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Assessment of the contamination level of the complex phosphate miner of Jorf-Lasfar effluents (El Jadida, Morocco) by measuring the DNA adducts in the Mussel (*Mytilus galloprovincialis*)

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Abstract

The levels of DNA adducts in hepatic tissue of the mussel (*Mytilus galloprovincialis*) were determined by ^{32}P-postlabelling. Mussels were collected at four sites located at different distances from the phosphate complex miner effluent of Jorf-Lasfar (at 17 km from El Jadida city) in Morocco. Results showed that total DNA adduct levels in all mussels ranged from 38-90 adducts per 10^9nucleotides.The mean adduct level (± standard deviation) in the mussel increases gradually toward the outlet of the effluent of the complex miner phosphate. The rate of DNA adducts detected at ST or control station (at 28 km of the complex) is 38 nucleotides per 10^9 nucleotides, while 52 adducts per 10^9 nucleotides were detected at station 1 (at 2 km of the complex), and 90 adducts per 10^9 nucleotides were detected at stations 2 and 3 (at 0.5 Km and 1 km from the discharge point of the complex, respectively). Moreover, DNA adducts of the mussels collected at all stations showed the same chromatographic profile including 11 minor and 3 major spots. Their rate of DNA adduct vary between 0.2 and 33.5. These results obtained during this investigation suggest that the effluents of many heavy industries (Energy, Metallurgy and Chemistry/Husbandry) are more likely to produce genotoxic damage to mussels.

Keywords: Mytilus galloprovincialis, DNA adducts, ^{32}P-postlabelling, Jorf-Lasfar Complex, Morocco

1. Introduction

Environmental carcinogenic contaminants found in soil, air, water or sediments can represent a significant genotoxic hazard to the ecosystem and to human health [1, 2, 3, 4].Mussels are a good bio-indicator species and a suitable sentinel species for pollution monitoring in water ecosystems due to their large geographical repartition, ecologic niche and their cumulative power of pollutants which explains their presence in heavily polluted areas inhabited by fish [5]. These advantages have led several countries to incorporate determination of contaminants in mussels' tissue in their national surveillance programs. Among the variety of natural and anthropogenic contaminants in the aquatic environment many are genotoxic, i.e. capable of interacting with the genetic material. It is now widely recognized that the marine environment contains a variety of natural and anthropogenic chemical compounds some of which are capable of interacting with the genetic material of aquatic organisms [6].

The field of genetic toxicology currently offers a wide array of methodologies for the assessment of the impact of genotoxic exposure on aquatic species. Molecular techniques have the potential to allow the determination of 'biomarkers' that could ultimately predict the genotoxic impact of exposure to environmental contaminants, thus the major biomarkers of DNA damage is the measurement of DNA adducts in target cell or tissues [7].

The genotoxicity of some environmental contaminants arise from their ability to form covalent bonds with DNA molecules [8]. These reactions leading to form addition products or adducts. This chemical damage could ultimately leads to mutation and malignant transformation of the cell [9].

The measurement of DNA adducts is therefore used to assess the genotoxic and carcinogenic potential of chemicals as well as to characterize the exposure of organisms to contaminated environments [10].

Till now, many studies on the measurement of DNA adducts in mold by ^{32}P-postlabelling technique were carried out to evaluate the degree of contamination of the aquatic environments [11].

The Moroccan coast extends over a length of 3500 km. There is a significant heritage for Morocco because of its rich fauna and flora, but unfortunately it is continually assaulted by a growing population, an important industrial concentration (chemical, textile, tannery, power plant ...). In the present study, our aim was to determine the levels of DNA adducts in the hepatopancreatic tissue of *Mytilus galloprovincialis* to evaluate the contamination level of the complex miner phosphate of Jorf-Lasfar effluents.

Jorf-Lasfar area is located 25 km south of the city of El Jadida (Morocco) and contains a complex of chemical industry named Morocco phosphorus III and IV whose releases are discharged directly and continually in sea water using two manifolds. The cooling rejection (Rc): effluent liquid, consisting of sea water used for cooling the levels of sulfuric workshops, for maintaining the vacuum in the boilers and for washing the filters and the principal rejection (Rp): effluent liquid-solid-rich phospho-gypsiferous residues. The latter are recovered at the outlet of the step of filtration (**Figure 1**).

In the present study, our aim was to determine the levels of DNA adducts in the hepatopancreatic tissue of *Mytilus galloprovincialis* to evaluate the contamination level of the complex miner phosphate of Jorf-Lasfar effluents (away 17 km from El Jadida city, Morocco).

2. Materials and methods

2.1. Mussels collecting

Mussels were collected at four stations on sea Atlantic from the province of El Jadida, Morocco. The control station (ST) located 10 km North of the city of El Jadida, far different sewage outfalls, station 1 (S1), station 2 (S2) and station 3 (S3) are located at 27 km, 2 km, 1 Km and 0.5 Km from the complex phosphate miner of Jorf-Lasfar (**Figure 1**). Twenty mussels of the species *Mytilus galloprovincialis* were collected per station in June (breeding season is between March and June). The mussels were taken at low tide in the intertidal zone in the lower intertidal. Within one hour of the capture, the mussels belonging to the class size 3-4 cm were dissected, and the hepatopancreatic tissues were carefully removed before being transferred individually in cooled containers. They were then stored at -80°C until DNA extraction.

2.2. Purification of DNA

Hepatopancreatic DNA of *Mytilus galloprovincialis* was extracted and purified as described previously [12]. Essentially, the mussels were crushed in liquid nitrogen, and 2 to 3 g of crushed hepatopancreatic tissue powder were incubated at 65°C for 60 min in SET buffer (100 mM NaCl, 20 mM EDTA ; 50 mM Tris base, pH 8.0) with 20% SDS. 40 milliunits (mU) of proteinase K were added and the mixtures were incubated at 37°C for another 2 h. Proteins were precipitated by adding 6M potassium acetate (pH5.0).

The samples were kept on ice for 30 min before being centrifuged for 10 min at 10 000 x g at 4°C. The samples were thereafter submitted for 1 min to phenol : chloroform : isoamylalcohol (25 : 24 : 1; v : v : v) then 1 min for the chloroform : isoamyl alcohol (24 : 1; v : v). DNA was precipitated in cold ethanol, spooled out, centrifuged for 10 min and rinsed twice with 70% ethanol. To eliminate RNA, 1 U of RNAse and 5U of RNAse T1 were added to 100 mg of nucleic acids and incubated at 37°C for 1hr. The extraction procedure was repeated to eliminate proteins from the samples. DNA was then dried under vacuum and stored at -85°C until use.

2.3. ^{32}P-postlabelling technique

The ^{32}P-postlabelling technique used for DNA adducts detection was that described by Reddy and Randerath [13] with minor modifications [14]. Briefly, DNA (6µg) was digested at 37°C for 4 hr with 183 mU micrococcal nuclease and 12 mU spleen phosphodiesterase in a reaction mixture (total volume 10 µl) containing 20 mM sodium succinate and 10 mM$CaCl_2$, pH 6.

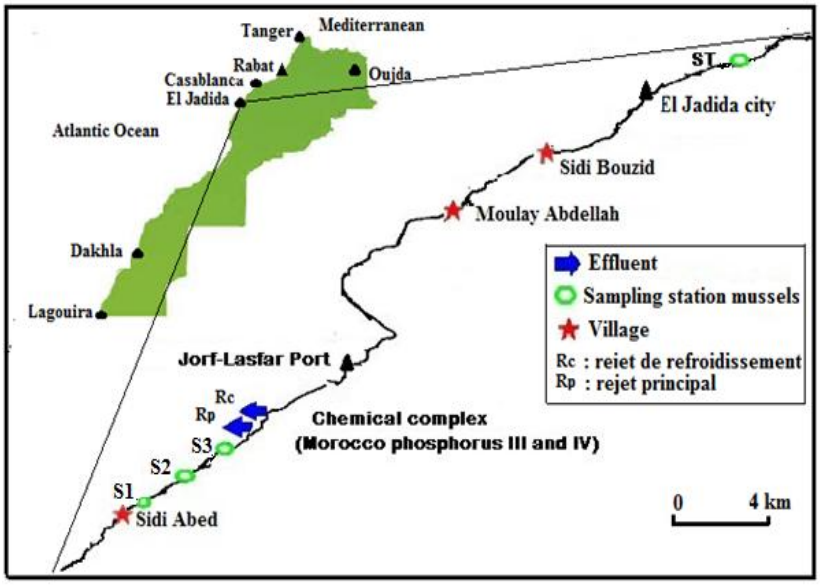

Figure 1: Geographical location of the study area

Digested DNA was treated with nuclease P1 (6 µg) at 37°C before ^{32}P-postlabelling, performed as follow : to the nuclease P1 treated solution, 2 µl of bicine buffer solution pH 9.8 and 4.5 µl of a mix containing 17 pmol[γ-^{32}P] ATP (100 µci) and 2.8 U T4-polynucleotide kinase were added. The mix was incubated for 45 min at 37°C. Normal nucleotides, pyrophosphate and excess [γ-^{32}P]ATP were removed by chromatography on polyethyleneimine cellulose plates in 2.3 M NaH_2PO_4 pH 5.7 (D1) overnight.

Origin areas containing labeled adducted nucleotides were cut out and transferred on to polyethyleneimine cellulose plate, which was run in 4.77 M lithium formate and 7.65 M urea pH 3.5 for 4.5 h (D2). Two further migrations (D3 and D4) were performed perpendicularly to D2. The solvent for D3 was 0.6 M NaH_2PO_4 and 5.95 M urea pH 6.4 for 3 hr and the solvent D4 was 1.6 M NaH_2PO_4pH 6.0 for 2-3 h. Radioactive adduct spots identified by autoradiography were excised from the chromatograms and their radioactivity counted by Cerenkov counting. Knowing the specific activity of ATP and taking into account the ^{32}P decay, the total levels of adducts were calculated by adding the radioactivity of the individual spots on the chromatograms. The results are given in relative adduct levels (adducts/10^9 normal nucleotides).

Three repetitions of the analysis of DNA adducts rate were performed. The statistical analysis was performed with the software STATITCF. If the differences between the means are significant, we used the test of Newman Keuls and for the comparison of averages. The probability threshold for acceptability or rejection of the hypothesis of equality of averages is fixed at 5%.

3. Results and discussion

The chromatograms of DNA adducts from the hepatic tissue of mussels taken at 4 stations from the effluent of complex phosphate miner Jorf-Lasfar (Morocco) and in blank runs in which DNA was omitted are shown in **Figure 2** (**Figures 2A to 2E**). DNA adducts of mussels collected at these stations have the same chromatographic profile (**Figures 2B to 2E**). They have 14 spots including 11 minor (numbers 1, 2, 3, 4, 5, 6, 7, 8, 9, 12 and 13) and 3 major spots (numbers 10, 11 and 14). Their rate of DNA adduct vary between 0.2 and 6 and between 5 and 33.5, respectively (**Figure 2F and table 1**).

Table 1 : Number of DNA adducts / 10^9 nucleotides per spot *in Mytilus galloprovincialis collected* at 4 stations (2B to 2E)

Spot Number	1	2	3	4	5	6	7
Adducts/10^9nucleotides	0.2 – 1.1	0.7 – 3	0.6 – 2.5	1 – 3.5	0.8 – 1.5	1.3 – 6	0.8 – 2
Spot Number	8	9	**10***	**11***	12	13	**14***
Adducts/10^9 nucleotides	0.5 – 1.5	0.8 – 4.7	**18 – 33.5**	**7.4 - 13**	0.7 - 3	0.2 - 2	**5 - 13**

** The major spots*

Table 2 shows the DNA adduct amounts of mussels taken at 4 stations from the effluent of complex phosphate miner Jorf-Lasfar (Morocco). They show a gradual increase from $38/10^9$ nucleotides in mussels collected near El Jadida city (at 27 km from complex phosphate miner) considered as control station (ST) (**Fig. 2B**), to $52/10^9$ nucleotides in mussels collected at Station 1 localized at 2 km from complex phosphate miner (**Fig. 2C**), to $90/10^9$ nucleotides in mussels collected in S2 and S3 stations localized at 1 Km and 0.5 Km from complex phosphate miner effluent (**Figures 2D and 2E**), respectively.

The analysis of variance showed that differences in the DNA adduct levels is significant ($P < 0.05$).

Moreover, the diffuse diagonal zones of radioactivity shown on all chromatograms are characteristic of an exposure to complex mixture of DNA reacting substances. These latter are emitted by several industrial plants established in complex phosphate miner Jorf-Lasfar such as energy industries, metallurgy and chemistry / Husbandry.

Table 2 : DNA adduct levels in the mussel (*Mytilus galloprovincialis*) collected at different distances from the complex phosphate miner Jorf-Lasfar

Figure 2	**Site**	**Adducts/10^9 nucleotides**
2B	ST	38 ± 2 c
2C	S1	52 ± 3 b
2D	S2	90 ± 4 a
2E	S3	90 ± 5 a

Although the number of DNA samples used in our study was limited, the results clearly indicate that is possible to demonstrate a genomic damage on *Mytilus galloprovincialis* on the dose-response**.** More studies on the kinetics of the formation and repair of DNA adducts and the choice of the control station is needed to completely assess the situation. The mussels should be collected in clean water (away from the discharge point) or pushed in aquariums under controlled conditions.

Although several studies have concentrated predominantly on the indirect assessment of the biological impact of genotoxin exposure, or the assessment of terminal events, such as the induction of *neoplasia* in selected marine species, recent advances in molecular techniques have now enabled the direct detection of DNA damage induced by genotoxins in selected marine species [7].

If several studies on the measurement of DNA adducts in mussel by the ^{32}P postlabelling technique were worldwide carried out to evaluate the degree of contamination of the aquatic environments [15, 16, 17]. Among the advantages of this method over other methods of DNA adducts evaluation, one is undoubtedly its high sensitivity (1 adduct/10^9 normal nucleotides). Furthermore, its non-specificity makes it possible to estimate, with the choice of appropriate chromatographic solvent systems, the effects of complex mixtures of environmental pollutants [11].

Our paper gives the first results on the DNA adducts determination in mussels to evaluate the contamination degree of aquatic organism by effluent of complex phosphate miner Jorf-Lasfar (Morocco).

Recently, there has been an increase in interest in techniques of greater specificity that are capable of detecting individual genotoxin–DNA adducts. Several studies have already indicated that DNA adducts could be used as an indication of the exposure of aquatic organisms to environmental genotoxin [18, 19].

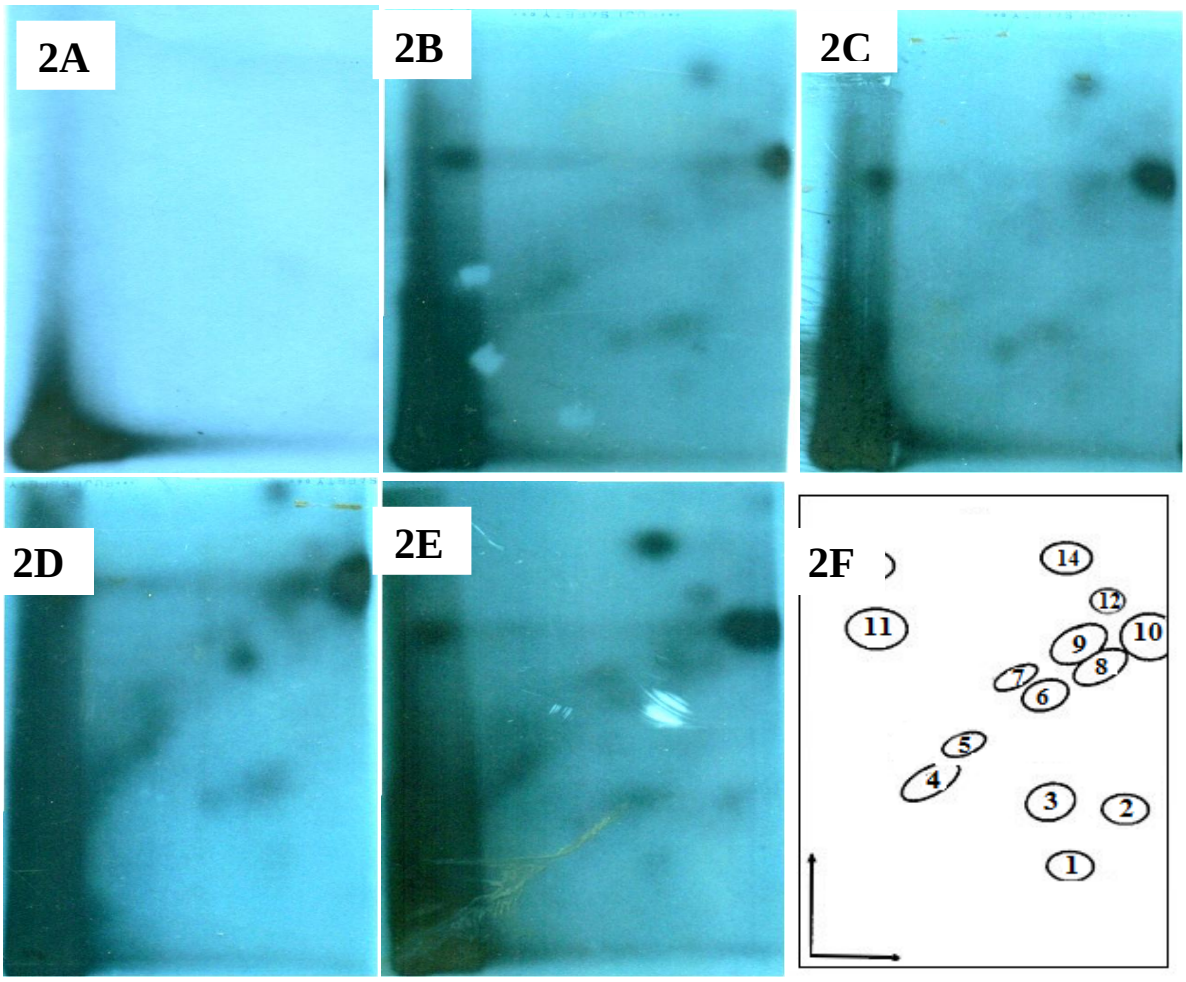

Figure 2 : Autoradiogramms of the DNA adduct patterns from *Mytilus galloprovincialis* collected at 4 stations (2B to 2E) from the effluent of complex phosphate miner Jorf-Lasfar (Morocco), 2A : reaction mixture without DNA (blank) ; 2B : station ST (38 adducts/10^9 nucleotides) ; 2C : S1 (52 adducts/10^9 nucleotides) ; 2D : S2 (90 adducts/10^9 nucleotides) and 2E : S3 (90 adducts/10^9 nucleotides) ; 2F : Chromatographic position of DNA adducts (spot) on the chromatogram.

Levels of DNA adducts in a tissue can thus be considered an exposure biomarker because they can reflect the internal dose of a contaminant, its metabolic activation and tissue distribution of metabolites. The choices of mussels as bio-indicators species are several. Indeed, they are sessile filter feeders capable of accumulating large amount of pollutants and especially metallic and hydrophobic contaminants such as polycyclic aromatic hydrocarbons [20, 21, 22, 23]. In Morocco, several studies have shown that the metal concentrations of Cd, Hg, Cu and Zn in mussels collected from the sea coast near the Jorf-Lasfar Complex Phosphate are very high, far exceeding international standards [24, 25]

Our findings on the genotoxicity of the Jorf-Lasfar Complex Phosphate Miner effluents are very interesting and should be followed by the assessment for DNA adducts in other aquatic species. It will be also interesting to assess the human exposure by extending the impact of the gases emitted by complex phosphate miner of Jorf-Lasfar on the human health by measuring the rate of DNA adducts in white blood cells of workers and inhabitants people of different location from the Complex Phosphate Miner.

Conclusion

The ^{32}P-postlabeling technique remains a very sensitive method for the measurement of DNA adducts *in vitro* and *in vivo*. It evaluates environmental genotoxic impact without prior knowledge of the identity of pollutants. In this study, the ^{32}P-postlabelling method was used to monitor the pollution in the Jorf-Lasfar Complex Phosphate Miner effluents. The method was used for the first time in Morocco for the assessment of DNA damage in the

mussel (*Mytilus galloprovincialis*) sampled from the effluents of the Industrial complex. Results demonstrated a gradual increase of DNA damage as far as the samples are collected near the complex phosphate miner. These findings suggest that *Mytilus galloprovincialis* could be used as a bio-indicator for pollution monitoring and should spur authorities to establish a monitoring program to assess the situation completely.

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