whereas the first site was more contaminated with organic compounds. In this case, it is preferred for alga to grow under mixotrophic conditions and use organic compounds as the sole carbon source.

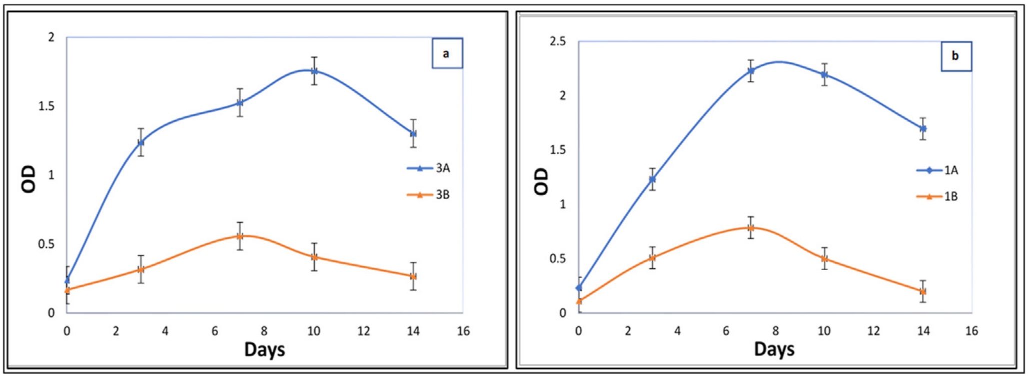

Figure 1. Growth curves of immobilized (A) and suspended (B) *Chlorella* sp cells measured as optical density 600 nm. (a) Growth on sample one for metals and metalloid bioremediation experiment. (b) Growth on sample three for organic compounds biodegradation experiment.

Melo et al. (2018) proved that when *C. vulgaris* was grown under mixotrophic conditions, the cellular productivity increased, and it becomes more effective to remove agro-industrial by-products. The highest growth rate of *C. vulgaris* was obtained when grown under mixotrophic conditions than when grown under photoautotrophic conditions (Abreu et al., 2012). Bansal (2019) investigated whether the growth of *C. vulgaris* and *C. protothecoides* were promoted under mixotrophic conditions when using glycerol as a carbon source. When *Chlorella* spp. was grown mixotrophically on glucose, it produced superior biomass concentration than heterotrophic and photo-autotrophic conditions (Cheirsilp and Torpee, 2012). The results indicated that immobilized cells were higher in growth than suspension cells in both two sites. *C. vulgaris* immobilized by sodium alginate produced a higher amount of cells than suspension cells (Abu Sepian et al., 2019; Rushan et al., 2019). The immobilization technique can offer higher micro-algal cell density, which is useful for diminishing lag period (Ide et al., 2016), due to it being less sensitive to stress conditions (Lee et al., 2020).

Results in Table 1 showed that the effect of seawater was taken for both sites on chlorophyll-a, Chlorophyll-b, and Carotenoids contents and the content of both immobilized and suspension *Chlorella* sp cells. Chlorophyll-a contents are more promote in suspension *Chlorella* sp that was grown in seawater taken from site 3 within ten days. Contaminations in site 3 were more abundant with metals and metalloid and less content of hydrocarbons, so the alga was grown under photoautotrophic conditions. Chlorophyll-a in autotrophic was promoted by alga growth, which revealed the production of necessary pigments by *Chlorella* sp for photosynthesis, the only pathway for the metabolism of phototrophic microalgae (Mohammad Mirzaie et al., 2016).

The same trends were observed for Chlorophyll-b contents but within seven days with suspended alga (Table 1). After 10 days of cultivation, Chlorophyll-*a* and *b* were decreased. This decrease may be due to the decrease in nutrient content in media such as nitrogen and phosphorus. Chlorophyll contents decrease could be due to decreasing nitrogen in media (Li et al., 2008). The highest level of carotenoid contents of *Chlorella* sp was 666.14 μg mL^{-1} recorded on the 14th day with suspension cells grown in the seawater sample taken from site one, followed by of *Chlorella* sp that was grown on the same days but in site three. Both sites on day 14 of growth had the stress conditions such as site three that had the most contamination by metals, site one was mostly contaminated by organic compounds, and when incubations period to day 14, the nutrients of media decreased and accumulation of toxic compounds.

Green alga such as *Chlorella* can be overproducing secondary carotenoids under stress culture conditions like nitrogen limitation, cultivation period, and salt stress (Santhosh et al., 2016). A high amount of carotenoids were produced by *S. platensis* after 7 and 11 days of incubation with various concentrations of oil (El-Sheekh et al., 2013). The three-way ANOVA, shown in Table 2, demonstrated the variable among different sites, alga treatments, and the incubation periods related to Chl *a*, Chl *b*, and carotenoid. The results indicated that there was a significant interaction among sites, alga treatments (immobilized and suspended), and incubation times in relation to pigments contents in *Chlorella* sp. In site 1 there were significant interactions among the types in treatments (suspension, alga, and immobilized) and incubations periods and also in case of site three (Table 3).

3.2. The bioremediation of metals and metalloid

The results of metals and metalloids concentrations analysis of site 3 demonstrated that when applied suspension *Chlorella* sp and immobilized *Chlorella* sp, heavy metals were completely disappeared. The removal efficiencies of these metals were affected by their initial concentrations. *Chlorella* sp presented a high efficacy in removing 100% of Sb, As, Se, and Zn. This finding is consistent with the work of Zou et al. (2020) where their results showed that *C. vulgaris* was highly efficient in removing Se and Cr collectively and separately. The bioremediation process was effective using both suspended and immobilized *Chlorella* sp cells. Thus, our results may also be explained by enhancing the growth rate of *Chlorella* sp during the exponential phase. This result is in agreement with

Table 1. Mean ± SEM levels of Chlorophyll-a, Chlorophyll-b, and Carotenoids contents of immobilized and suspended cells during two weeks in both sites.

		Immobilized cells				Suspended cells			
		3rd	7th	10th	14th	3rd	7th	10th	14th
Chl-*a*	Site 1	1.7590 ± 0.090^{de}	2.8603 ± 0.145^{g}	0.7746 ± 0.279^{a}	0.7261 ± 0.279^{a}	3.8327 ± 1.000^{i}	1.6714 ± 0.069^{d}	2.7945 ± 0.145^{g}	2.9531 ± 1.000^{j}
	Site 3	1.0438 ± 0.271^{b}	1.4280 ± 1.000^{c}	1.7205 ± 0.069^{d}	0.9944 ± 0.271^{b}	3.5895 ± 1.000^{h}	2.3724 ± 1.000^{f}	8.6548 ± 1.000^{k}	1.8361 ± 0.090^{e}
Chl-*b*	Site 1	3.3528 ± 0.376^{i}	3.5713 ± 1.000^{o}	1.4355 ± 1.000^{e}	0.5768 ± 1.000^{c}	5.4971 ± 1.000^{n}	4.171 ± 1.000^{j}	4.3690 ± 1.000^{k}	2.3913 ± 1.000^{g}
	Site 3	1.8529 ± 1.000^{f}	2.6673 ± 1.000^{h}	3.2932 ± 1.000^{i}	0.2538 ± 1.000^{b}	6.2402 ± 1.000^{l}	7.2719 ± 1.000^{m}	2.60 ± 1.000^{a}	1.2563 ± 1.000^{d}
Car	Site 1	220.2 ± 1.000^{g}	474.3 ± 1.000^{l}	97.3609 ± 1.000^{b}	78.9526 ± 1.000^{a}	426.77 ± 1.000^{n}	276.65 ± 1.000^{h}	298.87 ± 0.428^{i}	666.14 ± 1.000^{o}
	Site 3	137.04 ± 1.000^{d}	171.18 ± 1.000^{h}	213.34 ± 1.000^{f}	106.36 ± 1.000^{c}	417.44 ± 1.000^{k}	297.05 ± 0.428^{i}	479.23 ± 1.000^{m}	555.77 ± 1.000^{n}

*Values in the same column with different letters are significantly different at $p \leq 0.05$ according to three-way ANOVA followed by Duncan's test.

Table 2. Three-way variance (ANOVA) among site, alga treatments and incubation days on the Chl *a, b* and carotene contents of *Chlorella* sp.

Source	Chl-*a*			Chl-*b*			Carotene		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
Intercept	1	105.372	.000	1	10.684	.047	1	577500	.000
site	1	3.122	.087	1	57.299	.005	1	4863	.000
Alga treatments	1	9.104	.005	1	1.911	.261	1	86380	.000
Incubation periods (days)	3	1.222	.318	3	19.783	.000	3	975.415	.000
site * alga treatments	1	.033	.858	1	.031	.870	1	33.563	.000
site * Incubation periods (days)	3	2.821	.054	3	.010	.998	3	7232	.000
Alga treatments * Incubation periods (days)	3	4.216	.013	3	.415	.755	3	15530	.000
site * alga treatments * Incubation periods (days)	3	5.290	.004	3	4506	.000	3	6406	.000

Table 3. Tests of between-subjects effects in related to site dependent pigments contents of alga (*Chlorella* sp).

	Source	Chl a			Chl b			Carotene		
		df	F	Sig.	df	F	Sig.	df	F	Sig.
Site 1	Intercept	1	31770	.000	1	45760	.000	1	257600	.000
	Alga treatments	1	3539	.000	1	881.459	.000	1	31070	.000
	days	3	258.122	.000	3	3537	.000	3	4744	.000
	Alga treatments * incubation periods	3	1356	.000	3	2527	.000	3	15550	.000
Site 3	Intercept	1	36.213	.000	1	42420	.000	1	358700	.000
	Alga treatments	1	2.559	.129	1	3673	.000	1	67640	.000
	Incubation periods (days)	3	1.876	.174	3	5078	.000	3	2816	.000

Li et al. (2019), who studied the biotreatment of mixed wastewaters with MnO_2 industry by *C. vulgaris*. However, heavy metals (Cu, Cr, Pb, and Cd) were removed from dyes by *C. vulgaris* was significantly enhanced when endophytic bacterial strain MN17 inoculum was applied (Mubashar et al., 2020). Marine green alga *Chlorella* sp. NKG16014 exhibited the highest elimination of Cd due to cell adsorption and intracellular accumulation (Matsunaga et al., 1999). Sorption capacities of heavy metals such as Cu, Zn, Cd, and Ni by *C. vulgaris* were attained at the lowest biomass concentration (Abdel-Hameed, 2010) The metals and metalloids in the current study's contamination levels can be correlated to contamination caused by the port activities.

3.3. The biodegradation of petroleum hydrocarbons

Results in Supplementary Table S3 investigated the organic compounds that were existent in five sites in Dhiba port. The results demonstrated that site no .1 was much contaminated by hydrocarbons, so it was shown for applied *Chlorella* sp and immobilized *Chlorella* sp for possible bioremediation and cleaning. Results in figures 2a,b, and Table 4 revealed the effect of *Chlorella* sp and immobilized *Chlorella* sp on removing organic compounds that exhibited in site one. Both treatments were effective in the biodegradation of hydrocarbons but the highest biodegradation rate of organic compounds was observed with immobilized *Chlorella* sp. Muñoz et al. (2003) suggested that the microalgae release biosurfactants that could improve phenanthrene degradation. Madadi et al. (2016) recommended using *C. vulgaris* and surfactants to treat wastewaters from petroleum industries. *C. vulgaris* had a high ability in remediation of crude oil hydrocarbons within 14 days (Xaaldi Kalhor et al., 2017). The results showed a complete absence of the previous hydrocarbons and a presence of new compounds. These new compounds may be due to the conversion of hydrocarbons into intermediate compounds (Okoh, 2006). This result is in agreement with El-Sheekh et al. (2013) who proved the ability of the *C. vulgaris* to degrade n-alkane and PAHs. Several studies established the vital role of *C. vulgaris* in the biodegradation of PAHs in the ecosystem (Abdel-Shafy and Mansour 2016; Wang and Zhao 2007).

Results indicated that immobilized *Chlorella* sp was more efficient to degrade organic compounds. This cells immobilization technology would accelerate the nutrient uptake rate of microalgae for improving the efficiency of seawater treatment. Immobilized *Chlorella* sp cells under optimal

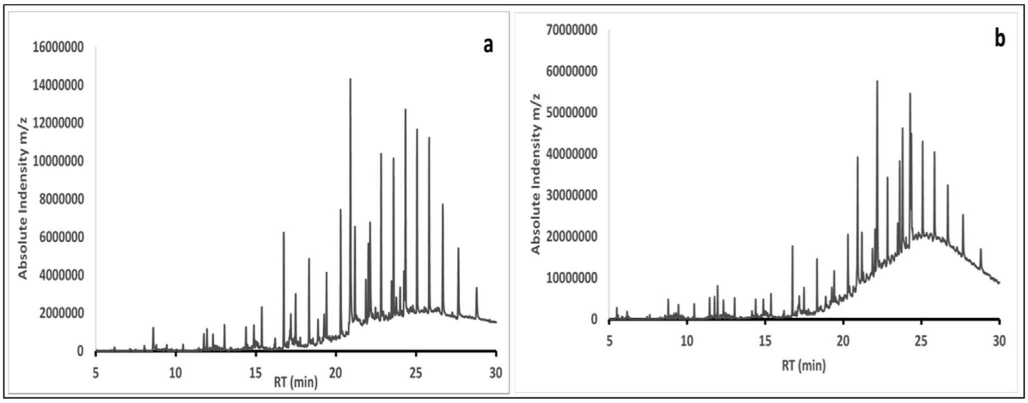

Figure 2. GC/MS chromatogram of residual organic compounds after 14 days of incubation. (a) with immobilized *Chlorella* sp. cells. (b) with suspended Chlorella sp. cells.

Table 4. Concentrations in ppm of organic compounds after experiment with both suspended and immobilized *Chlorella* sp. cells.

Compound Name	Molecular formula	Suspended *Chlorella* sp.	Immobilized *Chlorella* sp
7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	8.054702	2.845451
1-Docosene	$C_{22}H_{44}$	ND*	3.646065302
9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	15.82382	6.322348652
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	37.79498	3.058358
Hexatriacontane	$C_{36}H_{74}$	20.76870683	7.992108
Tetrapentacontane	$C_{54}H_{110}$	40.48991343	3.985724123
Tetratriacontane	$C_{34}H_{70}$	8.444043	ND
n-Heptadecanol-1	$C_{17}H_{36}O$	5.708477	ND
Octacosanol	$C_{28}H_{58}O$	8.281868	ND
13-Docosenamide	$C_{22}H_{43}NO$	26.94995	ND
Tetracosane	$C_{24}H_{50}$	5.985861	ND
Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{25}H_{46}O_6$	14.97093	ND

* ND-Not detected.

conditions are effectively efficient in eliminating nonylphenol from contaminated water (Gao et al., 2011). Liu et al. (2012) reported that immobilized *Chlorella sorokiniana* GXNN 01 was vital species for use in wastewater treatment. Immobilized *C. vulgaris* was capable of removing NH_4 and N from wastewater (Fraile et al., 2005). Immobilized cells have amplified reaction rates due to superior cell density (Mallick, 2002).

4. Conclusions

Dhiba's port is a strategic location and one of the most vital ports in Saudi Arabia where human activities are expected to be increased when the NEOM project will release. There were some contaminations indicated by metals, metalloid and organic compounds that appeared in five sites of Dhiba's port. Suspension and immobilized microgreen alga *Chlorella* sp were proved efficient for bio-remediate metals and metalloid. Immobilized *Chlorella* sp was the most effective in removing heavy metals that existed in two sites than suspension alga, there are many intermediate compounds were found after treatments by both immobilized and fresh alga, but the number of compounds were less than found in water treatments. Harvesting beads from media is very simple, and could be applied in biofuel production after bioremediation processes. It should be repeated study every year on port at different sites that represent the port activates, used different algae, and many factors effects in bioremediation processes.

Declarations

Author contribution statement

Ragaa Hamouda: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Abrar Alhumairi: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Amna Saddiq: Contributed reagents, materials, analysis tools or data.

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Declaration of interest's statement

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