

Assessment of the Marine Ecotoxic State in the Moroccan Coastal Area Anza-Taghazout following the Installation of Two Wastewater Treatment Plants: A Multibiomarker Study using *Mytilus Galloprovincialis*

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Abstract

The aim of the present study is the first to evaluate the ecotoxic state of the marine environment in Anza-Taghazout coasts (Morocco) after installation of two Wastewater Treatment Plants using a natural population of marine bivalves *M. galloprovincialis*. These coasts are exposed to many discharges generating, thus, different sources of pollutants. These pollutants can modulate the physiological responses of marine bivalves to environmental stress. In this context, a multi-biomarker approach consisting of a battery of biomarkers evaluation was used to assess the response of these species to stress. In the whole soft tissues of *M. galloprovincialis*, four biomarkers were evaluated: Acetylcholinesterase (AChE), Glutathione S-transferase (GST), Catalase (Cat) and malondialdehyde activity (MDA). In parallel, physico-chemical parameters were measured in the marine water of Anza-Taghazout within three selected sites: S₁ considered as 'hotspot' located at Anza city; S₂ located near of Aourir city and the third site, S₃ 'reference' located in Imouran beach. Our results showed that activities of both Glutathione-S-Transferase and Catalase were higher in *M. galloprovincialis* collected from site S₁, but high values of Malondialdehyde and Acetylcholinesterase activities were observed successively at S₃ and S₂. Application of Integrated Biomarker Response index (IBR) was suitable for classifying the stress response in the *M. galloprovincialis* but did not allow to evaluate the level of the xenobiotic exposure in the studied sites. The statistical results did not show any significant differences between the three studied sites and therefore S₁ has recently become clean due to the installation of two wastewater treatment plants.

Introduction

In the last years, several studies were interested on the biomarker's activities in sentinel species as important tools to assess environmental quality of the coastal areas in the world. The use of biomarkers as molecular and cellular responses is the best approach for environmental biomonitoring and biomarkers represent the first signals and early index of environmental pollution (Livingstone et al. 1997). Many toxic chemicals effects were evaluated at different levels of biological organization in marine organisms by using a large number of biomarkers (Rank et al. 2007; Viarengo et al. 2007). Thus, biomarkers as quantitative measure of changes in biochemical, physiological or behavioural structure occurring an organism is an appropriate response to xenobiotic exposure such as metallic, organometallic and organic pollutants. Those measures in different parts of cells (tissues, organs or body fluids) provide an earliest warning signal of biological effect which precede sub-cellular or biochemical responses that occur at an organism or population level (Schettino et al. 2012; Hutchinson et al. 2013).

Since 2016, there has been a good ecological action in maritime coasts of Anza-Taghazout in the south coasts of Morocco. Indeed, these coasts suffered from pollution situation for many years. Two wastewater treatment plants (WWTPs) were installed; one located in Anza - city and the second one in Aourir - city. WWTP of Anza treat wastewater of industrial area of Anza, harbor of Agadir and domestic wastewater of Anza city. While, WWTP of Aourir treat wastewater of two cities; Aourir and Tamraght.

Maritime coasts of Anza-Taghazout, located north of Agadir and which plays a highly important socioeconomic role in Sous Massa region (Morocco), has an important income from maritime fishing and tourist activities. However, it represents polluted habitat due to exposure to toxic liquid effluents of different origins. It contains urban and industrial centers and more sources of anthropogenic pollution (Moukrim et al. 2000). Discharges of industrial wastewater have resulted in high concentrations of trace metals in the shellfish including Cadmium (Cd), Mercury (Hg), Copper (Cu), Zinc (Zn) and Plomb (Pb) in the Bay of Agadir (Moukrim et al. 2000; Banaoui et al. 2004) knowing that industrial and urban wastewaters generated a reel pollution and a present threat to marine ecosystems (Halpern et al. 2008). In addition, many studies identified bacterial pollution in seawater of industrial zone of Anza and Agadir beaches (Mimouni et al. 2002; Chaouay et al. 2014, El Mourabit et al. 2020). Pesticides used in agriculture in neighbouring farms were found also in Anza Beach and Oued Souss estuary located near of Agadir beach (Aagnaou et al. 2014, 2018).

All of these pollutants could be the origin of stress situation of marine organisms. This stress is due to environmental conditions and xenobiotic exposure. Stress factors, both natural and anthropogenic, may be a cause of physiological and structural alteration at many levels of the marine organisms. Our previous studies have indeed demonstrated contamination of Agadir coastal by various pollutants using biomarkers (Banaoui et al. 2004; Ait Alla et al. 2006; El Azzaoui et al. 2019). Use of a battery of biomarkers allows an integrated assessment of effects at chemical, cellular, sub-cellular, tissues and whole organism levels due to the dynamic equilibrium of the marine environment.

To closely monitor the impact of installation of the two WWTPs onto the beach's health of maritime coasts of Anza-Taghazout, mussels of *Mytilus galloprovincialis* can be used as a pollution indicator or bio-accumulator due to its large ecological advantages. In general, bivalves are a good bioindicators for pollution biomonitoring particularly the *Mytilus* spp. (Taleb et al. 2009). *Mytilus* spp. accumulate waterborne pollutants during filter feeding of water (Beyer et al. 2017) and they have an efficient detection of many pollutants in water systems Reichwaldt and Ghadouani (2016). Also, they are characterizing by a large geographical distribution, resistance to many contaminants, long life-cycle, easy to capture and to maintain under laboratory-controlled conditions, all of these properties give to these organisms to be a good standard choice for ecotoxicological testing during bioaccumulation monitoring process of many compounds (Teixeira et al. 2017b).

Biomarkers are classified according to events triggered by battery of biomarkers of exposure, effects or sensitivities (Lagadic et al. 1997). Recently, a study involving the determination of a battery of biomarkers in *Mytilus galloprovincialis* to assess environmental quality showed that the selected battery of biomarkers responded efficiently to the environmental changes and allowed an environmental assessment between seasons and sites (Cravo et al. 2009). Battery of selected biomarkers in our work included four biomarkers which are Acetylcholinesterase (AChE, biomarker of neurotoxicity), glutathione S-transferase (GST, biomarker of detoxification), Catalase activities and malondialdehyde levels (CAT and MDA; biomarkers of oxidative stress and lipid peroxidation).

AChE has been used as a biomarker in several organisms such as mussels (Doran et al. 2001) due to its sensitivity to many contaminants such as organophosphorus or carbamate compound contamination. These contaminants apply inhibition effect onto AChE activity in mussels. This inhibition is directly linked with the mechanisms of toxic action of anticholinesterase compounds Rodriguez-Fuentes and Gold-Bouchot (2004) and has been used as an effect biomarker after exposure to pesticides and to certain metals (e.i. lead and mercury) Bocquené and Galgani (1991). Recently, AChE has been identified as a suitable biomarker for incorporation into the OSPAR Coordinated Environmental Monitoring Program (CEMP) (SGIMC, I. 2011).

In addition, GST has been recently identified as a suitable biomarker for monitoring chemical pollution in highly productive marine coastal ecosystems (Vidal-Linán et al. 2010) and has been widely used as a biomarker of exposure to PAHs, PCBs and trace metals in invertebrates' organisms (Funes et al. 2006). GST are involved in the conjugation and detoxification of organic compounds (Phase II detoxification), which also play a protective role against oxidative stress by catalyzing a selenium independent glutathione peroxidase activity (Sheehan et al. 2001), and which catalyze the conjugation of the reduced form of glutathione (GSH) with xenobiotics that can then be detoxified (Blanchette et al. 2007).

Catalase is an oxidoreductase enzyme present in prokaryotes and eukaryotes cells (Kashiwagi et al. 1997), especially in invertebrates with high activity (Regoli et al. 2002) and which is involved in catalytic conversion of two harmful molecules of hydrogen peroxide (H_2O_2) into two molecules of water (H_2O), and one of oxygen (O_2) (Gonçalves-Soares et al. 2012). Indeed, molecular oxygen is used naturally in aerobic processes or in oxidative stress occasions which induce catalase activity to be overexpressed to reduce the excess of formed oxygen peroxide (Oruc et al. 2004). So, this enzyme increase might function as a compensatory tool to face oxidative alterations (Van der Oost et al. 2003).

To measure disturbances caused by excessive reactive oxygen species production in marine organism's cells, MDA has been used as good biomarker of oxidative stress (Almeida et al. 2005). MDA used as marker of oxidation of membrane phospholipids through lipid peroxidation (reaction of oxygen with unsaturated lipids) due to oxidative stress (Alexandrova and Bochev 2005) and it is considered as a second carbonyl degradation products of lipid peroxidation of cell membranes. Additionally, toxicity biomarkers, such as MDA, well-known lipid peroxidation products, have been also proposed to reflect the oxidative status of exposed species (Cossu et al. 2000).

Then, to have a good study of all biomarkers, an IBR index was added in this paper to improve the discriminatory power of the adopted multi-biomarker strategy. This technique is used by many researchers in this field (Beliaeff and Burgeot (2002); Sanchez et al. 2013; Devin et al. 2014). Therefore, the aims of the present study were 1) to measure the following activities of four biomarkers (AChE, GST, CAT and MDA) in *M. galloprovincialis* mussels, 2) to study multi-biomarker strategy using IBR index and 3) to better comprehend the health state of Anza-Taghazout beaches after the installation of two WWTPs.

Material And Methods

Study areas

The mussel *M. galloprovincialis* was collected monthly during two years of biomonitoring from January 2016 to December 2018 in three sites of Anza-Taghazout marine coasts. The first site called Anza beach (S_1) is located at 10 km in the north of Agadir city near to Anza WWTP and Agadir harbour. It is characterized many years ago by its alarming pollution state and quality of its seawater was in category D (Mimouni et al, 2002; Chaouay et al, 2014). The second one is Aourir Beach (S_2) which receive a treated discharge of Aourir WWTP and effluents of Aourir river during rainfall periods. It is characterized by surf activity and a moderate touristic activity. The third one is called Imouran Beach (S_3), and it is characterized by the existence of big touristic activities (camping cars for the rest of tourists, hotel units etc...). It should be noted that the two sites S_2 and S_3 are clean in comparison of S_1 (Table 1 and Fig.1).

Table 1 Description of sampling sites and GPS location.

Beaches	Acronym	Latitude (google map)	Longitude (google map)
Anza	S_1	30°26'59.4"N	9°39'43.4"W
Aourir	S_2	30°30'08.3"N	9°40'59.0"W
Imouran	S_3	30°30'29.2"N	9°41'16.0"W

Animal sampling

In this work, one specie of bivalves is used: *Mytilus galloprovincialis*. Each sample consisted of a pool of 12 mussel's specimens within the same size for each month to create homogenous group (864 specimens during two cycles). Collection was performed at low tide by hands from littoral sediments in the intertidal zone, packed immediately in seawater in plastic bottle, and stored at 4°C in ice-cold during transport to the laboratory. Once in the laboratory, the animals were kept at - 80 °C until day of analysis.

Environmental parameters

During our studies, many environmental parameters were monitored monthly such as temperature (T), salinity (S), pH, dissolved oxygen (DO) and electrical conductivity (EC) in three sites during two cycles 2017 and 2018. Environmental parameters were measured on site using a portable multiparameter Orion Star™ A329 Thermo SCIENTIFIC®.

Tissue preparation (fraction S_9)

The soft tissues are taken after thawing the mussels stored at - 80 °C using a solid scalpel and then dried on absorbent paper to remove excess water. The weight of the entirety tissue mass is taken using a precision scale (0.001). Then, these soft tissues are immersed in 100 mM of Tris buffer at pH 7.4 (Tris [hydroxymethyl] aminomethane) in an ice bath (4 °C), with ratio of 3 mL of Tris solution per 1 gram of

fresh tissue and immediately homogenized with an Ultra-Turrax (at 5000 rpm). Then, an aliquot of the homogenate obtained was centrifuged at 9000 g for 30 minutes and at 4°C in a centrifuge (Sigma 6-16KR) to separate the soluble and insoluble compounds. The supernatant obtained at 9000g constitutes the post-mitochondrial fraction or S₉ which is either directly used or stored at - 80 °C in Eppendorf tubes labelled until the biomarker assays. Biomarkers were measured by using a spectrophotometer.

Biomarker measurements

GST, catalase and acetylcholinesterase activities and MDA levels were measured according to the methods of (Habig et al. 1974, Aebi 1983, Ellman et al. 1961; Sunderman et al. 1985) respectively. Total proteins present in the S₉ fraction were determined according to the method of Lowry et al. (1951), with bovine serum albumin as a reference.

Integrated Biomarker Response

To combine all of the measured biomarker data into an integrative index, our results were analyzed using IBR methodologies Beliaeff and Burgeot (2002). These two authors have established a simple method summarizing biomarker responses that simplify the interpretation in biomonitoring programs. However, adopting this more recent development of the IBR procedure, the results are the mean of the possible values obtained by permutation of the biomarker data in the mathematical calculation represented by Radar graph (Devin et al. 2014).

Statistical analysis

All graphs of the present paper were prepared in Microsoft corporation® Excel 2016. The activities of biomarkers were expressed as average \pm standard deviation (SD). Study of Analysis of variance (ANOVA) and Tukey's test ($P < 0.05$) were performed using the StatSoft, Inc. (2014). STATISTICA (data analysis software system, version 12. www.statsoft.com). IBR stress index of all biomarker data (AChE, CAT, GST and MDA) was performed by using original method of Beliaeff and Burgeot (2002) ameliorated by Sanchez et al. (2013).

Results

Environmental results

Concentration of five environmental parameters are shown in Table 2. In the three sites studied, values of pH is ranged from 8.10 ± 0.19 in S₁ to 8.15 ± 0.14 in S₂, temperature from 19.69 ± 2.68 (C°) in S₃ to 20.24 ± 2.76 (C°) in S₁, electrical conductivity from 54.73 ± 2.04 ($\mu\text{S}/\text{cm}$) in S₁ to 55.45 ± 1.56 ($\mu\text{S}/\text{cm}$) in S₃, Salinity from 36.09 ± 1.00 (mg/L) in S₁ to 36.27 ± 0.96 (mg/L) in S₃ and values of DO₂ range from 7.89 ± 0.78 (mg/L) in S₁ to 8.09 ± 0.92 (mg/L) in S₂. Environmental results that we have found, follow the climatic conditions.

Table 2
Mean \pm SD, minimum and maximum of pH, temperature, EC, salinity and values at the three sites. (Mean \pm SD (min ; max))

Site	pH	T (C°)	EC (μ S/cm)	Sal (mg/L)	DO ₂ (mg/L)
S₁	8.10 \pm 0.19	20.24 \pm 2.76	54.73 \pm 2.04	36.09 \pm 1.00	7.89 \pm 0.78
	(7.2;8.54)	(14.6;27.6)	(46.28;58.69)	(30.02; 37,53)	(6.21; 10.55)
S₂	8.15 \pm 0.14	19.91 \pm 2.99	55,09 \pm 2.14	36.12 \pm 1.09	8.09 \pm 0.92
	(7.38;8.43)	(14;29.2)	(46.6; 58.82)	(30.02; 37.67)	(6.11; 10.53)
S₃	8.14 \pm 0.14	19.69 \pm 2.68	55.45 \pm 1.56	36.27 \pm 0.96	7.91 \pm 0.85
	(7.19;8.4)	(14; 26.2)	(50.1; 58.69)	(30.52; 37.72)	(5.98; 10.25)

Biomarkers Results

In our study, the dataset formed by four biomarkers was monthly registered between January 2017 and December 2018 during 24 months. Six pools (two whole soft tissues each) of mussels were used. The results of spatiotemporal variation and results of ANOVA Tukey HSD test ($p < 0.05$) at three studied sites are shown in Fig. 2. During the first cycle, CAT concentrations ranged from minimum value of $3.34 \pm 1.43 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins registered at July 2017 in S₂ to a maximum value of $234.80 \pm 42.37 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins at February 2017 in S₃ (Fig. 2a). While, CAT activity in 2018 ranged from minimum values registered in July 2018 in S₃ with $2.50 \pm 0.82 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins to high value $97.11 \pm 1.49 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins at October 2018 in S₁. Measured activities of CAT in mussel show a high significant difference between the three studied sites during two cycles except July 2017, September 2017, November 2017 and December 2017 ($p > 0.05$).

In addition, GST activity presented in Fig. 2b shows, that all months except January 2017, December 2017 and December 2018, show a high significant difference between the three studied sites ($p < 0.05$). During the first cycle, GST concentrations ranged from minimum value of $4.98 \pm 2.54 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins registered at August 2017 in S₁ to a maximum value of $47.95 \pm 1.86 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins at March 2017. Whereas in 2018, GST concentrations ranged from minimum values registered in May 2018 in S₂ with $17.11 \pm 2.31 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins to high value $71.07 \pm 5.87 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins in November 2018 in S₁.

Spatiotemporal variations of MDA (Fig. 2c) show that the higher activity in the three studied sites during 2017 was registered in February in S₃ ($106.87 \pm 4.32 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins) and the low activity was recorded in July in S₂ ($1 \pm 0.22 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins). In 2018 cycle, the low activity ($2.16 \pm 0.48 \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ proteins) was registered in January in S₃ against high activity (67.04 ± 18.42

$\mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins) recorded in October in S_1 . Study of significance between sites shows a high significant difference during two cycles except November 2017, May 2018 and December 2018 ($p > 0.05$).

AChE activity (Fig. 2d) oscillated from $0.95 \pm 0.32 \text{ nmole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins in November in S_1 to $16.54 \pm 1.13 \text{ nmole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins in April also in S_1 during 2017 and from $1.17 \pm 0.32 \text{ nmole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins in January in S_3 to $17.69 \pm 4.59 \text{ nmole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins in September in S_3 during 2018 cycle. No difference was observed between sites in August, September, October 2017 and June, July, October 2018 contrary to other months.

Results of study of the difference between two cycles 2017 and 2018 in the three studied sites is shown in Fig .3. We observed an excessive induction and a high significant difference of GST activity of means of three sites in 2018 compared with 2017 cycle (Fig. 3a). In contrast, no difference was observed between two cycles concerning catalase activity (Fig. 3b). Regarding MDA activities, no difference was observed in both S_2 and S_3 , whilst S_1 has a high significant difference increased from $8.65 \pm 1.51 \mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins in 2017 to $20.73 \pm 1.51 \mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins in 2018 (Fig. 3c). AChE activities, don't show any difference among two cycles both in S_1 and S_2 , whilst S_3 show a high significant inhibition in AChE activity during 2017 with $4.01 \pm 0.47 \text{ nmole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins compared to 2018 with $8.58 \pm 0.47 \text{ nmole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins (Fig. 3d). During the entire period of our study, the accumulation rate of the four biomarkers does not present a significant difference between mussels of the three studied sites ($p > 0,05$) (Table 3).

Table 3
ANOVA and Tukey HSD test of biomarkers results between sites (Mean \pm Std.Err).

Biomarkers	S_1	S_2	S_3	F	P-value
GST	30.53 ± 1.35	28.32 ± 1.35	26.60 ± 1.35	2.11	0.12
MDA	14.69 ± 1.53	16.59 ± 1.53	19.47 ± 1.53	2.45	0.08
CAT	37.42 ± 3.72	35.05 ± 3.72	35.94 ± 3.72	0.10	0.90
AChE	5.79 ± 0.39	6.70 ± 0.39	6.29 ± 0.39	1.36	0.25

IBR Results

The contribution of the four biomarkers to the IBR calculation differed according to pollution state of the three studied sites. The most sensitive responses could be visualized in the corresponding radar graph (Fig. 4). The results of IBR (Table 4) indicate a low value registered in S_1 (0.00 ± 0.00) and high one in S_3

(5.44 ± 0.04) during 2017 cycle. Conversely, low IBR was recorded in S_3 (0.00 ± 0.00) against high value (6.78 ± 0.00) registered in S_1 during 2018 cycle.

Table 4
Summary of IBR values

Sites	Years	IBR.mean	IBR.sd	IBR.min	IBR.max
S_1	2017	0.00	0.00	0.00	0.00
	2018	6.78	0.00	6.78	6.79
S_2	2017	2.12	0.40	1.68	2.46
	2018	0.13	0.05	0.09	0.18
S_3	2017	5.44	0.04	5.40	5.46
	2018	0.00	0.00	0.00	0.00

Discussion

The current study was carried out to evaluate effect of WWTPs installed on maritime coasts of Anza-Taghazout ecosystem since 2016. It is considered as the first ecotoxicological approach in this region to monitor all the effects related to WWTP installed on marine ecosystem life, using *Mytilus galloprovincialis* as sentinel species.

Abiotic factors may be a source of influence of biomarker activities. Many studies demonstrated the relationship between abiotic factors (Temperature, salinity, pH, oxygenation, water-type and substrate) and modification of biomarkers expressions (Vidal et al. 2002; Pfeifer et al. 2005). In addition, salinity influence considerably biochemical response of *Crassostrea angulate* oyster exposed to Arsenic (Moreira et al. 2016). Impact of Arsenic in the embryo-larval development of oysters are altered by changing in salinity and temperature (Moreira et al. 2018). Thus, changes of environmental parameters can heavily influence the enzyme activity of the biomarker (Whyte et al. 2000).

Regarding biomarkers activities shown in Fig. 2 and Fig. 3, biomarkers assessed in three representative sites in the present paper show a seasonal variability. Invertebrates use CAT as an enzyme of antioxidative defence and protection (Regoli et al. 2002). High levels of CAT registered in our studies may be related to an excess of H_2O_2 formed after using O_2 in aerobic process or oxidative stress occasions (Oruc et al. 2004). Reactive oxygen species (ROS) such as H_2O_2 were eliminated by CAT which convert two molecules of H_2O_2 into two molecules of H_2O and one of O_2 (Gonçalves-Soares et al. 2012). ROS can be very dangerous for constituents of cells and provoke damage of DNA (Frenzilli et al. 2001), lipid peroxidation (Viarengo 1989) and lysosomal alteration (Winston et al. 1996). The rise of CAT activity may suggest that *M. galloprovincialis* were subjected to oxidative stress, making it possible to conclude that is in agreement with work of Vidal-Linan et al. (2010) in polluted zone. Previous studies have shown that

Mytilus.sp presents an induction of CAT activity in response to organic pollution (Porte et al. 1991), trace metals (Duarte et al. 2011), polycyclic aromatic hydrocarbons (Orbea et al. 2006), chemicals compound present in domestic sewage discharge (Saenz et al. 2010), domestic wastewater and agriculture discharge (Abbassi et al. 2015). However, a decrease of CAT activity should not be considered as a signal of a good health state of the environment, but it can be also an index of the difficulties that have organisms in defending against oxidative stress (Regoli et al. 1995). In fact, decrease of CAT activity may be the results of exposure during long time to oxidative stressors. According to several studies which demonstrated that the antioxidant defence can be invaded due to prolonged periods of exposure to contaminants (Lowe and Fossato 2000; Stohs et al. 2000; Duarte et al. 2011).

Concerning AChE activity in Fig. 2d, it shows a seasonal variability as demonstrated by Taleb et al. (2007) and Elazzaoui et al. (2019). It must be recalled that synaptic transmission of nerve impulses requires the contribution of AChE enzyme and is inhibited by neurotoxic compounds like organophosphate and carbamate pesticides (Bocquené et al. 1998). Decrease of AChE in our study may be related to temperature conditions (Kirby et al. 2000). Also, both salinity and temperature could modify AChE activity in bivalves (Pfeifer et al. 2005). Many studies have demonstrated that metals inhibit unselectively AChE activity, while organophosphate and carbamate pesticides inhibit selectively AChE activity, leading to serious physiological deficiency in marine wildlife (Rickwood and Galloway 2004; Tsangaris et al. 2010). In addition, variation in AChE activity in mussels may be related to differences in sex, age, length, weight, or physiological conditions (Fairbrother et al. 1989).

Besides this, GST activities in *Mytilus galloprovincialis* present seasonal variations in the three studied sites. It is worthwhile to note that the highest enzyme induction found in our studies is explained by many factors. High induction of the GST activity reflects a stress state due to contamination by organic compost such as HAPs (Trisciani et al. 2012). In addition, dioxins may also induce GST activity (Van der Oost et al. 2003). This induction enters into phase II reactions biotransformation of chemical compounds (Sheehan et al. 1999) specially in digestive gland of mussels which is characterized by abundance of GST activity and which paly highest role in conjugation detoxifying metabolism (Canesi et al. 2007). In an industrialized area for example, active monitoring has demonstrated that mussels from the most polluted sites show a significant higher GST and GPx activities compared to the control site (Vidal-Linan et al. 2014). Drug cetirizine induces a decrease of GST activity in *M. galloprovincialis* (Coppola et al., 2017). While, trace elements, nanoparticles and a mixture of endocrine disruptor compounds including NP, endocrine disruptor bisphenol-A increase this activity (Canesi et al. 2008; Coppola et al. 2017). It is primordial to be taken in account that, mussels exposed continuously to a weak level of toxicity and which is not high enough to elicit an efficient response may be the origin of a weak response of GST due to acclimatization of mussels to these conditions (Wepener et al. 2008). Variations of temperature can also be the origin of the GST activities in *M. galloprovincialis* as studies of (Kayaa et al. 1999) have proved that temperatures below 20°C and above 35°C generated weakest activities of GST while highest activities were found at 30°C for the mussels of *M. galloprovincialis*. Variations of GST activity could be also linked to the mussel's reproduction cycle (Id Halla et al. 1997) and high levels of GST activity may be

due to restoration of reserves and gametogenesis periods while lower values coincide with spawning periods (Kayaa et al. 1999).

Induction of MDA during winter season months may indicate an oxidative stress situation due to high contamination in bivalves. The MDA content in combination with variations in the activity of the antioxidant enzymes expresses the lipid peroxidation at the cellular membrane phospholipids (Alexandrova and Bochev 2005). It is correlated to the contamination of the aquatic environment by polyaromatic hydrocarbons (HAP) and heavy metals (Labrot et al. 1996; Frouin et al. 2007). Lipid peroxidation is generated in aquatic organisms exposed to high concentrations of toxic substances, including HAPs (Downs et al. 2002). Existence of HAPs in the marine environment is due to atmospheric emissions, water-efficient effluents and surface runoffs (Simpson et al. 1996). Indeed, MDA is carbonyl degradation products of lipid peroxidation of cell membranes which reflect the oxidative status of exposed species after oxidative stress (Cossu et al. 2000; Blaise et al. 2002). The concentration of MDA has been largely used as a biomarker of disturbances caused by excessive production of ROS in the cells of marine organisms (Almeida et al. 2005). Seasonal variability of MDA concentration during our studies may also translate the relationship between reproductive cycle and oxidative stress in bivalves (Wilhelm Filho et al. 2001).

IBR method is used to estimate amplitude of contamination. Four measured biomarker data were combined into an integrative index namely IBR index (Beliaeff and Burgeot 2002). Mussels exposed to high concentrations of pollutants during our period of survey translate high IBR value observed in Radar graph both in S₃ in 2017 and in S₁ in 2018. Conversely to low IBR value registered in S₁ in 2017 and in S₃ in 2018 which explain exposing of mussels to low concentrations of contaminants. For example, studies of (Peric and Buric 2019) demonstrate that exposure of mussels *Mytilus galloprovincialis* to 5 mg/L of organophosphorous pesticide chlorpyrifos resulted in low IBR value, conversely to high IBR value registered at 15 mg/L of metal copper. In addition, (Bocquené et al. 2004) used IBR index to combine AChE, GST, CAT activities and MDA concentrations, on the common mussel *Mytilus edulis* in the coast of Brittany (France) and they found that IBR index was strongly related to MDA levels (Bocquené et al. 2004). Broeg and Lehtonen 2006, measuring IBR in eelpout (*Zoarces viviparus*) and blue mussel (*Mytilus sp.*) populations of the Baltic Sea, reported good accordance between IBR and tissue levels of organochlorine compounds and they have concluded that IBR becomes more robust when the number of biomarkers increases. Damiens et al. 2007, found good agreement between IBR values and copper, PCB concentrations in transplanted mussels but not with PAHs concentrations.

However, IBR is not suitable for giving an indication of the level of stress in the mussels; this IBR recognizes variations in the studied biomarkers, but not their biological meaning (Bouhallaoui et al. 2017). Beliaeff and Burgeot (2002), also concluded that the selection of an appropriate battery of biomarkers allow information to be summarized in the form of a multivariate data and can avoid false-negative responses obtained with a single biomarker. Interpretation of IBR data must be done carefully considering possible mutual interference of mixture components, often asynchronous and complex biomarkers responses and magnitude of response of each biomarker (Quintaneiro et al. 2015).

Conclusion

In conclusion, this ecotoxic assessment performed in Anza-Taghazout coasts has enabled us to confirm the ecological restoration of the health stat of Anza beach. The actual health state of S_1 may be due to installation of two WWTPs in the two cities of Anza and Aourir.

Furthermore, multibiomarker approach assessment has revealed a seasonal variability of the four biomarkers measured in soft tissues of sentinel species *M. galloprovincialis* at the three sites of interest. In addition to that, IBR approach allows us to good visualization of biological effect of contaminants on Mussels. This combination of four biomarkers data allows obtaining a compact means of representing data.

The lack of significant differences between the three studied sites is a good indicator that S_1 which had been qualified many years ago as the most polluted area within the region of Agadir coastline, has recently become clean.

To sum up, these ecotoxicological approaches with biomonitoring studies on site give us an idea about area health state after installation of the two WWTPs in Anza and Aourir and similar studies like ours could be a reference of positive impact of WWTP onto health state of maritime coasts.

Declarations

1. Ethics approval and consent to participate

“Not applicable” in this section,

2. Consent for publication

I give my consent for the publication of details of this work in the above Journal and article.

3. Availability of data and materials

The data that support the findings of this study are available from the corresponding author, [Youssef EL Mourabit], upon reasonable request.

4. Declaration of competing interest

All the authors of the present article declare to have no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations. The following article has not been published previously and that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the copyright-holder.

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6. Author contributions

All the authors declare to have participated in this study, with the following participation: Youssef EL MOURABIT, Abdellatif MOUKRIM and Aicha AIT ALLA: Experimental design and Methodology, Youssef EL MOURABIT and Mustapha AGNAOU: Experiment assays and analysis, all authors: Writing - review and editing.

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Figures

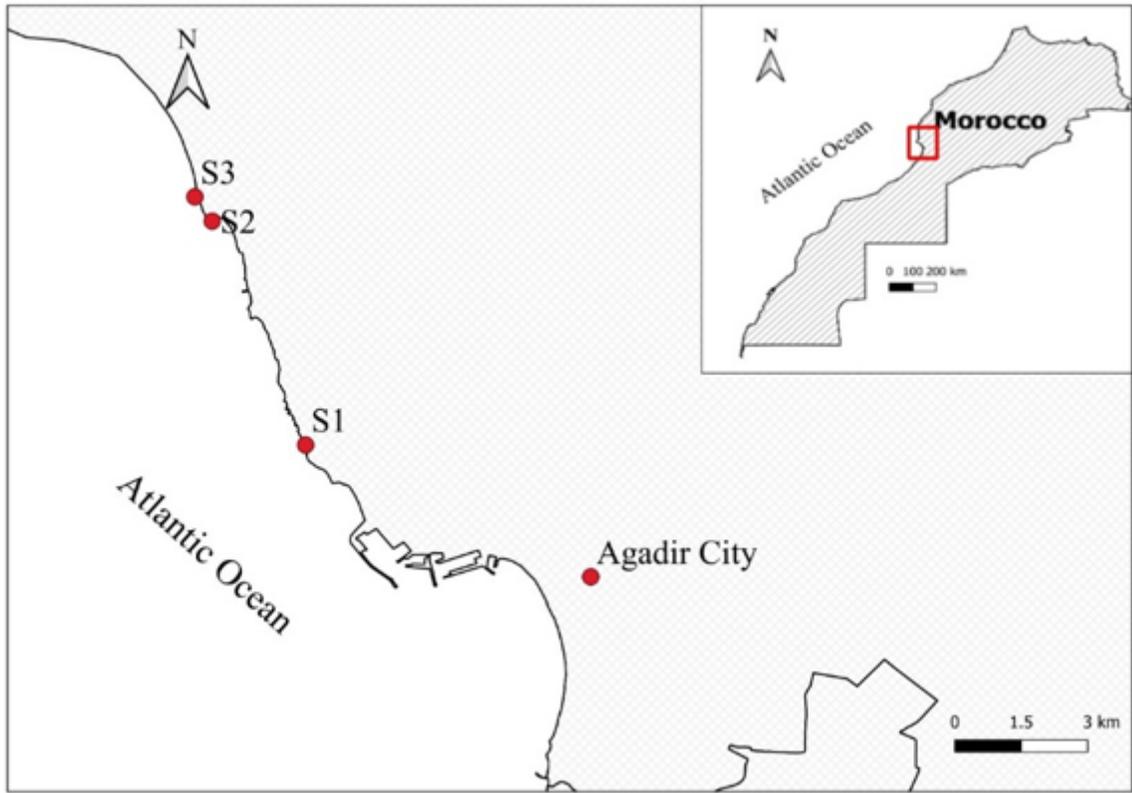


Figure 1

Location of the study area. S1) Anza beach. S2) Aourir beach. S3) Imouran beach. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

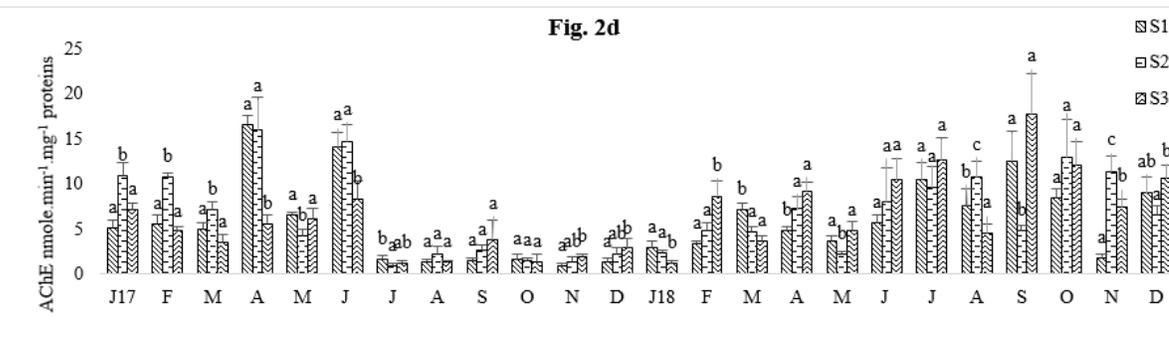
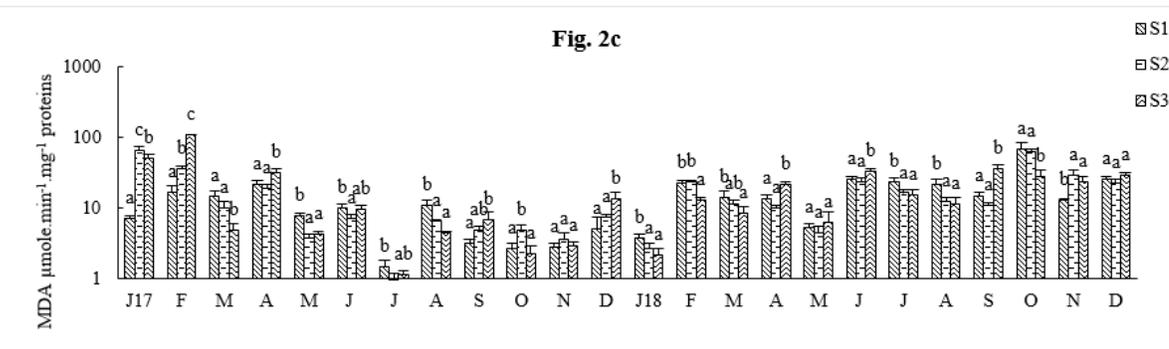
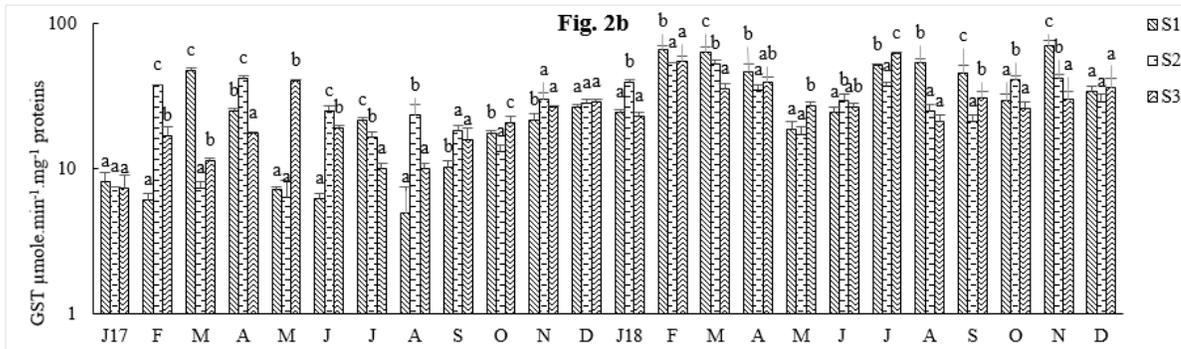
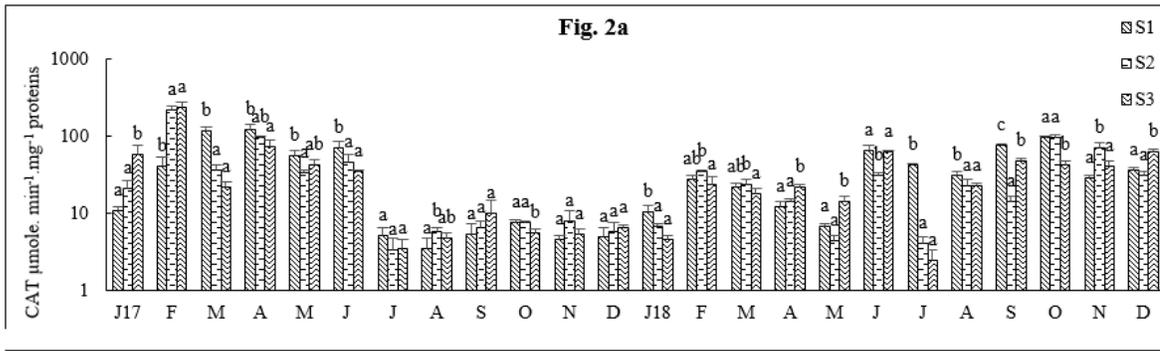


Figure 2

Mean enzymes levels of *M. galloprovincialis* in S9 of whole animal represented by site and months. a) Catalase activities, b) GST activities, c) MDA activities and d) AChE activities. The values indicate mean \pm SD, significant differences between sites were analysed by ANOVA (Tukey's post hoc test), groups with the same letters are not significantly different at the chosen level (5%).

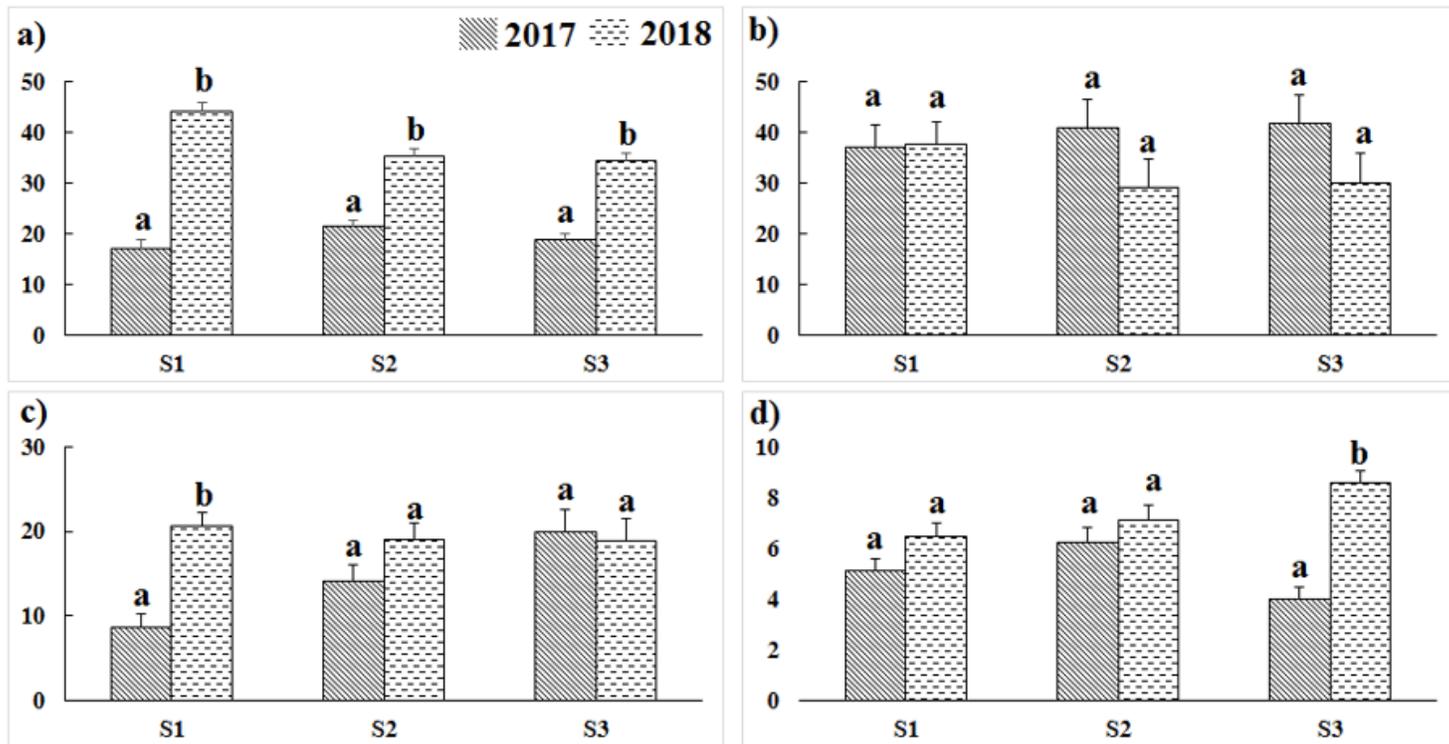


Figure 3

Annual Mean enzymes of *Mytilus galloprovincialis* in S9. a) GST activities, b) Catalase activities, c) MDA activities and d) AChE activities. The values indicate mean \pm SE, different letters mean significant differences at the chosen level (5%).

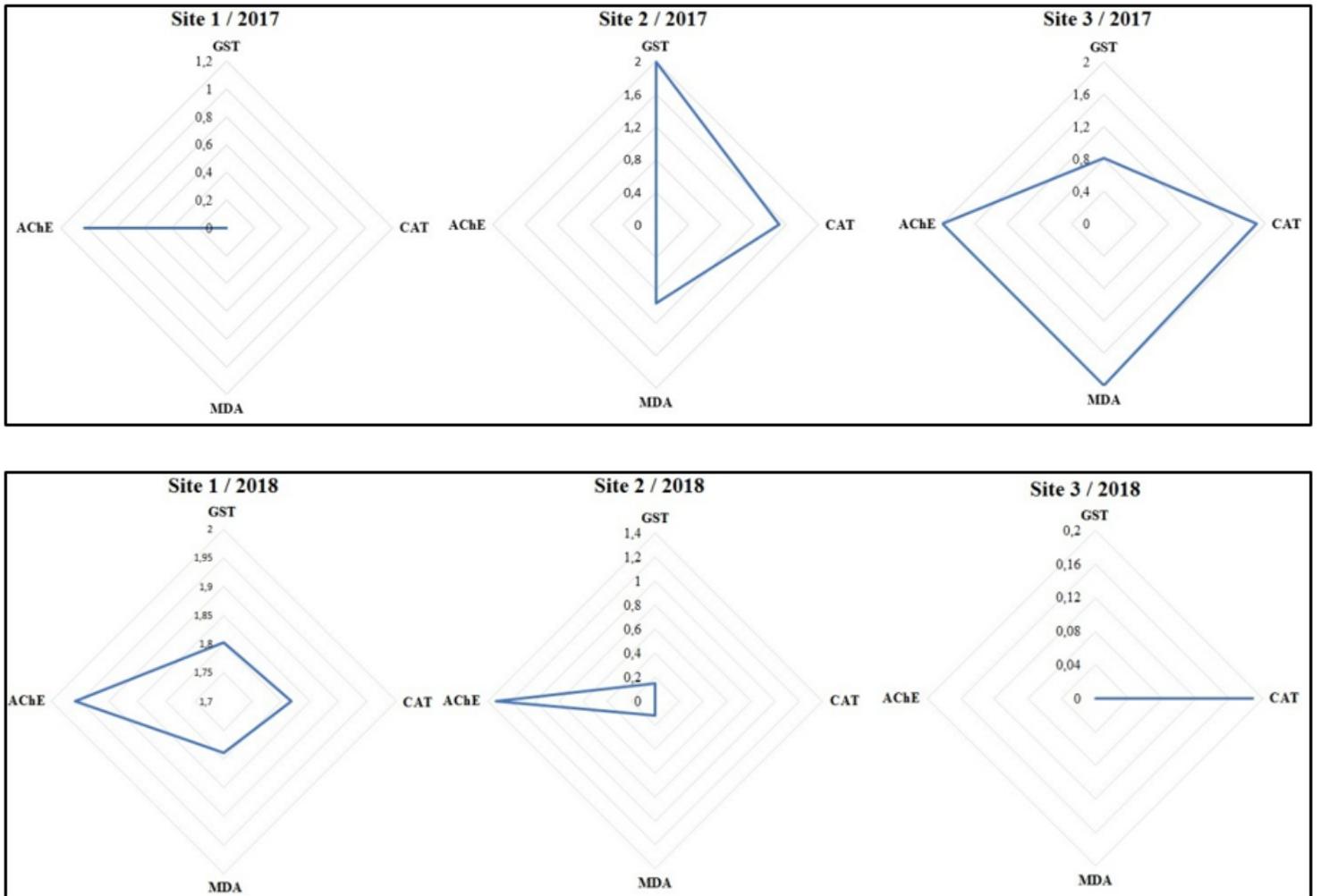


Figure 4

Radar graph of S1, S2 and S3 during 2017 and 2018