Evaluation of *Cladophora laetevirens* (Chlorophyta) and *Patella caerulea* (Mollusca) as bioindicators for heavy metals Pollution at Al-Hanyaa Mediterranean Coast, Libya.

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ABSTRACT

This study was designed to evaluate the new record seaweed Cladophora laetevirens (Dillwyn) Kützing (Chlorophyta: Cladophorales) and Patella caerulea Linnaeus (Mollusca: Gastropoda) as bioaccumulating agents. The samples of two species were collected from polluted and unpolluted (reference) sites from the rocky shores of Al-Hanyaa coasts, Libya. Zinc (Zn), cadmium (Cd), lead (Pb) and copper (Cu) were determined using voltammetric technique. The results exhibited that, Cu occupied the highest level (559.15 \pm 68.55 μ g g⁻¹ fresh weights) among metals accumulated by Cl. laetevirens in polluted site, followed by Pb and vice versa in unpolluted site, where Pb attained $179.71 \pm 31.19 \ \mu g \ g^{-1}$ fresh weight. Despite that Cd has minimal concentration levels (either in Cl. laetevirens or P. caerulea tissues, at both sites), it recorded maximal bioaccumulation factors (BAFs) for *Cl. laetevirens* in both sites. Metal pollution index (MPI) recorded in a descending order as: Cl. *laetevirens* > P. *caerulea* gut > P. *caerulea* muscle for both sites. Cadmium was positively correlated with Cu and Pb in both sites. The present data and their analysis proved the consistency, appropriateness, tolerance and reliability of *Cl. laetevirens* as a bioindicator regardless the metal concentration levels in seawater. These results confirmed that *Cl. laetevirens* is the strongest accumulator for Cu, Pb and Cd while *P*. *caerulea* is a potential accumulator for Zn.

Keywords: Cladophora laetevirens; Patella caerulea; heavy metals; Al-Hanyaa; Libya; bioindicators

INTRODUCTION

Heavy metal pollution is one of the most important challenges on the earth's surface at the present time, where the discharge of dangerous inorganic compounds of natural and anthropogenic sources, widely pollute the aquatic system (Marcovecchio, 2004). The same author reported that the contamination, with non-degradable heavy metals, is more threatened the estuaries and semiclosed coastal ecosystems, especially near highly populated or industrial areas.

Although some heavy metals are essential in trace concentrations for the protection of body metabolism, growth and general well being of living organisms, they can lead to poisoning of organisms at higher concentrations (Bradh, 2005). Heavy metals in sediment samples can be changed by organic matter content, grain size, soil composition, pH and oxidation-reduction potential. Furthermore, seawater may contain very low concentration levels of heavy metals (Förstner, 1985). Thus, it was not difficult to think about the use of organisms that adsorb and accumulate heavy metals to assess the contamination levels in any aquatic ecosystem. Organisms were found to be a potential biotechnological alternative method than

physicochemical methods (Zabochnicka-Swiatek and Krzywonos, 2014). So, the determination of heavy metal concentrations using algae is preferred than seawater and sediment samples, because of algal properties such as their distribution, size, longevity, presence at polluted sites, ability to accumulate metals and ease of identification (Conti and Cecchetti, 2003).

The coastal aquatic ecosystem of Al-Hanyaa region, Libya, has recently received much attention due to sewage influent into some of its sites. So, this investigation was carried out to evaluate the potential suitability of the new record marine benthic algae, *Cladophora laetevirens* (Chlorophyta), and its allied limpet *P. caerulea* (Gastropoda), inhabiting Al-Hanyaa coast, as bioindicators of heavy metals pollution.

MATERIALS AND METHODS

The study area

Al-Hanyaa is a coastal small city, situated on the northeastern Mediterranean coast of Libya. It lies at $32^{\circ}.45$ N and $21^{\circ}.30$ E, and has a rocky shore extends about 4 km along shoreline and suffers from sewage pollution in some sites. The bottom of the intertidal zones is characterized by several boulders of variable sizes in addition to fast area of flat rocks which provide good substrates for growing of algal mats and shelters for gastropod *P. caerulea* and other marine benthic organisms.

Two sites were chosen during this study. The first site, polluted, lies adjacent to a sewage outlet at $32^{\circ}50'44''N$ and $21^{\circ}31'12$ E. The second one is a reference (unpolluted) site, lies at $32^{\circ}50'5''N$ and $21^{\circ}30'22$ E and is far about 3Km westerly from the first site (Fig.1).



Fig. 1: Map of Al-Hanyaa, Libya on the Mediterranean Sea.

Taxonomy of the studied organisms

Cladophora laetevirens was identified using taxonomical keys; the World Register of Marine Species (WoRMS Editorial Board, 2013) and Abbott (1999). It is worth mentioning that *Cl. laetevirens* was documented as a new record on the Libyan coastline by El-Adl (2014). Whereas *P. caerulea* was identified using different taxonomical keys (Borradaile *et al.*, 1977).

Sample collection

Water samples from the reference and polluted sites were collected using sterile 1 liter plastic container. Samples of *Cl. laetevirens* and *P. caerulea* with the same ages, size and healthy in appearance were handpicked during low tide at winter 2014.

The collected samples were rinsed thoroughly with seawater on-site after collection, placed in plastic bags with information on dates and place, and transferred immediately to the laboratory as soon as possible. At the laboratory, samples were initially washed under a jet of tap water and rinsed in distilled water to remove any mineral particles or organisms.

Analytical methods

At the laboratory, *P. caerulea* was dissected where gut and muscles were separated. For analyses, one gram of each sample was digested in 20 ml nitric acid and 3 ml perchloric acid in Kjeldahl apparatus until white fumes appear and organism's tissues dissolved completely. The samples were allowed to cool after that, 10 ml of HCl (1:1) were added and complete digestion process continued till the solutions turn clear. Finally, distilled H₂O was added to digested samples till 50 ml according to AOAC (1980). The concentration of four metals (Zn, Cd, Pb and Cu) in seawater samples, *Cladophora* and *P. caerulea* (gut and muscle) have been measured using hanging mercury dropping electrode (HMDE), reference electrode (Ag/AgCl 3MKCl) and platinum auxiliary electrode, model VA 746 trace analyzer coupled to a model VA 747 stand, Metrohm co.

Indices of pollution:

The metal pollution index (MPI):

MPI was used to compare the total content of heavy metals at locations studied according to Usero *et al.* (1996) and represented by the following formula:

$$MPI = (Cf_1 x Cf_2 x Cf_3 \dots Cf_n)^{1/r}$$

Where $Cf_n = Concentration$ for metal in the sample.

Bioaccumulation factors (BAFs):

BAFs of the studied contaminants were calculated based on Harrahy and Clements (1997) formula: BAF = CS / Cw

Where CS = Concentration of the metal in the sample.

Cw = Concentration of the metal in water.

Statistical analysis:

The data were analyzed using SPSS statistical program. Pearson's correlation was used to measure the strength of the association between heavy metal concentrations in *Cl. laetevirens* and *P. caerulea*. ANOVA test was employed to differentiate between means.

RESULTS

Heavy metal concentrations in seawater samples

As shown in Table (1), the discharge of sewage pollutants lead to an increase in heavy metal concentration levels in seawater compared with those recorded from the reference site.

Table 1: Mean values of heavy metal concentrations detected in seawater samples collected from Al-Hanyaa city, Libya compared with the corresponding seawater standard values of EPA ($\overline{X} \pm$ SD μ g l⁻¹, n = 3).

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Metals/Sites	Site I (Reference)	Site II (Polluted)	EPA (2014)*
Zn	5.40 ± 1.20	7.40 ± 0.140	81
Pb	0.20 ± 0.05	0.40 ± 0.170	1.8
Cd	0.03 ± 0.004	0.07 ± 0.005	1
Cu	0.24 ± 0.06	0.60 ± 0.160	3.1

* EPA (2014) shows maximum contamination level in seawater sample.

The present results revealed that, the level of heavy metal concentrations in both reference and polluted sites showed the same trend, and was arranged in the following the order: Zn > Cu > Pb > Cd (µg g⁻¹ fresh weight). It is noticeable that Zn concentration levels were the highest among metals determined in both seawater types. However, the recorded concentration levels at both sites were very low than the standard values (Table 1).

ANOVA results verified that the variations of metal levels in seawater are very highly significant, regardless the type of sites (F=45.7467, df =3, P < 0.01, Table 2). Nonetheless, there is no significantly difference between the studied sites. The variance percentage of Zn (95.89 %) and Cu (3.12 %) may be responsible for this increment of significance.

10	ille 2. Two - way ANOVA	101 seawater	neavy	metals at Al-manyaa, Libya.					
	Source of Variance	SS	df	MS	F	P-value	F crit		
	Metals	56.75335	3	18.91778	45.7467	0.005277	9.276628		
	Sites	0.845	1	0.845	2.043366	0.248214	10.12796		
	Error	1.2406	3	0.413533					

Table 2: Two - way ANOVA for seawater heavy metals at Al-Hanyaa, Libya.

Heavy metal concentrations in Cl. laetevirens and P. caerulea

Heavy metal concentration levels accumulated in *Cl. laetevirens* collected from polluted and reference sites were higher than those detected in *P. caerulea* tissues, except Zn determined in *P. caerulea* gut (Table 3).

The results also showed that, the metal concentrations in *Cl. laetevirens* was arranged in the following descending order: Pb $(197.7\pm 31.19) > Cu (165 \pm 18.19) > Zn (44.00 \pm 14.35) > Cd (38.62 \pm 2.44 \,\mu g g^{-1}$ fresh weight) in reference site compared to Cu $(559.15 \pm 68.55) >$ Pb $(207.51 \pm 23.82) > Zn (135.39 \pm 14.00) > Cd (77.19 \pm 1.22 \,\mu g g^{-1}$ fresh weight) in polluted site. For *P. caerulea* gut heavy metals, Zn concentration was the highest level at both sites followed by Pb, Cu and Cd. These metals were represented in the following descending order: Zn $(133.44\pm 18.13) >$ Pb $(47.04\pm 4.24) > Cu (35.30\pm 4.22) > Cd (2.30 \pm 0.32 \,\mu g g^{-1}$ fresh weight) in reference site, increased remarkable with the same pattern to Zn $(241.93 \pm 41.37) >$ Pb $(60.37 \pm 3.29) > Cu (45.06 \pm 7.80) > Cd (4.28\pm 0.63 \,\mu g g^{-1}$ fresh weight) in polluted site.

In *P. caerulea* muscles, the concentrations of measured heavy metals were remarkably lower than those detected in gut (Table 3). The results exhibited high levels of Pb, followed by Zn, and Cu, and declined sharply for Cd, but with slight increase in polluted site. It is obvious that, *P. caerulea* gut has more ability to accumulate metals from the two sites more than muscle. In addition, *P. caerulea* gut accumulated a considerable concentration of Zn more than *Cl. laetevirens*.

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Sites		Reference site		Polluted site							
Species	Cl. laetevirens	Р. са	ierulea	Cl. laetevirens	P. caerulea						
Metals		Gut	Gut Muscle		Gut	Muscle					
Zn µg/g	44.00 ± 14.35	133.44 ± 18.13 18.26 ± 0.02		135.39 ± 14.00	241.93 ± 41.37	27.36 ± 3.60					
Pb µg/g	179.7 ± 31.188	47.35 ± 4.51	24.98 ± 0.54	207.51 ± 23.82	60.37 ± 3.29	32.41 ± 2.69					
Cd µg/g	38.62 ± 2.44	2.30 ± 0.32	1.46 ± 0.41	77.19 ± 1.22	4.28 ± 0.63	3.60 ± 0.56					
Cu µg/g	165 ± 18.187	35.30 ± 4.22	7.2 ± 2.10	559.15±68.55	45.06 ± 7.80	16.76 ± 1.75					

Table 3: Mean concentration levels of heavy metals ($\overline{X} \pm SD \ \mu g \ g^{-1}$ fresh weight) detected in *Cl. laetevirens* and *P. caerulea* living on the rocky shores of Al-Hanyaa city. Libya

One-way ANOVA verified that all metals, regardless the type of sites, were exceedingly significant at p<0.001 (Table 4). Also, the difference between *Cl. laetevirens* and *P. caerulea* was highly significant in both sites, except for *P. caerulea* gut in reference site (p>0.05). The calculated variance percentage showed that Cu

(73.34 %), Zn (14.45 %) and Pb (10.45 %) were responsible for the elevation in this significance. Also, the variance percentage of *Cl. laetevirens* (21.56 %) and *P. caerulea* gut (5.2 %) in polluted site may be responsible for an increase in species significance.

Species		SS	df	MS	F-value	P-level	Metals	SS	df	MS	F-value	P- level
Reference site												
Cl. laetevirens		52131.	3	17377.2	45.873	.000	Zn	132527.8	5	26505.57	53.39	.000
P. caerulea	gut	529.03	3	509.68	1.629	.258	Cd	14926.33	5	2985.27	2410.14	.000
P. caerulea	muscle	29886.	3	9962.29	84.083	.000	Pb	96872.44	5	19374.49	79.95	.000
Polluted site							Cu	691374.3	5	138274.8	153.71	.000
Cl. laetevirens		420797	3	140265.	102.698	.000						
P. caerulea	gut	109379	3	36459.7	69.162	.000						
P. caerulea	muscle	1459.5	3	486.52	82.525	.000						

Table 4: One-way ANOVA for metals and tested organisms at Al-Hanyaa, Libya.

Data illustrated in Fig. 2 (A and B), elucidated the ability of *Cl. laetevirens* to accumulate heavy metals efficiently than *P. caerulea*. BAFs for Cd was followed by Pb, Cu and Zn in *Cl. laetevirens* inhabiting the reference site, whereas, it followed by Cu, Pb and Zn in *Cl. laetevirens* affected by sewage discharge. Pb dominates the metals accumulated by *P. caerulea* (gut and muscle) in both sites.

For comparing the common effects of the total heavy metals on the two studied sites, MPI was calculated and represented in figure 3 (A&B). The results revealed that MPI values were higher in *Cl. laetevirens* than *P. caerulea* gut and muscle at both sites. Where, their values attained 84.25 and 186.6 μ g g⁻¹ for *Cl. laetevirens* comparing with 26.72 and 40.96 for *P. caerulea* gut and 8.32 and 15.21 for its muscle in reference and polluted sites, respectively (Fig. 3 A). The previous figure proves the comparable affinity of the two organisms in accumulating heavy metals at both sites, where this accumulation seems to proportion to the concentration of such metals in seawater.

Data plotted in Fig. 3B for MPI showed the positive correlation relationship between the two sites. This relationship emphasized the efficiency of the tested organisms for bioaccumulation of metals regardless their concentrations in the seawater ($R^2 = 0.991$). Reviewing Table (5), it was found that Cd positively correlated with Cu and Pb in polluted and reference sites at P < 0.01. Also, Cu significantly correlated with Pb in both sites.



Fig. 2: Bioaccumulation factors of heavy metals for *Cl. laetevirens* and *P. caerulea* collected from Al-Hanyaa rocky shore, Libya.



Fig. 3 A: MPI values for *Cl. laetevirens* and *P. caerulea* in polluted and reference sites. B: Correlation between MPI of both organisms in polluted vs. reference site at Al-Al-Hanyaa, Libya. n=3.

Polluted site						Reference site					
	Cd	Cu	Pb	Zn		Cd	Cu	Pb	Zn		
Cd	1	0.991**	0.976**	-0.064	Cd	1	0.9652**	0.9786**	-0.3228		
Cu		1	0.974**	-0.22	Cu		1	0.979**	-0.274		
Pb			1	-0.072	Pb			1	-0.223		
Zn				1	Zn				1		
** Correlation is significant at the 0.01 level					** Correlation is significant at the 0.01 level						

Table 5: Pearson correlation for heavy metals in polluted and reference sites at Al-Hanyaa, Libya.

DISCUSSION

Coastal waters receiving sewage and other effluents can have considerably higher heavy metals concentrations than that of the open sea. This fact can be applied on the studied area which suffered from different types of wastes either from the open sea like ship wastes or from the anthropogenic activities on land as well as the nature of rocks and its erosion rate.

The levels of metals investigated in polluted and reference water samples were lower than that of the maximum concentration levels recommended by EPA (2014). The data attained clarify that Zn concentration levels were higher than the levels of Cu, Cd and Pb in both water types. In accordance with Pérez-López *et al.* (2003), the results showed an increase in heavy metal levels in seawater by increasing the anthropogenic activities. Anyhow, heavy metal contaminated seawater may influence the marine organism's health. According to the findings of Chapman *et al.* (1996), algal species that failed to eliminate heavy metals through excretion, compartmentalization or metabolically immobilization subjected to adverse and toxic effects.

Epizoic and/or benthic *Cl. laetevirens* was found to storage higher levels of metals than *P. caerulea* tissues, except in the case of Zn because of the algal high negative surface charge and its affinity for heavy metal cations emitted through the anthropogenic metal emissions (Rao, 1986). The increments in metal levels in *Cl. laetevirens* increases the metal levels in *P. caerulea* gut through the food chain, this agree with Pérez – Lòpez *et al.* (2003).

It is obvious that, Zn was bioconcentrated in *P. caerulea* gut in considerable levels in both reference and polluted sites. This result was similar with that attained by Nott and Nicolaidou (1989, 1994) who referred to Mollusca as efficient bioindicators and that of Wang and Caihuan (2002) who reported that, marine gastropods are

known to accumulate high metal concentrations in their tissues, especially in the digestive glands.

Lozano *et al.* (2003) reported that, algal samples containing lower than 2 μ g g⁻¹ Cd of dry weight can be considered not polluted. Accordingly, *Cl. laetevirens* can be considered polluted as it contains 38.62, and 77.19 μ g g⁻¹ Cd of fresh weight in reference and polluted sites, respectively. *Cl. laetevirens* collected from reference and polluted sites contain high levels of Pb, Cu and Cd than *P. caerulea*. This increase in metal concentration levels may be attributed to the high affinity of *Cl. laetevirens* to accumulate metals, even in the relatively clean sea water. Also, the blooming and survival of *Cl. laetevirens* in clean and polluted sites, referred to the tolerance characteristics enabling it to monitor a wide range of pollution levels. The present results are in agreement with that attained by Al-Homaidan *et al.* (2011), who referred to *Cladophora* as a good indicator for various heavy metal pollution conditions.

In *Cl. laetevirens*, Pb exhibited the highest concentration levels of samples collected from the reference site, while Cu prevailed that collected from the polluted site. The increment in Pb accumulation in *Cl. laetevirens* may be attributed to sewage discharge and the anthropogenic activities. This agrees, to some extent, with Pérez – Lòpez *et al.* (2003), who reported that local industrial discharges and leaded petrol from cars and boats corresponded to Pb contamination. While the elevation in Cu levels may be depended on the nature of rocks and its erosion and the subsequent participation of their particles.

BAFs of Cd in *Cl. laetevirens* were the highest and similar in both sites, emphasizing its potentiality for biomonitoring this metal regardless its concentration, which reflects to some extent its inability for the excretion of that metal. In general, *Cl. laetevirens* can be used as an efficient biomonitor for Cu, Pb and Cd pollution, while *P. caerulea* can be used as a good indicator for Zn pollution. This result agrees with that attained by (Al-Homaidan *et al.* 2011).

In spite of, the lowest performance of MPI score in this algal species due to its low sensitivity, but it provides a representative picture for the marine environment. The results evidenced that MPI was the highest in *Cl. laetevirens* at reference and polluted sites, when compared to *P. caerulea* tissues. Comparing MPI for the two species, it can be concluded that *Cl. laetevirens* has a great capability for metal bioaccumulation than *P. caerulea*. These results were found to be similar to that attained by Pérez – Lòpez *et al.* (2003). The strong correlation, based on MPI values between polluted and reference sites, proved the potential suitability of *Cl. laetevirens* and *P. caerulea* to accumulate metals effectively regardless their concentrations, confirming the overall consistency in metal uptake at both sites.

The employing of Pearson's correlation and interaction between metals and their effects on *Cl. laetevirens* and *P. caerulea* draws attention to positive correlation of Cd with Cu and Pb. Likewise, Cu and Pb are significantly correlated with each other at both sites. Noted positive significant correlations among metals may reflect a common source of occurrence and indicative of similar biogeochemical pathways for subsequent accumulation in the two sites studied. Furthermore, ANOVA analysis reveals highly significant differences between metals at both of seawater and the studied species, while non-significant for the sites detected. Generally, the highly significant difference between *Cl. laetevirens* and *P. caerulea* emphasized that *Cl. laetevirens* may be the most suitable biomontor for heavy metal accumulation in clean and polluted sites than *P. caerulea*.

As a conclusion, algae are more appropriate bioindicators than their allied limpets. The results clearly pointed out the well documented possibility of using *Cl*.

laetevirens as a convenient monitoring tool for heavy metal contamination of coastal marine environment because of its consistency, appropriateness and reliable for monitoring.

Cl. laetevirens can be used as a good indicator of Cu, Pb and Cd pollution, whereas *P. caerulea* can be utilized as an indicator of Zn contamination. The results predicted that *Cl. laetevirens* is promising for heavy metals bioremediation than *P. caerulea*, due to its potential suitability for metals accumulation even at a very low concentration level of metals in seawater. However, *Cl. laetevirens* had a higher tolerance for sewage pollution than *P. caerulea*. The present results consider a data base and useful for future studies on pollution on Libyan coastal line.

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ARABIC SUMMARY

استخدام طحلب الكلادوفورا الأخضر والبطلينوس (الرخويات) كمؤشرات حيوية لتلوث المياة البحرية بالعناصر الثقيلة بسواحل البحر المتوسط - الحنية - ليبيا

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تم إجراء هذه الدراسة لتقييم تحمل طحلب الكلادوفورا (Cladophora laetevirens) والبطلينوس (caerulea) من الرخويات النامية بمياه السواحل الليبية للتلوث بالعناصر الثقيلة واستخدمهما كُمؤشرات حيوية لتراكم تلك العناصر بمنطقة الدراسة. ولقد جمعت العينات من موقعين أحدهما ملوث بمياه الصرف الصحى والآخر أختير كمنطقة ضابطة (غير ملوثة) على طول الساحل الصخري بمنطقة الحنية بليبيا. ولقد تم قياس مستويات عناصر الزنك والكادميوم والرصاص والنحاس باستخدام جهاز فولتميتر، وأظهرت النتائج أن تركيز النحاس النحاس أعلى مستوى من حيث وصل متوسطه إلى $m g^{-1}$ fresh النتائج أن تركيز النحاس النحاس أعلى مستوى من حيث وصل متوسطه إلى $m g^{-1}$ weight بين المعادن التي تراكمت في طحاب الكلادوفور ا في الموقع الملوث، ويليه الرصاص في ذات الموقع غير أنه سجل أعلى معدلات التركيز في الموقع الضابط (fresh weight g⁻¹ fresh weight ع 1.19 μg g⁻¹ fresh weight)، ثم النحاس. وعلى الرغم من التركيزات الضئيلة لعنصر الكادميوم سواء في الكلادوفورا أو البطلينوس في كلا الموقعين، إلا إنه سجّل أقصى معاملات للتراكم الحيوي (BAFs) في الكلادوفورا في كلا الموقعين. كما أوضحت قيم دليل التلوث بالمعادن (MPI) ترتيباً تنازليا على النحو التالي: الكلادوفورا >ً أمعاء البطلينوس > عضلات البطلينوس في كل من الموقعين وأشارت التحاليل الاحصائية إلى ارتباط الكادميوم إيجابيا مع النحاس والرصاص في المواقع الملوثة والنقية على السواء. كما وضحت النتائج والتحليل الإحصائي الفروق المعنوية الكبيرة بين تركيزات المعادن المختلفة وأيضًا بين الكائنات المدروسة، غير أنه لا يوجد أيّ دلالة معنوية بين المواقع، كما أثبت التحليل الإحصائي تحقق الثبات (consistency) والملائمة (appropriateness) والفاعلية (reliability) وقدرة الاحتمال (tolerance) لطحلب الكلادوفور المدي واسع من التلوث واعتباره دليلا حيويا فعالا، بغض النظر عن مستويات تركيز المعادن في مياه البحر. كما أكدت النتائج كفاءة طحلب الكلادوفورا في تراكم النحاس والرصاص والكادميوم وكفاءة البطلينوس في تراكم الزنك.