Impact Assessment of Diesel Oil on the Zhe Oyster (*Crassostrea plicatula*) Using RAPD Analysis

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Abstract: The lethal and sublethal effects of oils on aquatic organisms have been widely investigated, but the potential molecular impacts of oils on aquatic organisms are remaining unclear now. In order to realize the effects of diesel oil on the Zhe oyster, the RAPD (random amplified polymorphic DNA) technique was used. RAPD is a useful assay procedure for the detection of genotoxin-induced DNA damage and mutations. In the present study, the Zhe oysters were exposed to diesel oil at different concentrations and for different exposure periods. The results showed that the DNA band change in RAPD profiles of oysters following diesel oil treatment included loss of normal DNA bands, the appearance of new DNA bands and variations in DNA intensity compared to oysters not exposed to diesel oil. The effects of changes to GTS (genome template stability) were time- and concentration-dependent, the GTS of 10 mg/L was 82.46%, 80.70% and 63.15% in the 8, 16 and 32 days, the GTS of 20 mg/L was 75.44%, 71.93% and 56.14% in the 8, 16 and 32 days, the GTS of 40 mg/L was 73.68%, 70.18% and 56.14% in the 8, 16 and 32 days, respectively. The DNA polymorphisms detected by RAPD analysis could be used as a useful biomarker assay for the detection of genotoxic effects in diesel oil pollution on the oysters, and may be useful for environmental contamination risk assessment.

Key words: Diesel oil, Zhe oyster, RAPD, GTS, DNA damage, biomarker.

1. Introduction

Oysters, known as “milk of the sea”, are considered delicious, nutritious and protein-rich, with a variety of unsaturated fatty acids, amino acids, vitamins and minerals. The Zhe oyster (*Crassostrea plicatula*) is an important member of China’s coastal shellfish population and has a high economic value. It is considered a native species and is widely distributed in China, with the production of this oyster reaching 3.62×10^6 metric tons in 2002, accounting for 37.6% of the total annual marine molluscan yield [1]. Bivalves (such as oysters) are widely used as sentinel organisms for monitoring the concentration of selected pollutants in coastal environments, such as heavy metals, organo-chlorine compounds and petroleum

hydrocarbons have long been recognized as the most deleterious contaminants to biota in the world’s marine and estuarine waters, oysters have become good bio-indicators of environmental pollution in coastal and estuarine ecosystems because they, as filter feeders, bioaccumulate contaminants [2].

Over the past few decades, environmental contamination of water by oil has increased drastically. Oils (crude and fuel) spilled into the environment have multiple negative effects on aquatic organisms. Marine pollution due to oil and oil exploration pollution in ocean environments is an especially critical environmental concern [3]. Most previous studies have focused on the effects of oils on survival [4-11], morphological parameters [9, 11, 12], hematological physiological parameters [9, 12, 13], biochemical parameters [14-21] and histological parameters [13, 18, 19]. In addition, the molecular

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level changes of oil on aquatic organisms, such as micronucleus frequency, DNA repair, cytochrome P4501A, DNA adducts, DNA single strand breaks, microsatellite and AFLP loci were also studied [22-27].

Recently, the RAPD (random amplified polymorphic DNA) assay has been used to detect possible damage in the genomic DNA of organisms in ecotoxicology as a good biomarker [28-34]. The RAPD assay presents some advantages: RAPD assays lie in the PCR-based technique being easy identification of regions of amplification, deletion or rearrangement without prior information about the genome [34]; RAPD assays require very little source material and under certain circumstance and the analysis can also be performed non-destructively which can be useful for the screening of rare or valuable samples; RAPD technique is the high level of the overall sensitivity and relatively cheap and does not require the use of specialized and expensive equipment; RAPD method has the potential to detect a wide range of DNA damage [35]. However, there are no studies of oil contamination which use RAPD technology. In the present study, the principal objectives were to assess the impact of diesel oil contamination on the Zhe oyster in terms of DNA damage.

2. Materials and Methods

2.1 Animals

The oysters used in this study come from Xiangshan county (29°21'16.50" S, 121°58'46.72" W), Zhejiang province, in the People's Republic of China. The shell length was 4.03 cm $\pm$ 0.11 cm, and the shell width was 2.65 cm $\pm$ 0.19 cm. The shell weight was 10.24 g $\pm$ 0.54 g, and the oysters have been domesticated two weeks before testing.

2.2 Kinds of Fuel Oils and Preparation of Oil

In the present study, the fuel oil used was No. -20 diesel oil. The oil was supplied by the Institute of

Waterborne Transportation Science, administered by the Ministry of Transportation of the People's Republic of China.

The authors prepared mixtures of oil from fuel oil samples in a 2,000 mL beaker using 2,000 mg of diesel oil per Liter of clean seawater. The beaker was stirred continuously at a uniform speed for 30 min and then emulsified with an ultrasonic cleaner (DL-720A, made in Shanghai, China) for 8 h. In this way, the authors simulated the effects of oil spilled in the sea. TPH (Total petroleum hydrocarbon) analysis was performed using UV spectrophotometry [36]. The various concentrations were then diluted.

2.3 Experimental Design

The oysters were exposed to four concentrations of mixtures of diesel oil (0, 10.0, 20.0 and 40.0 mg/L) for 32 days. The oysters were fed with chlorella 200 mL a day, algae concentration was 3.5×10^6 cell/mL. There were 100 oysters in the 100 L tank per experimental group, 50 L mixtures of oil in each tank, and the temperature of the test was 24 °C $\pm$ 2 °C. Test water changed daily, and 10 oysters were taken from each group at 2, 8, 16 and 32 days. The samples were preserved with 95% ethanol and changed after three days. Each treatment was replicated three times.

2.4 DNA Extraction and RAPD Analysis

Genomic DNA was isolated from adductor muscle tissue (approximately 100 mg) using the standard phenol-chloroform method [37]. Subsequently, DNA was resuspended in a 50 μ L TE buffer (2 mL 1 M Tris, 40 mL 0.5 M EDTA, 158 mL ddH₂O, pH 8.0) and stored at -20 °C until use.

The RAPD assays were performed on the GeneAmp PCR System 9700 (Applied biosystems, Invitrogen, USA) in 25 μ L of total volume containing approximately 2 μ L of genomic DNA (10 ng/L), 1 μ L primers (10 μ mol/L), 1 μ L dNTP (2.5 mmol/L each), 0.2 μ L Taq DNA polymerase (5 U/ μ L), 10 $\times$ reaction buffer (100 mmol/L Tris-HCl,

pH 8.3, 15 mmol/L $MgCl_2$, 500 mmol/L KCl, 0.1 mmol/L EDTA, 5 mmol/L DTT, 50% glycerol, 0.1% Triton X-100), 18.3 μ L distilled water. The 30 primers used were 10 bp in length (S1-S30, Sangon Biotech, China). The RAPD protocol consisted of an initial denaturing step of 7 min at 94 °C, followed by 45 cycles at 94 °C for 1 min (denaturation), 37 °C for 1 min (annealing) and 72 °C for 2 min (extension), with an additional extension period of 10 min at 72 °C. For each amplification, a negative control was run for each primer. Reaction mixtures were stored at 4 °C prior to use. 3 μ L of each PCR product was electrophoresed in 1.5% agarose gels containing ethidium bromide for verifying the amplified fragment length with a DNA marker DL2000 (Takara, China). Images were captured using a high-resolution scan and digitalized images were counted directly for RAPD analysis.

2.5 Estimation of GTS (Genomic Template Stability)

Genomic template stability (%) was calculated as $100 - (100a/n)$, where a represented RAPD polymorphic profiles detected in each sample treated and n represented the number of total bands in the control. Polymorphism observed in RAPD profiles included disappearance of a normal band and appearance of a new band in comparison to control RAPD profiles [38]. To compare the sensitivity of this

parameter, changes in this value were calculated as a percentage of their control (100%). In addition, as the sampling time interval of 2 days is too short, there is no calculation of the GTS and compared with others.

3. Results

3.1 TPH Concentrations

Three different oil concentrations were set in this test, 10 mg/L, 20 mg/L and 40 mg/L (the actual concentration of oil), and with the corresponding TPH were 1.05×10^{-2} mg/L, 2.10×10^{-2} mg/L and 4.20×10^{-2} mg/L, respectively.

3.2 Effect of Diesel Oil Stress on RAPD Profile

Aimed at verifying the genetic effect of diesel oil contamination, the RAPD analysis was performed on DNA extracted from groups of 10 oysters from each replicate treated with diesel oil at concentrations of 0-40 mg/L. In total, 30 random 10-mer primers (Table 1) were used to amplify genomic DNA samples from the diesel oil-treated and control groups, and only 18 primers generated specific and stable results with a total number of 57 bands.

The RAPD fingerprints showed virtual differences between exposed oysters and control oysters. The changes included both loss and addition of bands compared with the control groups (Fig. 1). For example,

Table 1 Sequences of 30 primers used in this experiment.

No. of primers	Sequences (5'→3')	No. of primers	Sequences (5'→3')
S1	GTTCGCTCC	S16	TTTGCCCGGA
S2	TGATCCCTGG	S17	AGGGAACGAG
S3	CATCCCCCTG	S18	CCACAGCAGT
S4	GGA CTGGAGT	S19	ACCCCCGAAG
S5	TGCGCCCTTC	S20	GGACCCCTTAC
S6	TGCTCTGCCC	S21	CAGGCCCTTC
S7	GGTGACGCAG	S22	TGCCGAGCTG
S8	GTCCACACGG	S23	AGTCAGCCAC
S9	TGGGGGACTC	S24	AATCGGGCTG
S10	CTGCTGGGAC	S25	AGGGGTCTTG
S11	G TAGACCCGT	S26	GGTCCCTGAC
S12	CCTTGACGCA	S27	GAAACGGGTG
S13	TTCCCCCGCT	S28	GTGACGTAGG
S14	TCCGCTCTGG	S29	GGGTAACGCC
S15	GGAGGGTGTT	S30	GTGATCGCAG

Fig. 1 RAPD profiles of genomic DNA from adductor muscle of the Zhe oyster exposed to varying concentrations of diesel oil. The RAPD patterns were obtained using 10-mer primers (a) S8, (b) S11, (c) S21, (d) S26, (e) S27, (f) S28, (g) S29, and (h) S30. In (a), (b), (c), (d), (e), (f), (g) and (h), numbers 1-12: control, 20 mg/L diesel oil exposed for 2 days, 40 mg/L diesel oil exposed for 2 days, 10 mg/L diesel oil exposed for 8 days, 20 mg/L diesel oil exposed for 8 days, 40 mg/L diesel oil exposed for 8 days, 10 mg/L diesel oil exposed for 16 days, 20 mg/L diesel oil exposed for 16 days, 40 mg/L diesel oil exposed for 16 days, 10 mg/L diesel oil exposed for 32 days, 20 mg/L diesel oil exposed for 32 days, 40 mg/L diesel oil exposed for 32 days, respectively. M: molecular marker (2,000, 1,000, 750, 500, 250 and 100 bp from top to bottom).

the primer S8 showed the number of disappearing RAPD bands was greater at several concentrations of 10-40 mg/L for different days of exposure (Fig. 1a 8-1, 8-2). The primer S11 showed the number of

disappearing RAPD bands to be greater at several concentrations of 10-40 mg/L for different exposed days (Fig. 1b 11-2, 11-3) as well. On the other hand, some figures showed changes in the addition of bands

compared with the control groups, for instance, the primer S11 showed the number of adding RAPD bands was greater at several concentrations of 40 mg/L exposed for 2 days (Fig. 1a 11-1). Other figures showed the same phenomena, as shown in Fig. 1e, Fig. 1g 29-4 and Fig. 1h 30-2.

3.3 The Correlation between GTS and the Concentration of Diesel Oil

In Table 2, the modifications in RAPD profiles are shown as a percentage of their control [37]. Changes in the RAPD patterns were expressed as decreases in GTS, a qualitative measure reflecting the obvious change to the number of RAPD profiles generated by the diesel oil-contaminated oysters. GTS values calculated for 18 primers are presented. The GTS value was decreased with the increased time of exposure. For instance, the GTS of 10 mg/L was 82.46%, 80.70% and 63.15% in the 8, 16 and 32 days, respectively. There was significant linear relationship between GTS and the concentration of diesel oil. The regression equations were $y = 98.6x - 0.228$ ($R^2 = 0.9734$, y was GTS, and the x was concentration), $y = 98.664x - 0.266$ ($R^2 = 0.9769$) and $y = 94.299x - 0.434$ ($R^2 = 0.9023$) at 8, 16 and 32 days, respectively. In addition, although the GTS of 20 mg/L and 40 mg/L were 56.14%, the changes of RAPD profiles

were different, with the appearance of 12 new bands in the 20 mg/L group, and the disappearance of 22 normal bands in the 40 mg/L group (Table 3). This effect will be used to evaluate damage to oysters in cases of oil spillage.

4. Discussion

4.1 Application of RAPD Technique in Ecotoxicology

In the field of ecotoxicology, RAPD studies describe the RAPD changes such as differences in band intensity as well as gain/loss of RAPD bands [35]. The RAPD assay has been used to determine the mutagenic effects of heavy metal pollution on the model plant (*Arabidopsis thaliana*), DNA from plants exposed to heavy metals solution displayed polymorphic bands which were not detectable in DNA of unexposed plants [29]. RAPD assay can be used to qualitatively detect the kinetics of B(a)P-induced DNA effects in the water flea (*Daphnia magna*) exposed to 25 $\mu\text{g/L}$ and 50 $\mu\text{g/L}$ the main changes occurring in RAPD profiles produced by the population of *Daphnia magna* was a decrease and increase in band intensity compared with the control population [30, 31]. DNA changes in barley (*Hordeum vulgare*) seedlings induced by cadmium pollution using RAPD analysis and the results showed

Table 2 Changes of genomic template stability for all primers by diesel oil-contaminated oysters for different times (%).

No. -20 diesel oil concentration (mg/L)	8 d	16 d	32 d
0	100	100	100
10	82.46	80.70	63.15
20	75.44	71.93	56.14
40	73.68	70.18	56.14

Table 3 Changes of total bands in control, polymorphic bands and varied bands in diesel oil-contaminated oysters.

Exposure time (d)	No. -20 diesel oil concentration (mg/L)						
	0		10		20		40
	a + b	a	b	a	b	a	b
8	57	5	5	6	8	8	7
16	57	5	6	10	6	9	8
32	57	11	10	12	13	3	22

a: appearance of new bands; b: disappearance of normal bands; a + b: polymorphic bands compared with control

that the changes occurring in RAPD profiles of root tips following Cd treatment included variation in band intensity, loss of normal bands and appearance of new bands compared with the normal seedlings and the genomic template stability (a qualitative measure reflecting changes in RAPD profiles) was significantly affected at the above Cd concentration [32, 33]. The similar effects was found in rice (*Oryza sativa*) contaminated by Cd [34]. Therefore, the comparison between “unexposed” and “exposed” genomes show that RAPD analysis can be used to evaluate how the environmental pollutants modify the structure of DNA in living organisms.

4.2 RAPD Technique in Diesel Oil Pollution

The RAPD assay presents many advantages and the RAPD assay is very reliable after optimization [31]. Therefore, the RAPD method has the potential to detect DNA damage (e.g. DNA adducts DNA breakage) as well as mutations (point mutations and large rearrangements) [35]. In the present study, the DNA damage in oysters stressed by the diesel oil was very clear, for example, we selected changes in the primers, as indicated by arrows, in comparison to a control group, which showed changes including loss and addition of bands compared with the control groups (Fig. 1). DNA damage leads to the instability of the genomic template. In this way, previous studies demonstrated that the GTS parameter picked up significant effects of B(a)P and Cd exposure [32-34, 38]. The RAPD assay was considered as a semi-quantitative assay [39] and another people use it as a qualitative rather than a quantitative method [31]. One problem is that the number of primers and characteristics of bands of RAPD for statistical significance in GTS. At present, there are 2-14 primers used in RAPD tests, obtaining a total number of bands ranging from 51-180 [30-34, 38]. In the present study, we selected 18 primers in RAPD tests and got 57 bands (Table 3). Therefore, the number of primers is not the most important factor, the total

number of RAPD bands and polymorphic bands are the key factors in the present study. There is a clear tendency of the concentration of pollutants to be proportional to the number of RAPD polymorphic bands. For instance, different polymorphic bands were detected at each concentration of Cd for different primers, and the value of polymorphisms was $p(\%) = 34.5\%$, 40.9% and 44.5% for 30, 60 and 120 mg/L Cd, respectively [32]. In the present study, the authors found that not only exposure concentrations but also exposure times of pollutants were proportional to the number and polymorphisms of bands (Table 3). The number of RAPD polymorphic bands is exactly the number of loss and addition of bands compared with the control groups, and is the basis of the GTS calculation.

5. Conclusion

RAPD (Random amplified polymorphic DNA) is a useful assay procedure for the detection of genotoxin-induced DNA damage and mutations. This study showed that the DNA band changes in RAPD profiles of oysters following diesel oil treatment included loss of normal DNA bands, the appearance of new DNA bands and variations in DNA intensity compared to oysters not exposed to diesel oil. This study also showed that the change of GTS showed a dose-dependent and time-dependent tendency to the diesel oil. Therefore, a RAPD assay was quantitative and could be used as an investigation tool for environmental toxicology, as well as a useful biomarker.

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