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RAPD-Contaminant Indicative Bands Induced by Sodium Lauryl Sulfate of Economic Fish *(Sardine Aurita)* from Libyan Coasts

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Abstract

The presence of sodium Lauryl sulfates (SLS) as synthetic detergents, in marine environment arises mainly from its presence in complex domestic and industrial effluents as well as its release directly from some applications (e.g., oil dispersants and pesticides). It has been reported that SLS is toxic and affects survival of aquatic animals such as fishes, microbes like yeasts and bacteria. It is also toxic to mammals like mice and humans but to a lesser extent. Many studies have shown that the direct discharge of untreated wastewater into aquatic environment containing anionic surfactants causes significant damage to the aquatic environment due to the growth of algae that consume oxygen needed for the life of aquatic organisms. These damages in the aquatic environment are caused not only by the presence of anionic surfactants (SLS), but also by their interaction with other pollutants in the aquatic environment, which increases their toxic effect on aquatic organisms. Such detergents, however, became a public nuisance because they were neither soluble nor biodegradable. The present study intended to evaluate the fate of SLS as synthetic detergents on Tripoli coastal site of Libya, and quantifying their impacts on fish *(Sardine aurita)* DNA mutation, compared with other species through literature review. Our results provide functional evidence for genome toxicity of fish, of the DNA mutation caused by detergents effluents. This study provides the rationale for a simple genetic test to identify the impacts of detergents on aquatic ecosystem of Libyan coastal region.

Keywords: Polymorphism; Contaminants' indicative bands; RAPD marker; SLS; Sardine aurita.

1. Introduction

The aquatic ecosystem was a combination of interconnected species that are reacting to each other of habits and habitats. The discharging of many different types of synthetic chemicals (*i.e.*, fertilizers, pesticides, and detergents) into the marine environment causes several adverse effects on biodiversity of aquatic ecosystem [1, 2]. Further, the chemical contamination of aquatic ecology affects algal population which is primary producers wills directly affects the consumers, as the food web is disturbed which leads to the depletion of aquatic biota. Hence the release of chemicals or effluent from the industries into the environment has to be restricted up to certain limit, [3].

Besides, the measurements of biological impact are accomplished using either species-specific responses to toxicants [4], or impacts on higher levels of organization from individuals to populations, and so on.

Genetic bottleneck, is a reduction in genetic polymorphism at the population level. Most contaminants show specific mechanisms of toxicity, for example, impairing the nervous system or the liver. These effects may acutely or chronically reduce population sizes in wildlife by weakening individuals, culling young, or initiating disease processes, for example [5]. In addition, many are genotoxic, which is, directly damaging to DNA. Chronic genotoxicity may induce gamete loss, reduce longevity, or cause developmental delay or tumorigenesis [6], any of which may lead to population reductions, thus reducing the number of genetic lineages in populations. Sweeping or systematic reduction in genetic variability has been called a bottleneck effect [7, 8] and could lead to inbreeding depression [9, 10], which could exacerbate the effects of direct contaminant damage by further reducing overall population size and ultimately genetic polymorphisms.

However, ecotoxigenomics can be defined as toxicant induced changes in the genome of the organisms. Further, genotoxicity refers to the effect of toxicants on the DNA structure of germ cells, spermatozoa and egg cells. In principle, genotoxicity means that any effects observed are trans-generational effects that occur via changes in DNA structure, [11]. Molecular genetic techniques have proven very effective at examining large amounts of genetic information.

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1.1. Selection of Genetic Markers

At this early stage in our understanding of the effects of contaminant exposure on genetic patterns in field-exposed populations, we are not able to predict, a priori, which of the possible outcomes is most likely (*i.e.*, bottleneck, selection, mutation). Some authors have suggested that bottlenecks are the most likely outcome of chronic contaminant exposure in natural populations [12]. It is certainly the pattern that is easiest to test for using any highly variable, neutral marker, and that has been observed most frequently so far. However, these facts are confounding, and methods must be sought to test for a variety of possible effects and more confidently attribute patterns to their probable causes, [13].

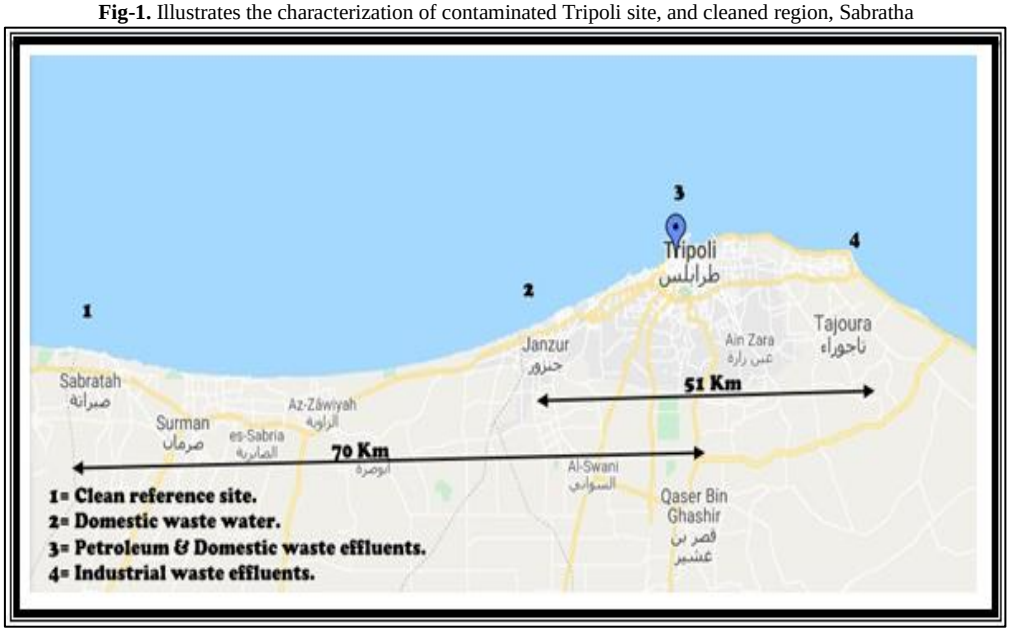
Detergents are cleaning products derived from synthetic organic chemicals. Surfactants are the components mainly responsible for the cleaning action of detergent [14]. It is used widely in both industrial and domestic premises to wash cloths, equipment, installations, heavy-duty machines and vehicles.

The chemical components of detergents are often called surface-active agents or surfactants because they act upon surfaces. A common feature of detergents is that they are made up of comparatively large molecules (Molecular weight over 200 g). One part of the molecules is soluble in organic material and the other part is soluble in water, [15]. Today the detergents are globally highly market competitive; with different competed brands to get more customers and consumers, [16].

Sodium lauryl sulfate (**SLS**), also known as sodium laurel sulfate or sodium dodecyl sulfate, is an anionic surfactant commonly used as an emulsifying cleaning agent in household cleaning products (laundry detergents, spray cleaners, and dishwasher detergents).

Although, Libya is located on the Mediterranean coast, with a length of about 1950 km, it is suffering from huge different quantities of contaminants. Tripoli city is the capital of Libya; and about 4 million habitants living in this city. This study is taking place for Tripoli coast which lies between Tajora and Janzour, as in figure 1 which illustrated the characterization of contaminated Tripoli site, and cleaned region, which is Sabratha.

Fig-1. Illustrates the characterization of contaminated Tripoli site, and cleaned region, Sabratha



Globally, the seriousness of sea water pollution is increasing day by day along with the damage caused by various pollutants, especially oil and chemicals for the marine environment and its natural resources that have direct or indirect impact on human. In addition, Tripoli coastal site receives different types of pollutants, as industrial and domestic wastewater effluents. Most of these pollutants are untreated, which include organic, in organic, heavy metals, and detergents.

While, many studies have shown that the direct discharge of untreated wastewater into aquatic environment containing anionic surfactants causes significant damage to the aquatic environment due to the growth of algae that consume oxygen needed for the life of aquatic organisms. These damages in the aquatic environment are caused not only by the presence of anionic surfactants (**SLS**), but also by their interaction with other pollutants in the aquatic environment, which increases their toxic effect on aquatic organisms. The contaminated waste water input of Libyan coastal marine ecosystem, affect aquatic ecosystem health. It accumulates on tissues and muscles, since is capable of disrupting the endocrine system of animals, including fish, wildlife, and humans due to an interference with the normal mechanisms of hormone action, [17].

The sources of detergents on the Libyan coastal site are:

- Domestic wastewater: untreated effluents discharged into the sea.
- Marine petroleum refinery: detergents used as dispersants for boilers and tankers.
- Industrials effluents: untreated wastes were discharged to the coastal site.

Integrating tests for new mutations into study design and marker selection will help genetic ecotoxicologists to come to an understanding of which, if any, of the mechanisms of genetic change may be the biggest threat to natural populations.

The present study sought to investigate the effects of chronic sub lethal exposure to **SLS**' contamination by studying the genetic variation of high probability of finding contaminant-indicative RAPD pattern in a resident and economic fish.

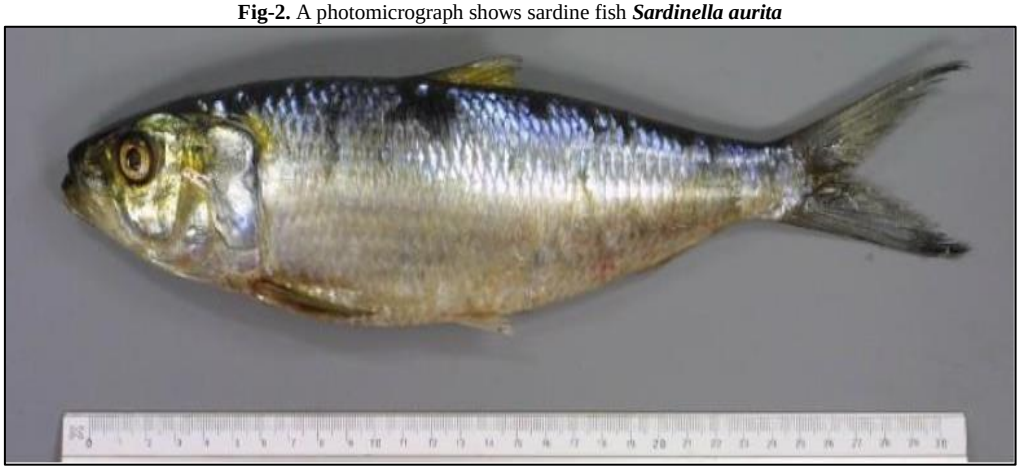
2. Materials and Methods

The present study is conducted in the western part of the coast in the Libyan cities of Tripoli and Sabratha. Tripoli coast is polluted mostly with organic pollutants, and the Sabratha is the cleaner part of Libyan coast. Twenty four samples of fish (***S. aurita***), 4 samples/ month for a period of 6 months of muscle tissue, of sardine's fish were collected.

In Libya, different waste products and certain chemical wastes found at Tripoli beach contain many substances that are considered toxic. Environmental contaminants can produce irreversible and often deleterious effects in organisms under controlled laboratory conditions. However, very little is known about the effects of chronic exposure to low levels of multiple contaminants pervasive and persistent in the environment. This study is conducted in the western part of the coast in the Libyan cities of Tripoli and Sabratha. Tripoli coast is polluted mostly with **SLS** detergents as organic pollutants, while, Sabratha is more clean part of Libyan coasts as a reference site.

The random amplification multiple image RAPDs technique of DNA is used, to identify the effect of exposure of sardine fish to pollutants. It is compared to the reference samples, using RAPD-PCR based technique.

Fig-2. A photomicrograph shows sardine fish ***Sardinella aurita***



2.1. DNA Extraction

The DNA was extracted from muscles fish tissue using lysis buffer containing 2% CTAB [18] and incubated with proteinase K (2 µg/ml) for 2 hrs., following extraction with phenol: chloroform (1:1) and chloroform: isoamyl alcohol (24:1) and incubation with ribonuclease A (350 µg/ml). The DNA of both host and parasite individuals was precipitated by adding absolute ethanol (100%).

After centrifugation (16000 g), ethanol was removed and DNA pellets left overnight at room temperature. The pellet was resuspended in TE (10 mM; Tris, 1mM; EDTA, pH 8.0) and the DNA concentration and purity was determined by 2% agarose gel electrophoresis using the gel photo-documentation system DC 120 Zoom Scanner (**Eastman Kodak, NY, USA**).

2.2. DNA Amplification by RAPD-PCR

The method of Simpson, *et al.* [19] was used, with small modifications. Two nanograms of genomic DNA obtained from the fish samples were amplified with Thermal Cycler (**Techni-England**) using RAPD-PCR based technique.

Amplification reactions were performed in a 25 µl final volume containing: 11.0µl H_2O, 25µl 1x PCR Master Mix (**Promega**), 1.0µl of 0.4 µl each arbitrary primer and 0.5 µl DNA (50ng/µl). Specific details for each primer are given in Table 1. RAPD primers were purchased from Operon technologies (**Operon Technologies, Alameda, CA, USA**). Only one primer (OPA-20) was amplified successfully of OPA kits (1-20).

Table-1. Sequences of RAPD primer successfully amplified in both SLS-exposed and unexposed fish population

Primer	Sequence
OPA-20	5'-GTTGCGATCC-3'

The amplification conditions were as follows:

- 1 cycle at 95°C for 5 min.
- 2 cycles at 95°C for 30 sec, at 30°C for 2 min and at 72°C for 1 min.

- 33 cycles during which the annealing temperature was changed to 40°C and the time of the extension step was increased to 5min during the final cycle.

The PCR products were stored at -20°C. Agarose gel electrophoresis (2%) and Ethidium bromide staining - Four microliters of each DNA amplification reaction was added to 2.5µl sample buffer (0.125% Bromophenol blue, 0.125% xylene cyanol, and 15% glycerol) and the mixture was submitted to 2% Agarose gel electrophoresis in TBE (2 mM Tris-borate, EDTA, pH 8.0) at 60 volts. The gels were stained with Ethidium bromide for 2 min, destained with deionized water for 10 min.

Polymorphism analysis: Genetic variability of the susceptible and resistant individuals was evaluated by analyzing the electrophoretic band patterns obtained on the gels and by determining the coefficient of similarity as described by Dice [20].

The stained gel was captured and scored using Imager& Scorer Software (Total lab software program, ver. 12.3). The frequency of each band was calculated as (number of bands/ fish with a particular band) / (total number of fishes/ fish in the sample). The nomenclature of the bands is indicated by the name and number of the primer (designated by the manufacturer) followed by the molecular length of the band in subscript. For example, band $OPA_{11(1.360)}$ is amplified using primer OPA_{11} and is 400 base pairs (bp) long.

2.3. Statistics

Each RAPD-DNA profile was scored, independently, and the amplicons of RAPD amplification of each sample was repeated in cases where scorings were not in complete agreement. A measure of the genetic similarity of an individual sardine fish to others collected at the same site was obtained by determining the fraction (f) of markers it shared with other fish from the same site using the following equation:

$$f = 2\left(\frac{m_{xy}}{m_x + m_y}\right)$$

Where, *mxy* is the number of bands any two samples share and *mx* and *my* are the number of bands amplified in organism *x* and organism y, respectively [21].

3. Result and Discussion

The fish samples collected from the two compared populations, have two different genetic similarity. The samples of February and March, were more homogeneous of similarity (94.3%) between fish population from SLS-exposed Tripoli beach, while samples of the genetic similarity measurement by (87%) from the unexposed fish population of Sabratha beach.

Fig-3. A photomicrograph of DNA fragments by RAPD technique and agarose gel electrophoresis of SLS exposed (1-6) compared with unexposed fish samples (7-9). Arrows show SLS- indicative bands (2, 5); M=DNA ladder DM3200

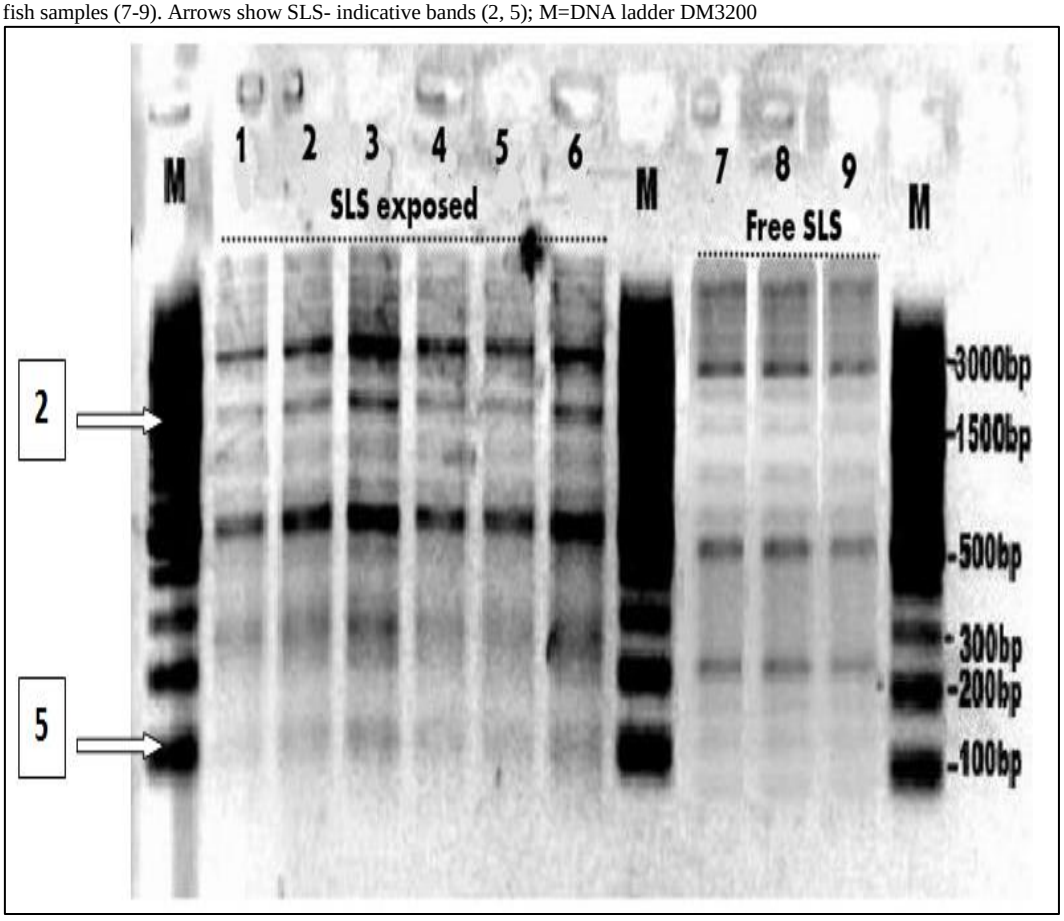


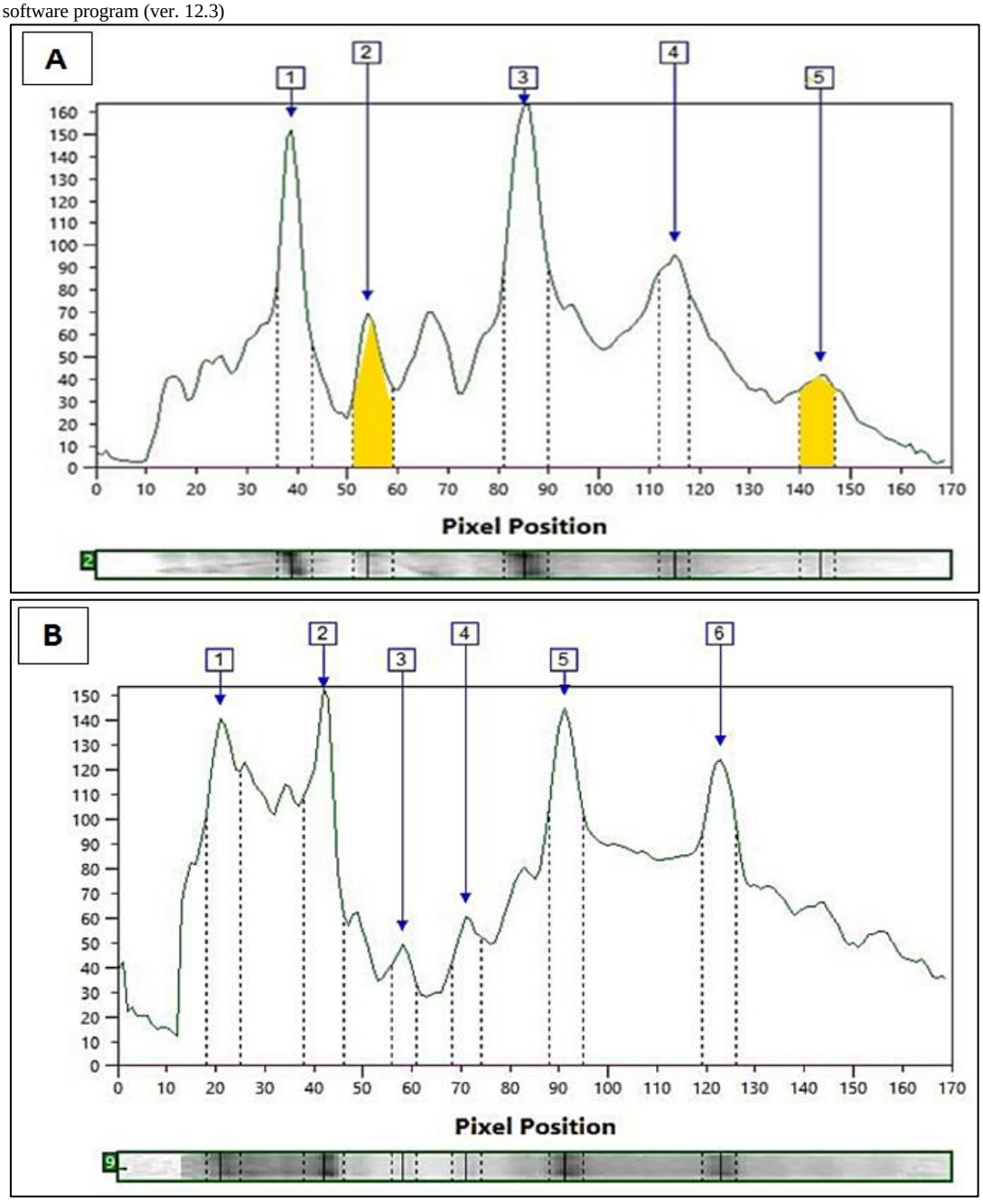
Table-2. The incidence of monomorphic, polymorphic, and conserved RAPD bands in both SLS-exposed and unexposed fish populations using primer kits (OPA_{20})

Populations	SLS exposed fish			Free SLSfish		
RAPD Bands	**Monomrphic**	**Polymorphic**	**Conserved**	**Monomrphic**	**Polymorphic**	**Conserved**
1	-	-	-	5250bp	-	-
2	3100bp	-	3100bp	3100bp	-	3100bp
3	2000bp*	-	-	-	-	-
4	-	-	-	1550bp	-	-
5	-	-	-	800bp	-	-
6	450bp	-	450bp	450bp	-	450bp
7	220bp	-	220bp	220bp	-	220bp
8	120bp*	120bp	-	-	-	-

*SLS exposed indicative bands

Table 2, summarize the incidence of scored monomorphic, polymorphic, and conserved RAPD bands in both SLS exposed and impacted fish populations. The average number of RAPD bands in SLS-exposed fish population reduced to be 5 bands only, while, the profile of RAPD bands in the unexposed population remains by 6 bands. There were only 3 conserved bands between the two different populations ($OPA_{20\ (3100)}$, $OPA_{20\ (450)}$, $OPA_{20\ (220)}$). There was only one polymorphic bands ($OPA_{20\ (120)}$) and two characterized SLS-indicative bands in the exposed population ($OPA_{20\ (2000)}$, $OPA_{20\ (120)}$).

Fig-4. Chart profiles to show RAPDs indicative bands (2and 5) of SLS exposed (A), compared with unexposed fish samples (B), analyzed by Total lab software program (ver. 12.3)



Environmental stress is just one of many potential causes for reduction of standing variation within a population. Also, a reduction in genetic diversity is not necessarily the only selective response to anthropogenic stressors. Nonetheless, the analyses of genetic diversity levels in rusty sardine fish described here suggest that RAPD-PCR may be a useful measure of population health through its ability to detect significant decreases in genetic diversity between sites affected by common anthropogenic stressors and very similar but unaffected reference sites. As such, RAPD-PCR-based measures of a population's genetic diversity have the potential to be the basis of a valuable alternative or augmentation to conventional assessments of environmental insults.

Molecular genetic data fall into 1 of 3 categories: sequence data, codominant allele data, or dominant allele data. Sequence data is the most informative, being both an absolute reflection of DNA patterns, and genealogical (*i.e.*, reflecting historical lineage relationships). Codominant markers, in which both alleles at a locus can be scored (*e.g.*, microsatellites, allozymes), are somewhat less informative. This is because, although DNA variation can be seen by different band mobilities, bands have greater potential for homoplasy (alleles that appear identical but have evolved from different ancestors) than do sequence data, and the data are not genealogical.

Dominant loci allow only scoring of a band (locus) as being present or absent (*e.g.*, AFLP, RAPD, DNA fingerprinting). These marker types are generally the least powerful because much information is lost when one does not know the nature of the absent allele (*e.g.*, its size, whether it amplified). On the other hand, dominant allele techniques often generate many more data points (loci) than the others.

In a series of papers, Theodorakis, Shugart, and others [22], examined molecular genetic change using RAPDs, allozyme variation, and a genotoxicity assay, DNA strand break analysis, to assess the effects of radionuclide exposure on mosquito fish (*Gambusia affinis* and *G. holbrooki*) in ponds in Tennessee. Western mosquito fish, *G. affinis*, from 2 contaminated and 2 reference sites were examined using 12 allozyme loci and 15 polymorphic RAPD primers [23].

RAPD dissimilarity index values were found to be higher at contaminated than at reference sites. Genetic distance values calculated from both the RAPD data at one of 8 polymorphic loci, indicated that contaminated populations are more similar to each other than to any of the reference sites. Each of these measures indicated that contaminated populations were genetically divergent from reference populations.

Several specific RAPD bands and a high average number of bands were only found in exposed populations. The so-called contaminant-indicative bands (CIBs), and the average number of RAPD bands, all correlated positively to fecundity in these fish.

Theodorakis and Shugart [24], correlated these CIBs with incidence of DNA strand breaks induced in these fish in laboratory exposures. The analysis showed some association between CIBs and reduced incidence of strand breaks, implying a possible selective advantage correlated with the possession of CIBs. In order to test the hypothesis that the CIBs were related to adaptation to radionuclide exposure, authors surveyed for the presence of these bands in another mosquito fish species (*G. holbrooki*) from a separate radionuclide-contaminated drainage [25].

They found a greater frequency of 3 of the CIBs in fish from the contaminated sites. They followed this survey with a Southern blot analysis of these bands and those identified in *G. affinis*, and found them to be homologous. Authors inferred from this result that these CIBs were very likely to be selectively advantageous loci within the 2 species of mosquito fish.

Three studies used the RAPD technique to assess nuclear DNA variability in populations exposed to contaminants, [26-28]. Nadig, *et al.* [26], compared several measures of variability in redbreast sunfish, *Lepomis auritus*, from 4 mercury-contaminated sites and 2 reference streams. A subset of primers indicated reduced genetic variation at, or increased similarity of, contaminated sites when site types were pooled. None of the indices reflected the gradient of contamination present at the contaminated sites. These suggestions of correlation between reduced genetic variability and exposure are strengthened in this study, however, by correlation with the biomarker and tissue chemistry data that confirm fish exposure, and the data generated in a related study that demonstrated DNA damage in exposed fish. This example shows the importance of providing correlative data that potentially link genetic patterns to damage from contaminants. However, although the reliability of the RAPD technique can be strengthened by rigorous standards and controls, newer marker types are currently coming into favor that share some significant advantages with RAPDs, but that have been shown to be more easily rendered repeatable [29, 30], and thus that could provide stronger results in similar studies.

4. Conclusion

Based on the results of the study the paper shows that how the use of random amplification technique of DNA molecules, and multiple RAPDs profile, to identify the effect of exposure to pollutants on Sardine are capable of determining the sensitivity of fish muscle tissues, compared with reference one's, by using PCR.

By integrating population genetic approaches into the current program, the Ecological Significance Indicators lead to advancements in assessing ecological risk at contaminated waste sites. One particular useful application in this regard is the development of population-based biomarkers. This is a reflection of perturbations at higher levels of biological organization. It can be used as early-warning indicators of such perturbations. The other beneficial use is the characterization of previously unidentified loci that could be used as novel individual- and population-level biomarkers of genotoxicant exposure and effects. This demonstrates that how Ecological Significance Indicators for the Estimation of DNA amplicons from RAPD-PCR based technique can be assessed in a robust manner for two fish populations (***S. aurita***) and proven very effective at examining large amounts of genetic information related to the discrimination of SLS-contaminated and reference fish populations.

There was a tendency to get a high probability of finding contaminant-indicative patterns if they are present. Of course, these data sets should be combined with good exposure documentation, careful experimental design, and markers of effect, where possible.

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