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The Use Of a Battery Of General Biomarkers to Discern The Effect Of Sewage Primary Treated On Two Population Of *Donax trunculus* Located Outside Of M'zar Wastewater Plant Outfall

Zaina IDARDARE[1], Maryem NADIR[2], Aicha AIT ALLA[2], Abdellatif MOUKRIM[2]

[1] Laboratory of Marine Biology and oceanography
Higher Institute of Maritime Fisheries (ISPM)
Agadir- Morocco
idardarezaina@yahoo.fr

[2] Laboratory of Aquatic Systems: Marine and continental ecosystems
Faculty of Sciences, Ibn Zohr University
Agadir, Morocco

***Abstract*—Wastewater treatment plants (WTP) are common sources of a wide mixture of chemicals entering the aquatic environment. We have investigated the impact of WTP on two populations of *Donax trunculus* situated outside of the WTP outfall by using antioxidant stress parameters (catalase (CAT) and Gluthation S-transférases (GST)), Acetylcholinesterase (AchE) and malondialdehmyd (MDA). In addition, we have determined the distribution of the species along the Bouadisse beach. AchE, Oxidative damage and antioxidant enzymes had discerned the effect of the wastewater primary treated. The density showed also clear cut distribution in relationship with distance from the WTP outfall. The sites situated in the proximity of WTP outfall and the southernmost site showed decreased densities.**

Keywords—Donax trunculus; Biomarkers; wastewater treatment plant Outfall; Pollution; Density; Bouadisse Beach

I. INTRODUTON

Urban population growth is an interrupted and accelerating phenomenon in many cities in developing countries in recent decades. The Agadir city and its surrounding area (second economic pole in Morocco) are representative in this respect. In fact, the population of the metropolitan area rose from 105,000 inhabitants in 1971 to more than 598,020 inhabitants in 2014 and expected to reach 833,360 by 2030. Parallel to the considerable efforts deployed to satisfy the population's water demand and sanitation, in addition to the industrial and tourist's water supply, large amounts of wastewater are produced. In fact, the Agadir city produces each day more than 50,000 m^3 of wastewater (RAMSA data). After November 2002, the wastewater of Great Agadir has been directed to M'zar wastewater treatment plant located 10 km south of Agadir city. Wastewaters are treated in anaerobic ponds and discharged in the Bouadisse Beach.

The treated wastewater, as well as the raw sewage, have been shown to contain a wide range of natural and anthropogenic substances, including pharmaceutical products, heavy-metals, ammonia, pesticides, endocrine disruptors and polycyclic aromatic hydrocarbons [1, 2, 3], many of which have also been measured in the receiving environment [4].

Because of the fact that sewage treatment plant are frequently associated with the release of xenobiotics, many effects are observed in aquatic species (fish and freshwater mussels) exposed to treated and untreated municipal wastewater, such as, increase of metallothioneins and mixed-function oxidase activity, DNA damage, vitellogenin induction, lymphocyte proliferation and decrease of phagocytic activity [5,6,7]. These molecular and cellular responses could be used to assess the quality of the aquatic ecosystems [8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20].

The main objective of this study was to investigate the impact of the M'zar plant outlet on the Bouadisse beach by measuring biomarkers (Acetylcholinesterase, Catalase, Glutathion S-transferases and Malondialdehyd) and the density of two populations of *Donax trunculus:* one situated in the south of the M'zar plant outlet and another in the north.

II. MATERIALS AND METHODS

A. Sampling sites

A 10 km coastline of the Bouadisse beach was chosen for the study. This beach is located at south of the Oued Souss estuary and continues to northern of Sidi Toual. After the set up of the M'zar wastewater treatment plant, sewage sludge primary treated was discharge directly in the area. The resulting effluents were thus not totally cleared of suspended solids and still contain micropollutants such as bacteria and contaminants, some of which may be harmful to humans and aquatic organisms.

Six sites were selected along the shoreline to investigate the impact of the WTP outfall: four sites located in the north of the WTP discharge zone and spaced 1 km of each other and two sites situated in the south "*Fig. 1*". The first southern site (S5) was at 4 km away from WTP outfall.

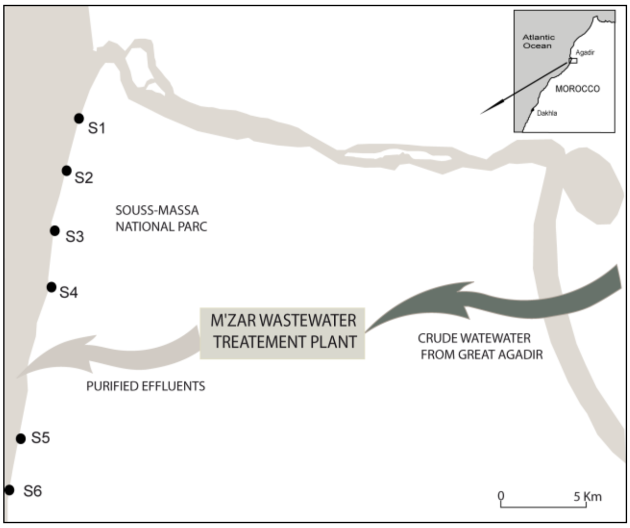

Fig. 1. *Location of sampling sites along the Bouadisse beach*

B. M'zar wastewater treatment plant (WTP)

In November 2002, a huge project is established by RAMSA based on the satisfied results harvested from the Ben Sergao pilot plant, wastewater treatment through dune sand infiltration-percolation is underway at M'zar dune, a suburb of Agadir. The first treatment is by anaerobic stabilization pond. The plant is conceived to treat wastewater by infiltration-percolation of 50,000 m^3/day of highly concentrated effluents in twenty-four infiltration basins, with a flow of 10,000 m^3/day, was consisting of 2 m thick eolian sand. The eight anaerobic stabilization ponds (6,000 m^3 for each basin and a theoretical residence time of 2.5 days; depth of 4.30 – 6.60 m) are used while the ninth is being drained. They allow 40–50 % reduction of suspended solids and organic matter; however, they do not remove the salts and pathogens "*Table 1*".

If the sand filters process efficiency is demonstrated, the concentrations of the soluble salts in the wastewater coming from the cannery industries are high and exceed the standards of wastewater treated reuse. Furthermore, the nitrate concentrations are significant "*Table 2*".

C. Biochemical parameters analysis

Clams were collected in autumn and spring 2005 at low tide and transported alive to the laboratory where they are kept at -30 ° C until analysis.

The animals were dissected. Six replicates of two individuals were homogenized using Ultra-Turax T25 in 1:3 (w: v) 0.1M Tris-HCl buffer (pH 7). Homogenates were centrifuged at 9,000 g for 30 min. All preparation procedures were carried out at 4ºC. The post-mitochondrial fractions (S9) were stored at - 80 °C until biochemical assays.

All assays were carried out in duplicate. AchE activity was assayed by the method of Ellman et al. [21]. The GST assay was performed using a modified CDNB method based on Habig et al. [22]. CAT activity was determined as described by Aebi [23] at 25 °C in phosphate buffer (50 mM pH 7.4) with hydrogen peroxide (3 mM) as substrate. Total protein content was measured by the Lowry method [24] using Bovin Serum Albumin (BSA) as reference standard material. Lipid peroxidation was evaluated by assaying malondialdehyde (MDA) following the method of Sunderman [25].

The activities are expressed in nmol/min/mg of proteins. MDA content is expressed as nmol/mg of proteins. Means ± standards deviation (SD) were calculated for each site and per season. The comparison of enzymatic activities among sampling stations, at each sampling date was determined by one-way analysis of variance (1-way ANOVA), followed by a Fisher least significant difference (LSD) multiple-comparison test using STATISTICA version 6.

D. Donax density

The density of *D. trunculus* was evaluated on four seasons, autumn, winter, spring and summer 2005. Clams survey was conducted upstream and downstream of the outfall plant. At each sampling station, four random sampling points were selected along an axis perpendicular to the transect line. At each sampling point, a 0.25 x 0.25 m quadrat was laid down. The sediment was dug out to a depth of 30 cm [14, 26, 27, and 28] and sieved through a 1.0 mm mesh sieve. Specimens were collected alive and placed in plastic containers. In the laboratory, specimens were killed by the addition of 8% formalin. The density is expressed in number of individuals per m^2.

TABLE I. MICROBIOLOGICAL AND PHYSICOCHEMICAL QUALITY OF RAW SEWAGE, POND EFFLUENT, AND PERCOLATED WATER (RAMSA DATA)

Parameter	Unity	Raw sewage	Pond effluent	Percolated water	Overall removal efficiency
pH		7.19	7.17	7.29	neutral
Conductivity at 20°C	µS/cm	3999.10	4069.76	3991.44	ns
Turbidity	NTU	743.67	345.37	3.57	99.52
COD	mg/O2	1921.44	837.08	55.47	97.11
BOD5	mg/O2	1033.41	308.37	7.08	99.32
MES	mg/l	682.23	369.92	5.37	99.21
Tot. P	mg/l	24.02	23.26	10.78	55.13
PO_4^{--}	mg/l	16.36	18.20	10.07	38.47
NTK	mg/l	141.04	144.76	6.22	95.59
NH_4	mg/l	127.05	146.46	3.95	96.89
NO_3^-	mg/l	1.17	1.11	419.00	
NO_2^-	mg/l	0.05	0.07	14.64	
Cl^-	mg/l	914.44	914.74	835.78	8.96
Na^+		582.87	560.46	532.78	8.59
K^+	mg/l	54.61	45.55	43.24	20.82
Detergents	mg/l	6.53	6.04	0.38	94.15
Fecael col. at 44°C	u/100 ml	1.0^{E+08}	4.5^{E+06}	1.3^{E}+04	99.99
Tot. Col.	u/100 ml	1.7^{E+08}	3.0^{E+07}	7.1^{E}+04	99.96
Fecael Strp. at 44°C	u/100 ml	1.8^{E+07}	8.1^{E+07}	4.3^{E}+03	99.98
Tot. Germ at 22°C	u/100 ml	1.9^{E+08}	3.10^{E+07}	5.0^{E}+04	99.97
Parasites	30	1	0		100.00

TABLE II. WASTEWATER QUALITY AT INFLOW AND OUTFLOW OF THE DECANTATION AREA (RAMSA DATA)

		Decantation area inflow	Decantation area outflow
Parameter	***Unity***	***Mean***	***Mean***
Temperature	°C	23.85	22.73
Conductivity at 20°C	µS/cm	4518.50	4375.20
Turbidity	NTU	766.22	353.33
PH		7.15	7.04
COD	mg/O2	2174.90	1015.20
BOD5	mg/O2	1133.30	357.90
COD/BOD		2.11	2.94
MES	mg/l	822.60	492.20
NTK	mg/l	155.90	150
NH_4	mg/l	140.40	154
NO_3^-	mg/l	0.83	1.19
NO_2^-	mg/l	0.02	0.002
Global N	mg/l	156.09	150.27
Tot. P	mg/l	24.70	24.49
PO_4^{--}	mg/l	19.78	21.62
Cl^-	mg/l	1032.80	962.00
SO_4^-	mg/l	123.88	24.04
Na^+		682.40	675.70
HCT	mg/l	8.43	4.19
Detergents	mg/l	10.96	9
S^{--}	mg/O2	3.94	0
Germ tot at 22°C	u/100 ml	5.93^{E+09}	3.10^{E+08}
Tot. Col.	u/100 ml	1.84^{E+08}	1.47^{E+07}
Fecael col. at 44°C	u/100 ml	1.89^{E+07}	1.88^{E+06}
Fecael Strp. at 44°C	u/100 ml	1.27^{E+07}	3.22^{E+06}

III. Results and Discussion

A. *Biochemical responses*

Biomarkers are often applied in environmental quality assessment, both in marine and freshwater biomonitoring surveys, frequently using molluscs as sentinel species [29, 30, and 31]. However, natural fluctuations due to endogenous factors and abiotic parameters, and the existence of historical contamination often make difficult to interpret field results [32, 33] in relation to punctual pollution and to establish cause-effect relationships. The aim of this work was to investigate whether antioxidant and acetylcholinesterase enzymes and the residue of oxidant damage (MDA) can provide information about the sites impacted by the WTP effluents release compounds

In the present study, AchE activity of clams from all the sampling sites, except from S1, increased from autumn 2005 to the winter 2005 "*Fig. 2 and Table 3*". Furthermore, this increase was not important in the sites most close to the plant outfall (S4 and S5). The change on AchE activity from autumn to spring were statistically significant ($p<0.001$) "*Table 3*". Moukrim et al. [15] have pointed out that the same species showed high values of AchE activities recorded in summer but were lower in spring, while GST activities were highest in the autumn-winter period. For lipid peroxidation, lower MDA content were recorded in winter with high values in the spring-summer period.

TABLE III. A post hoc LSD test results indicate which stations encountered statistically significant differences between seasons, and in which season the data differs between stations, sharing a common letter for the post Hoc test that is not significant ($p< 0.05$)

Biomarkers	Season within site			Site within season						
AChE	S1	Aut^{a}	Spr^{b}							
	S2	Aut^{a}	Spr^{b}							
	S3	Aut^{a}	Spr^{b}	Autumn	S1^{a}	S2^{a}	S3^{b}	S4^{b}	S5^{b}	S6^{b}
	S4	Aut^{a}	Spr^{b}	Spring	S1^{b}	S2^{b}	S3^{b}	S4^{b}	S5^{b}	S6^{a}
	S5	Aut^{a}	Spr^{b}							
	S6	Aut^{a}	Spr^{b}							
GSTs	S1	Aut	Spr							
	S2	Aut	Spr							
	S3	Aut	Spr	Autumn	S1^{b}	S2^{b}	S3^{b}	S4^{a}	S5^{b}	S6^{b}
	S4	Aut	Spr	Spring	S1^{a}	S2^{a}	S3^{a}	S4^{bc}	S5^{bc}	S6^{bc}
	S5	Aut	Spr							
	S6	Aut	Spr							
CAT	S1	Aut	Spr							
	S2	Aut	Spr							
	S3	Aut	Spr	Autumn	S1^{a}	S2^{b}	S3^{b}	S4^{b}	S5^{b}	S6^{a}
	S4	Aut	Spr	Spring	S1^{a}	S2^{b}	S3^{b}	S4^{b}	S5^{b}	S6^{b}
	S5	Aut	Spr							
	S6	Aut	Spr							
MDA	S1	Aut	Spr							
	S2	Aut	Spr							
	S3	Aut	Spr	Autumn	S1^{a}	S2^{ac}	S3^{b}	S4^{b}	S5^{b}	S6^{bc}
	S4	Aut	Spr	Spring	S1^{a}	S2^{b}	S3^{b}	S4^{c}	S5^{b}	S6^{b}
	S5	Aut	Spr							
	S6	Aut	Spr							

The decrease of AchE activity at S1 indicates that clams were exposed to anti-cholinesterase agents coming from the Oued Souss estuary that drains agricultural areas. It is noteworthy to precise that at this survey time (spring), it was rainy period and a mixture of xenobiotic had been released in the mouth of Oued Souss estuary.

Generally, low AchE activities were recorded in the sites most close to the WTP outlet (S4 and S5). These sites were persistently exposed to the anti-cholinesterase agents released by sewage treatment plant. Similar results on AChE activity were reported in several field studies using bivalve species. Dellali et al. [34] reported a decrease on AChE activity from *Mytilus galloprovincialis* affected by urban and industrial pollution and hydrocarbons from marine traffic. A significant decrease on AChE activity was also reported in the clam *Ruditapes decussates* affected by an input of non-treated wastewaters and heavy metals contamination [34] and also in *D. trunculus* sampled in the Anza, site affected by urban and industrial wastewaters [15]. AChE activity has been widely used as a biomarker of exposure to organophosphate and carbamate pesticides in several field studies [35, 36]. Recently, several field and laboratory studies show evidences that other types of contaminants such as metals, surfactants [37] and compounds in complex mixtures [15,34] may also inhibit the AChE activity of several bivalve molluscs.

GST activities showed a tendency to increase in the sites located in the vicinity of the WTP outfall (especially S4, S5). This increase is suggestive of an increase of exposure to inducers of these enzymes drained by domestic and industrial primary-treated effluents. In vertebrates, induction of specific GST following exposure to diverse agents such as PAHs, PCBs and organochloride pesticides has been well documented [38, 39]. Although metals are not natural substrata for these enzymes, recent studies have shown in vivo GST induction in fish exposed to heavy metals [40]. However, conflicting results have been reported regarding GST induction by these sorts of contaminants in mussels [41, 42]. In *Mytilus sp*., GST isoenzymes have already been purified and characterized [43, 44] and recent studies carried out with *M. edulis* showed that different GST isoforms may be induced as a function of the nature of the pollutants [43, 44]. Biomonitoring surveys and field caging experiments in contaminated sites have yielded inconsistent results concerning inducibility of GST activities in fishes and mussels. While some field studies have pointed out good responses of GST to organic pollution [45] others have detected little or no response [46]. The discrepancies between these findings may be due to differences in the pollution type and load in each case. Moukrim et al. [15] reported an induction of GST activities in *D. trunculus* populations from Anza beach (north of Agadir bay) exposed to domestic and industrial effluents

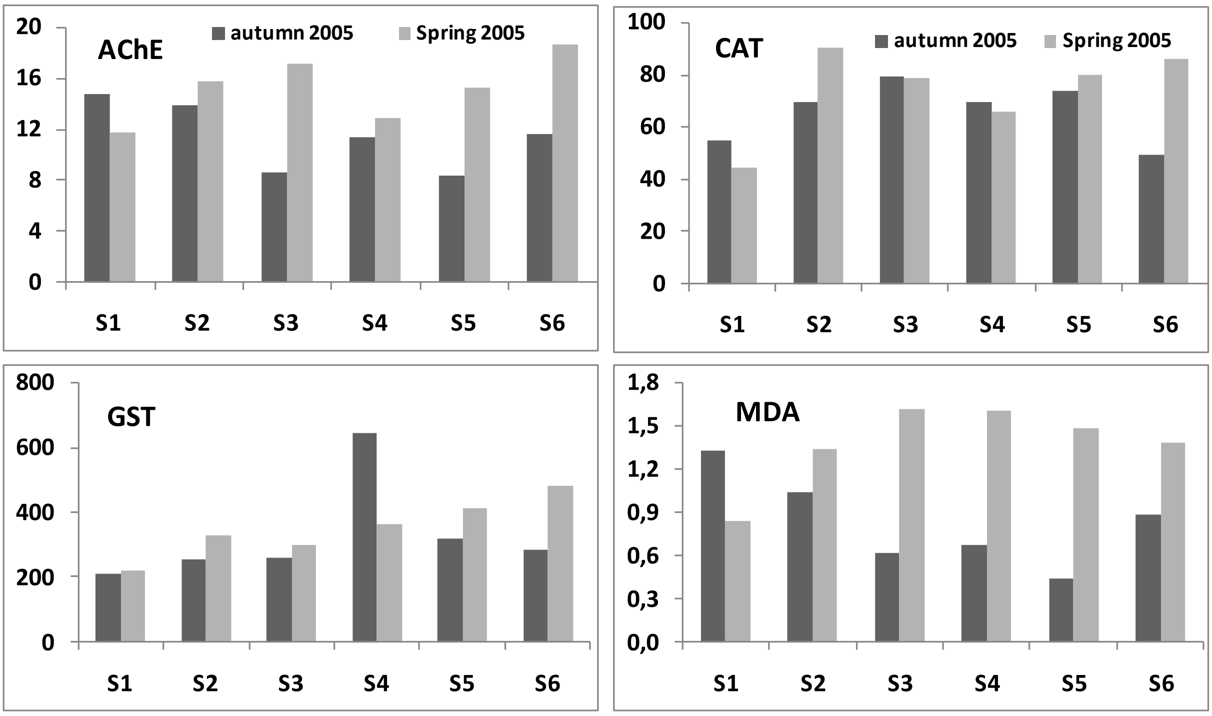

Fig. 2. *Seasonal variation of GST, AChE and CAT activities expressed in (µmole/min/mg Proteins) and MDA content (nmole/mg proteins)in whole tissues of D. trunculus from the different sites at Bouadisse Beach*

For Catalase activity, with the exception of S1 over the study periods and S6 in autumn, considerable and slightly similar concentrations are recorded in the remaining sites. This biomarker shows no trend in relation to the WTP outfall. However in the literature, high levels of CAT were recorded in the area subjected to the wastewater impact such the levels previously reported for *D. trunculus* in the Anza beach [15] and in other clams like *R. decussates* investigated in the sites where these animals are faced to an oxidative challenge [47,48]. Furthermore, elevated CAT activity was recorded in the fish caged in the vicinity of the WTP effluent [49, 50].

At the beginning of this study, significant levels of MDA were displayed in sites not impacted by the plant sewage effluents (S1). However, in spring 2005 considerable MDA levels were recorded in southern sites and reached a peak in S4 located in the vicinity of outlet plant (643.42 ± 252.92 nmol/mgP). Elevated lipid peroxidation levels were observed by [51] in cichlid fish and by [15] in *D. trunculus* taken from polluted sites, compared to clean sites. Reference [52] and [53] have demonstrated increases in MDA levels in the gonads of white suckers exposed to pulp and paper mill effluents as well as municipal sewage treatment plant effluents.

B. Donax trunculus density in the Bouadisse beach

In this study, seasonal variations in the density have been illustrated in the "*Fig. 3*". The high densities were recorded in autumn and summer 2005. The observed seasonal variability could be related to seasonal variations in water temperature. Lower temperatures during autumn and winter would be the reason for the seasonal migration of the species towards their lower levels of distribution. As temperature fluctuations are smaller in water than in sand, the species might move to its lower respective distribution levels, towards the water, in order to avoid extreme variations and achieve an optimal temperature (thermoregulation). Seasonal variability in the zonation pattern of sandy beach macrofauna has been attributed to other reasons, such as aggregated responses for reproduction [54,55] or variations in other key physical variables such as sediment water content, sandy beach erosion and accretion [56, 57].

In the addition to toxicant in WTP effluent, the reproductive stage may be affected by bacteria and protozoans often present below the WTP outfall [58]. This may be especially pertinent in the M'zar plant which only treated at the primary level while this study has occurred, which may lead to an abundance of undesirable bacteria and protozoans below the outfall.

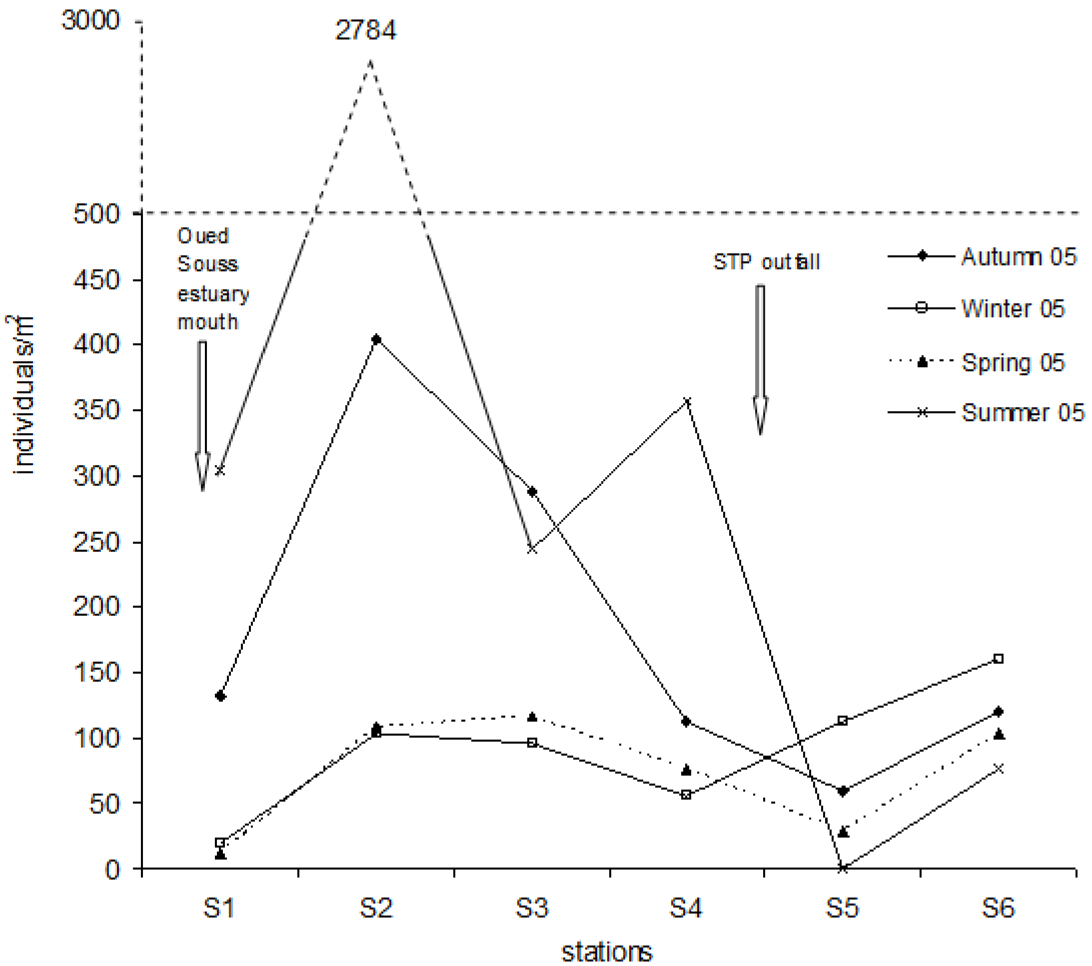

Fig. 3. *Population abundance of D. trunculus in the Bouadisse beach in Autumn, winter, spring and summer 2005*

In this work, *D. trunculus* showed a marked longshore variability in abundance along the Bouadisse beach. The clams were abundant in the upper stations "*Fig. 3, Table 4*". Peak densities were at station S2 ($p< 0.001$) in the all survey reaching a maximum of 2784 clams per m^2 in summer 2005. As distance from the canal diversion decreased densities were declined. Clams were not present at station S5 (4 km south of the WTP discharge zone) in the summer 2005 and were at decreasing abundance at S4 which was comparable to station S6. The absence of organisms close to the wastewater discharge might be ascribed to physico-chemical unsuitability of the habitat; and scarcity of food availability. Concerning the former, the great amount of suspended solid and organic matter (approximately 50-60% from a flow of 50,000 m^3/ day) carried by the sewage discharge, and the constant erosion of the site would suggest a constantly changing environment, and a less suitable habitat for the clams which caused a decrease in their density near the wastewater discharge zone, especially if we know that *D. trunculus* is generally associated with poor organic enriched areas [59]. These hypotheses are supported by our field observations: during the summer 2005 when a dyke was built to protect the outfall construction which led to isolation of the northern stations from the WTP effluent, a repopulation of S4 has begun. In addition to that, the clam's densities become important in S5 and S6 while the WTP discharge zone was dry in winter

In terms of hydrodynamic, topographical, and sedimentological characteristics, there are no alongshore trends in either beach slope or mean sand particle size in the intertidal zone of Bouadisse beach. However, an alongshore current (canaries current) moves predominately from the north to the south. This current was documented by several authors for its important role in the pollutants distribution along the Moroccan coast [60, 61]. Addressing the specific aspect of this current, in this present study, *D. trunculus* density exhibits a substantial directional distribution along the beach in relationship with the dispersion of the pollutants carried by the primary treated wastewater characterised by higher range in the values of salinity, protozoans and of coliform bacteria "*Tables 1 and 2*" and that the sites southernmost from the outfall are the most affected by the WTP input. For this raison low densities were found in S5 and S6.

TABLE IV. THE ANOVA TEST TO COMPARE THE SIGNIFICANCE OF THE VARIATION OF *D. TRUNCULUS* DENSITY BETWEEN SEASONS AND SITES

	SS	DF	MS	F	p
Sites	7199320	5	1439864	237,5316	0.00
Seasons	4848093	3	1616031	266,5936	0.00
Sites*seasons	15940339	15	1062689	175,3098	0.00
Error	436448	72	6062		

In summary, this study shows quite clearly that WTP effluents can release a lot of substances that can be harmful to aquatic life. The uses of biochemical parameters are sufficient to discern the difference between pollutant and clean sites and to provide indication of deleterious effects of contaminants on the biota. As we have discussed above, our results from antioxidant enzyme measurements had shown a clear response pattern; different parameters have shown increases in the oxidative and antioxidative stress and a decrease in AchE in the site close to the WTP effluent. It is also interesting to note that the use of density parameter to investigate the impact of WTP input was of great importance to show the difference between sites polluted (S4, S5 and S6) and the sites moderately clean in the north (S1, S2 and S3)

Conclusion

The Bouadisse beach is formerly pristine before the construction of the sewage treatment plant. The aim of the work was to determine the possible impact of the WTP effluents on the biochemical parameters and the density of *D. trunculus*. Globally, in regard of the parameters studied, clams sampled in the Bouadisse beach differed systematically between upstream and downstream sites, regardless of the sampling periods. The density of the population and the Biomarkers indicate an alteration in the site located in the vicinity of the outlet plant and also in the southernmost sites following north-south gradient.

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