THE USE OF BIO-MOLECULAR SUBSTANCE OF MARINE BIOTA AS AN ALTERNATIVE EARLY INDICATOR OF OIL POLLUTED ENVIRONMENT:

A NEW APPROACH FOR MONITORING CONSIDERATION

by
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I. INTRODUCTION

Oil enters the marine environment from various sources, for example from the accidental and intentional release of petroleum wastes during the production, transportation, refining and the use of this fossil fuel, domestic/industrial oily waste discharge and others. Oil from tanker spills, which is considered to occur rarely, usually only affects the coastal environment if prevailing winds and currents are directed onshore. The presence of stranded tar-balls on beaches has been reported in some areas due to the tanker routes nearby. A report of Lemigas and CNEXO (1984) showed that several sandy beaches on islands in the vicinity of Malacca Strait (Riau Islands), Makassar Strait (Langa beach) and in Kepulauan Seribu (Pulau Pari and Pulau Tikus) were polluted by stranded tar-balls by as much as 7.8 – 67 g/m (net weight) from 1982 survey and between 4.8 – 494 g/m from the 1984 survey. This report indicated that the heavy range of oil pollution increased from 1982 to 1984. Results of laboratory analysis on tar samples using gas chromatography indicated that four different types of oil were present viz.: crude oil residue, tanker sludge residue, fuel oil residue and weathered crude oil residue. Unfortunately, no similar data are available after the 1984 survey even the current oil pollution has occurred in the vicinity of Pulau Pabelokan and Pulau Pramuka in 2004.

Although oil pollution from refinery run-off has been estimated to be smaller compared to accidental tanker spills, such run-off will directly affect the coastal environment if the effluent is not managed properly. These industrial and refinery effluents, which usually flow into the coastal zone, result in relatively low levels of pollution for a long period of time. As a consequence the delayed effects of such pollution will occur. In this case marine organisms might be affected or stressed physiologically rather than killed under such a regime. Furthermore, increasing activities of processing units of a refinery from Atmospheric Residue Hydro Demetalisation (ARHDM) and from Residue Fluid Catalytic Cracking (RFCC) may result the increasing discharge in a significant number of volume. The discharge volume of liquid wastes and its quality depend on the quantity and type of crude as a feed stock. If the feed stock derived from naphtenic oils or heavy oils or high sulphur oils, care should be taken to the water disposal for the sake of environmental protection. In monitoring activities for the refinery effluent, the standard has been renewed since 1996 by the Ministerial Decree of the Minister of Environment No. 42/MENLH/10/1996 in the Appendix IV and Appendix V. Although the available treatment plant system is being used satisfactorily, but in some cases, several parameters including hydrocarbon contents still exceed the standard quality of the above Ministerial Decree. On the other hand, oil and grease contents as one of the parameters from the receiving bodies that should be analyzed have been designated, as stated in the Government Regulation No. 82/2001 and in the Ministerial Decree of Minister Environment No. 51/2004, instead of petroleum hydrocarbons. But from the basic scientific point of view, the oil and grease contents have the different meaning from the petroleum hydrocarbon contents in terms of chemical formula and its impacts on the aquatic biota. Oil and grease contents include the expression of the oils derived from biological products such as the fatty acids from aquatic organisms, palm oils and others. Unlike in the petroleum hydrocarbons, in plant oils/oil and grease no toxic compounds are found in its H-C chains viz.: vanadium, nickel, phenols, sulphide, aromatics, mercaptans, etc.

Since the impacts of petroleum hydrocarbons to the aquatic animals are not able to compare to those impacts of plant oils/oil and grease even at the same concentration, so that the oil and grease content seem to be in-significant and irrelevant to the petroleum activities. Total petroleum hydrocarbon contents in water and poly

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aromatic contents (= naphthalene, phenanthrene, dibenzthiophene and its alkylated homologues) are more significant than oil and grease.

The aquatic (marine) biota such as bivalves can take up oil into their tissues, which is at low concentration in the water (Blumer *et al*., 1970) by ingestion. Ingestion of hydrocarbons causes cell tissues to become stressed and undergo a series of often ir-reversible biochemical and cellular changes. The changes manifest themselves as alterations in the animal physiology and therefore represent good indicator of xenobiotic bio-accumulation (Moore and Lowe, 1985). The characteristic cellular defence mechanisms in all organisms studied to date under environmental stress involves the induction of certain bio-molecular substance which constitutes protein compounds which Atkinson and Walden (1985) called as heat shock proteins (hsp) or stress proteins (sp).

It is evident that synthesis of families of proteins of 60 kDa (kilo Dalton) and 70 kDa molecular weight (hsp 60 and hsp 70) and other stress proteins by cells of all organisms occurs in response to a wide variety of environmental stressors e.g. elevated temperatures, heavy metals, thiol reactive agents and amino acid analogues (Lindquist, 1986; Mizzen *et al*; 1989; Sanders, 1990). At least 30 stress proteins have been identified by gel electrophoresis (Anderson, 1989), and mainly they have molecular weights between 22 to 110 kDa (Schlesinger *et al*., 1982). Hsp 90, hsp 70 and hsp 60 are predominant in all prokaryotes and eukaryotes. A group of low molecular weight proteins (hsp 20 – 30) is also commonly found as shown by Burdon (1987).

Since bivalve mollusks are sessile, plentiful, inexpensive and relatively easy to maintain in the laboratory, their use is becoming important in monitoring programs and toxicological studies. Compared to mollusks, fish are expensive and prone to secondary stresses (such as handling and infection by fungi and bacteria) and they can avoid the polluted area. Many coastal areas in Indonesia produce several kinds of commercially valuable shellfish such as cupped oysters (*Crassostrea* sp.), scallops (*Pecten* spp), blood cockles (*Anadara granosa*, L), clam (*Tridacna* spp.) and green mussels (*Mytillus viridis*, L). Unfortunately there is little information on the use of these species as bio-indicators of oil pollution, particularly with respect to bio-molecular substance examination as a tool for monitoring activities. Electrophoresis is usually implemented for the examination of glycoproteins, phosphoproteins, enzymes, etc. It sems that the use of biological substance through electrophoresis method in oil pollution monitoring is a "new" breakthrough that needs to be considered.

The aim of the study was to propose an alternative method in environmental monitoring particularly at sub lethal effects through the use of bio-molecular of suitable shellfish in Indonesia as bio-indicator of oil pollution. The examination of the substance can be carried out by One-dimensional SDS - PAGE (Sodium Dodecyl Sulphate – Poly acrylamide Gel Electrophoresis) method.

II. THE UPTAKE OF PETROLEUM HYDROCARBONS ON MARINE BIVALVE MOLLUSKS

Invertebrates are generally less mobile than fish. Therefore, invertebrates may take-up hydrocarbons in relatively greater quantities, particularly if such organisms are sessile or bottom-attached invertebrates. This is because these organisms cannot avoid the polluted environment. The presence of pollutant hydrocarbons in tissues of natural populations suggests that these organisms are able to accumulate hydrocarbons from water, food or sediments.

Marine mollusks, as examples of the benthic epifauna, are evidently able to accumulate hydrocarbons even at sub-lethal concentrations. Stegeman and Teal (1973) concluded that petroleum and chlorinated hydrocarbons can be transported across molluss gill membranes, suggesting that this route can be a major route of uptake. Of course the hydrocarbons can be released after the animals are returned to the oil-free environment though the release of hydrocarbons from their tissues depends on several factors. Those factors are summarized, according to some authors (e.g. Fossato and Canzonier, 1976; Neff, *et al*, 1976; Neff and Anderson, 1981; Farrington, *et al*, 1982.), as among others:

- Type of hydrocarbon compounds (aromatics or n-alkanes).
- The molecular weight of the compounds.
- The biological half-life of the compounds.
- The concentration of hydrocarbons as a pollutant
- Length of exposure.
- The size of organism to be a victim.
- Physiological condition of the animal.

Moreover, the physiological effects of petroleum hydrocarbons on mussels at sub-lethal concentration in long period of time could lead to disturbed and reduced rates of metabolism and substantial affects on growth rate and reproduction. The disturbance on the reproduction system could lead the decrease of population.

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Accumulation of aromatic hydrocarbons appears to depend primarily on the partitioning of hydrocarbons between water and the tissue lipids. The aromatic hydrocarbons from petroleum are deposited in the muscle where lipids are present, but the major site of lipid deposition might be in the liver because this digestive organ is the main site that carries out lipid metabolism. It means that a higher fat content in marine mollusks correlate with higher concentrations of hydrocarbons in their tissues. The binding of hydrocarbons to tissue lipid is by hydrophobic interactions and not by covalent bonding. The bioaccumulation factor increases in proportion to the increase in molecular weight of the aromatic hydrocarbons. When returned into oil free seawater, marine animals rapidly release the accumulated hydrocarbons from their tissues. The high molecular weight aromatic hydrocarbons are released more slowly than the low molecular weight hydrocarbons (Neff, *et al.*, 1976).

An evaluation about concepts of bio-concentration in the tissues of aquatic animals has been given by Baron (1990). He indicates that bio-concentration is the process of accumulation of waterborne chemicals by fish and other aquatic animals through non-dietary routes. The bio-concentration factor (BCF) is a proportional constant relating the concentrations of a chemical in water to its concentrations in the aquatic animal at steady state equilibrium; it can therefore be used as an estimate of a chemical's propensity to accumulate in an aquatic animal. Bio-concentration cannot always be predicted solely by hydrophobicity, but other factors such as physiological control of uptake, biotransformation, distribution, intra and interspecies variation in BCF and environmental conditions should also be considered.

III. THE INDUCED STRESS PROTEINS IN MARINE MOLLUSKS

In marine invertebrates, studies on heat shock proteins or stress proteins are still limited. Most studies aimed at development of a bioassay of pollution have focussed on induced free amino acid (FAA) ratio, and enzyme examinations. The use of Taurine (T) : Glycine (G) ratio of the bivalves molluscs, which has been proposed by Livingstone (1985) as an indicator of stress from responds to a number of natural and man-made environmental stressors, is still less-reliable and doubted.

The microsomal mixed-function oxygenase (MFO) enzymes are part of the metabolic system that, for some animals, can be used as an adaptive mechanism for tolerating chemical pollution. The MFO system contains at least two protein components: an iron-containing haem protein called cytochrome P-450 and a flavoprotein called cytochrome-c reductase. Other studies have shown that the complete MFO detoxication system is present in bivalves (Ade *et al.*, 1982). Benzo(a)pyrene hydroxylase has been reported in *Crassostrea virginica* (Anderson, 1978a. 1978b) and *Mytilus edulis* (Stegeman, 1980, 1981a, 1981b). Other enzyme activities have also been demonstrated, namely epoxyde hydrase in *Mytilus edulis* (Bend *et al.*, 1977). Induction of the cytochrome P-450 has also been observed in *Mytilus galloprovincialis* (Guillaume *et al.*, 1984). Unfortunately, there is a lack of information on the interpretation to those enzymes and FAA related to protein molecular weight in gel-electrophoresis. None of the above are stress proteins.

Valuable information on stress proteins as an indicator of pollution by heavy metal (Cu) in *Mytilus edulis* was produced by Sanders *et al.* (1991). The mussels were exposed in the laboratory for 7 d to a range of Cu concentration (0 – 100 mg/liter). Results of quantitative immunobloting assay analysis indicated that the abundance of hsp 60 was greater in tissues exposed to elevated Cu concentration than in Controls.

The study on determination of stress proteins synthesized under heat shock treatment and zinc exposure on temperate and tropical anthozoans (coral builders) was carried out by Syukri (1991). A protein molecular weight of 97 kDa was produced by *Anemonia viridis* and *Actinia equina* (British sea-anemones) in response to a shift from 15° to 28°C for 2 hours. In *Actinia equina* also produced other proteins under stress as follows: 30, 42 and 72 kDa while in *Anemonia viridis* produced the 35 kDa protein. Exposure of *A. equina* to a 1000 µg/l zinc ($ZnNO_3$) for 6 hours resulted in the novel of 35 and 147 kDa proteins. These experiments indicated that exposure to different pollutants enhanced synthesis of different molecular weight proteins. Also, the suite of induced proteins depends on the species of organism. These findings agree with Schlesinger's (1986) statement that the precise molecular weight of stress proteins may vary among different organisms.

The information on induced stress proteins of marine organisms resulting from oil pollution was reported by Wibisono (1992). Results of the study showed that from performing bioassay in blue mussel, *Mytilus edulis* exposed in oil from Forties Blend (BP) in concentration ranging between 10 – 100,000 ppm compared to those which were directly extracted from the field as a clean environment reference, indicated that after 14 d treat-

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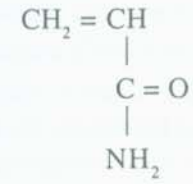
ment, a protein of 35 kDa appeared which was absent in control treatment (0 ppm treatment) and field controls. From LC_{50} data it was evident that the death of animals occurred only after day 14, at levels of 10 % at 100,000 ppm and 10 % at 10,000 ppm. It seems that the Forties Blend crude oils used as a pollutant has a low toxicity level. Though the band of 35 kDa molecular weight protein in the gel is not very thick, this is obviously produced after 14 d treatment (or at least between 7 d and 14 d) at all concentrations of oil. This protein might be considered as a molecular indicator of oil pollution and might be used as an early warning system.

IV. BASIC PHILOSOPHY

The general basic philosophy has been given by Hames (1990). He indicates that any charged ion or group will migrate when placed in an electric field. Since proteins carry a net charge at any pH other than their isoelectric point, they will also migrate where their rate of migration will depend upon the charge density. The application of an electric field to a protein mixture in solution will therefore result in different proteins migrating at different rates towards one of the electrodes. Zone electrophoresis is the final process where the mixture of molecules to be separated is placed as a narrow zone or band at a suitable distance from the electrodes such that, during electrophoresis, protein of different mobilities travel as discrete zones which gradually separate from each other as electrophoresis proceeds. There are some disadvantages to zone electrophoresis in free solution. First, any heating effects caused by electrophoresis can result in convective disturbance of the liquid column and disruption of the separating protein zones. Second, the effect of the diffusion is constantly to broaden the protein zones and this continues after electrophoresis has been terminated. To minimize these effects, zone electrophoresis of proteins is rarely carried out in free solution but instead is performed in a solution stabilized within a supporting medium. To reduce the deleterious effects of convection and diffusion during electrophoresis, the supporting medium allows the investigator to fix the separated proteins at the final position immediately after electrophoresis and thus avoid the loss of resolution, which results from post-electrophoresis diffusion. The most popular of supporting media are in current use being sheets of paper or cellulose acetate, materials such as silica gel, alumina, or cellulose which are spread as a thin layer on glass or plastic plates, and gels of agarose, starch, or polyacrylamide.

Paper, cellulose acetate, and thin layer materials are relatively inert and serve mainly for support and to minimize convection. In contrast, the various gels not only prevent convection and minimize diffusion but in some cases they also actively participate in the separation process by interacting with the migrating particles. These gels can be considered as porous media in which the pore size is the same order as the size of the protein molecules such that a molecular sieving effect occurs and the separation is dependent on both charge density and size. The pore size of starch and polyacrylamide gels have the same order of size as proteins molecules and so these do contribute the sieving effects. However, the use of starch gel is highly dependent on the quality of the starch gel itself, which being prepared from a biological product, is not always reproducibly good and may contain contaminants, which can adversely affect the quality of the results obtained. On the other hand, polyacrylamide gel, as a synthetic polymer of acrylamide monomer, can always be prepared from highly purified reagents in a reproducible manner provided that the polymerization conditions are standardized. In addition, polyacrylamide gel has the advantages of being chemically inert, stable over a wide range of pH, temperature, and ionic strength, and is transparent. Finally, polyacrylamide is better suited to a size fractionation of proteins since gels with a wide range of pore sizes can be readily made. So based on the above considerations and other reasons, polyacrylamide gels have become the medium of choice for zone electrophoresis of most proteins while starch gels have been widely used for the limited analysis of isoenzymes.

There are two types of electrophoresis examinations, which can be used being one dimension and two dimension of polyacrylamide gel electrophoresis. Although two-dimensional gel separations of proteins have the highest resolving power, one-dimensional polyacrylamide gel electrophoresis is still the most widespread form of the technique since it offers sufficient resolution for most situations coupled with ease of use and the ability to process many samples simultaneously for comparative purposes (Hames, 1990).



Acrylamide monomer

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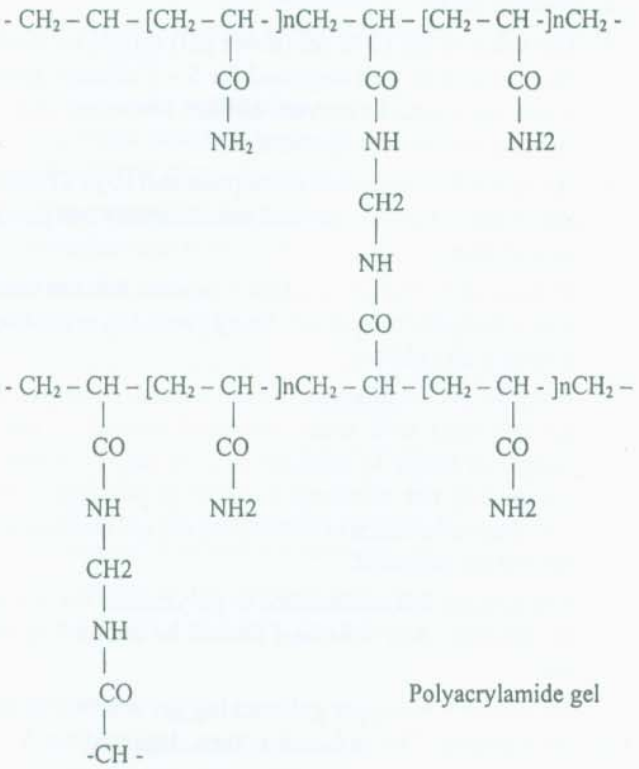

V. MATERIALS AND METHOD

A. *Test Organisms*

The marine bivalve mollusks e.g. green mussel (*Mytilus viridis*), may be collected at low tide from the inter-tidal zone of the presumably polluted shore. On the other hand the same species of animals should also be collected from any other place as a reference site of clean environment (field control). During collection, just four organisms from presumably polluted area and four animals from field control can be taken randomly and sacrificed for stress protein examination. After blotting and desecting the whole tissues of each of two organisms of each origin are placed in a sterile polythene labelled vial filled with homogenizing buffer that contained phenylmethyl sulfonyl fluoride (PMSF) as a protease inhibitor. Then the vials are kept in a polystyrene box filled with ice to freeze the samples. The proteins examination is carried out in the laboratory later by electrophoresis.

B. *Sample preparations*

The whole animal tissues from the field are taken out from polystyrene box as soon they arrive at the laboratory and the tissues are homogenized in a clean sterile glass homogenizer mixed with 5 ml of homogenizing buffer at 4°C (chilled on ice) for 5 minutes.

The homogenizing buffer had the following composition:

- 1.0 M Tris Buffer pH 7.2
- 1 % SDS
- PMSF
- Distilled water.

The supernatant from the homogenizer is poured into a clean small eppendorf tube and centrifuged at 13.224 g for 60 minutes at 4°C. A quantity of 0.25 ml supernatant is taken out and poured into a clean eppendorf mixed with 0.25 ml SDS sample buffer. The eppendorf is then immersed in boiling water to denature the proteins for 5 minutes and then kept at –20°C. The SDS sample buffer according to Laemli (1970) consists of :

- 0.5 M Tris HCl pH 6.8
- glycerol
- 10 % SDS
- β - mercaptoethanol
- 0.05 % (w/v) bromophenol blue (in water)
- distilled water.

C. *Chemical Reagent*

a. Acrylamide/Bis (30 % T, 2,67 % C)

Care should be taken since acrylamide and bis-acrylamide are potent neurotoxins by skin absorption or inhalation of monomer powder.

b. 4 % (w/v) SDS (Sodium Dodecyl Sulphate).

c. SDS Lower Gel Buffer pH 8.8 stored at 4°C

d. SDS Upper Gel Buffer pH 6.8 stored at 4°C

e. 10 % (w/v) Ammonium persulphate (it is used to polymerize the acrylamide)

f. N, N, N', N' – tetramethylethylendiamine (TEMED) (used as an accelerator of the polymerization process between acrylamide and bis-acrylamide by catalyzing the formation of free radicals from ammonium persulphate).

g. Electrolyte (Running Buffer).

This solution is made by mixing 9 g Tris base + 43.2 g Glycine + 3 g SDS. This mixture is diluted with 300 ml distilled water and stored at 4°C. 100 ml of this solution is taken and added with 400 ml distilled water and warmed to 37°C before use. This dilution can be used for 3X electrophoretic run.

h. Standard solution

The standard protein (polypeptides) solution contained protein markers as follows:

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	Mol. Weight
- Phosphorylase b of rabbit muscle	97.4 kDa
- Bovine serum albumin (BSA)	66.2 kDa
- Hen egg white ovalbumin	45.0 kDa
- Bovine carbonic anhydrase	31.0 kDa
- Soybean trypsin inhibitor	21.5 kDa
- Hen egg white lysozyme	14.4 kDa.

1 µl SDS-PAGE standard proteins (low-range) as indicated above are diluted with 20µl SDS sample buffer, and mixed well in a clean eppendorf with a syringe.

i. Ethanol 90 % p.a.

j. Acetic acid glacial

k. Methanol p.a.

D. *Equipments & Instrumentations*

a. Load on top balance

b. Fume cupboard

c. Refrigerator

d. Electrical centrifuge

e. Vaccum pump

f. A set of micro syringe

g. Whatman no. 1 filter paper

h. Glass trays

i. Mini gel Protean vertical dual slab cell electronic instrument, as shown in Figure 1.

j. Power supply.

k. Glass apparatus such as Erlenmeyer flask, Beaker glass, pipette, micro-pipette, Hamilton syringe with needle, homogenizer, etc.

l. Rubber hand gloves.

E. *Procedure for running a one-dimensional SDS-PAGE*

1. Cleaning the instruments

- All parts of the instruments should be cleaned with soap and water then, rinsed with distilled water.
- The glass plates, plastic comb and spacers should be wiped with 90 % ethanol to ensure good gel adhesion to the glass. After a final swab with an ethanol soaked tissue held in a gloved hand, the plates are allowed to air-dry.
- The cleaned sandwich glass with a pair of rubber spacers inserted between the glasses are installed on to the sandwich clamp assemblies then, aligned in the alignment slot of the casting stand. The rubber gasket provides a leak-proof seal without grease.

2. Pouring gels

- The solution for 15 % gel (lower gel) should be made. This solution is then degassed for 5 – 7 minutes using a vaccum pump to prevent bubble formation and to increase polymerization rates.
- 100 µl 10 % Ammonium persulphate and 10 µl TEMED are added to the solution and mixed rapidly but gently by swirling.
- Without delay the gel solution is pipeted into the sandwich glass until the pointer, being careful to avoid trapping any air bubbles.
- After the gel solutions have been poured, overlayer the gel solutions with water saturated butan-2-ol just a couple of drops to exclude oxygen and to ensure a completely flat meniscus as soon as possible. Great care should be taken in overlaying the gel solution and no mixing occurred.
- The gels are left undisturbed to polymerize for at least 40 minutes. Any vibration should be avoided at this stage.
- Meanwhile, the upper gel/stacking gel solution should be prepared. This solution is then degassed for 5 – 7 minutes.
- After degassing the stacking gel solution is then added with 50 µl 10 % Ammonium persulphate and 10 µl TEMED by mixing gently.
- Pour the stacking gel solution immediately into sandwich glass just near the spacer on top of the lower gel and insert the plastic comb which all the teeth of the comb are covered by solution.
- The stacking gels are left undisturbed for about 30 minutes to allow the polymerization process.

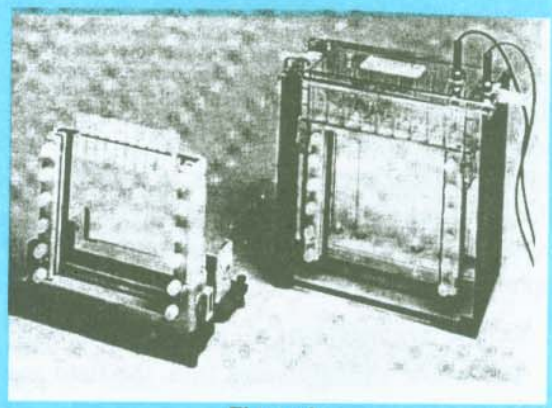

Figure 1
A mini gel vertical dual slab cell electrophoresis apparatus

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- Remove the comb carefully to expose the sample wells then rinse all wells completely with SDS running buffer.
- Remove the assemblies with the gels from the casting stand attached to the inner cooling core, where the shorter glass facing inside of the core.
- The core with the gels are placed into the lower buffer chamber and filled with running buffer solution.

3. Sample Loading

- 5 μl Standard molecular weight proteins is loaded into the centre of the well series of the sandwich gels using 10 μl vol. Hamilton syringe.
- An optimal amount of sample from field control (as a clean reference) is loaded at the side(s) of the sandwich gels. Meanwhile the amount of sample from polluted environment is loaded into the remaining wells.
- The needle of the syringe is inserted and immersed down to just ± 1-2 mm. from the well bottom before delivery.

4. Running the gels

- The electrical supply should be set up to 200 V. and the current should be 50 mA and begin the electrophoresis.
- The sample is run until the standard protein reaches the bottom of the gel sandwich.

5. Removing the gels

- Turn off the power supply after electrophoresis is complete.
- Remove carefully the inner cooling core from the lower chamber and pour off the running buffer.
- Release the clamp assembly and the glass plate sandwich is also removed.
- Push out one of the spacers of the sandwich to the side of the plates without removing it.
- The spacer can be twisted gently so that the upper glass plate pulled away from the gel.
- The gel can be removed by grasping gently from the two corners of the gel and lifted off.
- Stacking gels may be discarded before staining.

6. Staining and de-staining the gels

- Coomasie Brilliant Blue R-250 powder is used as staining material. The staining gel (dye) in 30 % methanolic solution must be made.
- The gels are immersed in a glass tray filled with staining solution and shake gently for 2 hours at room temperature.
- After staining, excess stain can be removed from the gel to allow protein bands to be seen clearly by soaking it in 50 % de-stain solution in de-ionized water and left overnight to obtain completely clear background.
- The stained bands in the gels then can be stored in water or to be photographed if needed.

F. ***Quantification of molecular weight of protein band from the polluted environment***

Results to be obtained are then compared to the standard molecular weight of protein bands upper or lower nearby and an estimate on molecular weight protein of tissues sample(s) from polluted environment should be made using the simple formula as follows:

$$Mw = \frac{A}{B} \; x \; C$$

Where: Mw = Molecular weight of protein to be observed (KDa)

A = distance migrated by the protein band to be observed (cm).

B = distance migrated by standard molecular weight protein band (cm) at the upper or lower nearby the band to be observed.

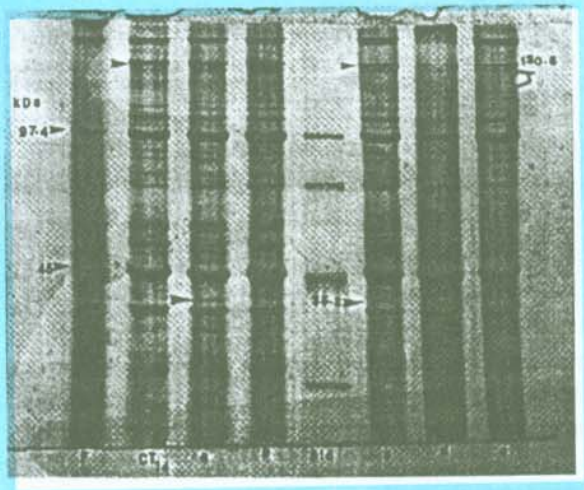

Figure 2
Molecular weight protein band (35 KDa) in gel electrophoresis of bivalve tissues *Mytilus edulis* from oil polluted environment (quoted from: Wibisono, 1992). Std. = Standard protein bands; CT_{14} = Control treatment after 14 d; F = Field control as clean reference

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C = Standard molecular weight (KDa) protein of comparative band.

VI. CONCLUSION AND SUGGESTION

The band of 35 KDa molecular weight proteins might be considered as a molecular indicator of oil pollution at sub lethal concentration. The biological substance of marine bivalve mollusks and its analytical method (one-dimensional poly acrylamide gel electrophoresis) can be applied as a new approach for monitoring consideration in petroleum activities since the impacts of low levels of pollution for long period of time does not always clearly appear. Though the above method is usually used for the examination of most protein (polypeptides), but the use for oil pollution monitoring constitutes a "new" sound technology to be offered. Environmental monitoring for sub- lethal effects is very important in minimizing the impacts particularly on the degrading aquatic animals population prevention in the sustainable development atmosphere. There are also several advantages in the implementation of this method e.g.:

- No need of so many expensive instrumentations.
- The time consumed for the implementation of this method is estimated to be less than a week.
- It can be considered as a tool for early warning system.
- The molecular weight of biological substance (protein) can be quantified by using a very simple formula.

It is suggested that the analysis on physical and chemical properties of stream (receiving bodies of effluent discharge) should be completed with total petroleum hydrocarbon contents and or poly aromatic contents other than the oil and grease contents to support better environmental information within the area of petroleum activities.The marine bivalve mollusks in Indonesia as suitable shellfish that can be applied for this purpose are *Mytilus viridis* (*kerang hijau*) and or *Anadara granosa* (*kerang darah*) which are species indicator of choice in the tropics.

REFERENCES

1. Ade, P., Banchelli Soldiani, M.G., Castelli, M.G., Chiersara, E., Clementi, F., Fanelli, R., Funari, E., Ignesti, G., Marabani, A., Oronesu, M., Palmero, S., Pirisino, R., Ramundo Orlando, A., Silano, V., Viarengo, A. and Vittozzi, L. (1982). Comparative biochemical and morphological characterization of microsomal preparations from rat, quail, trout, mussel and *Daphnia magna*. In : Cytochrome P – 450. Biochemistry, Biophysics and Environmental Implications. E. Hietanen, M. Laitinen and O. Hainninen (Eds.), Elsevier, Amsterdam. pp. 387 – 390.
2. Anderson, R.S. (1978 a). Developing an invertebrate model for chemical carcinogenesis : Metabolic activation of carcinogens. Comp. Pathobiol. 4, 11 – 24.
3. Anderson, R.S. (1978 b). Benzo(a)pyrene metabolism in the American oyster *Crassostrea virginica*. EPA Ecol. Res. Ser. Monogr. (EPA-600/3-78-009).
4. Anderson, R. (1989). Early warning of stress. New Scientist, 122 : 50 – 52.
5. Atkinson, B.G. and Walden, D.B. (1985). Changes in Eukaryotic Gene Expression in Response to Environmental Stress. Academic Press, Orlando, Florida.
6. Baron, M.G. (1990). Bioconcentration. Env. Sci. Technol. Vol. 24, nr. 11. pp 1612-1618.
7. Bend, J.R., James, M.O. and Dansette, P.M. (1977). In vitro metabolism of xenobiotics in some marine animals. Ann. N.Y. Acad. Sci. 298, 505-521.
8. Blumer, M.G., G. Souza and J. Sass (1970). Hydrocarbon pollution of edible shellfish by an oil spill. Mar. Biol. 5 (3) : 195-202.
9. Burdon, H.R. (1987). Temperature and animal cell protein synthesis. Soc. Exp. Biol. pp. 113 – 133.
10. Farrington, J.W., A.C. Davis, N.M. Frew, and K.S. Rabin (1982). No. 2 fuel oil compounds in *Mytilus edulis*. Retention and release after an oil spill. Mar. Biol. 66, 15 – 26.
11. Fossato, V.U. and W.J. Canzonier (1976). Hydrocarbon uptake and loss by the mussel, *Mytilus edulis*. Mar. Biol. 36, 243 – 250.
12. Guillaume, J.R., Carles, D., Leveau, M., Bertrand, J.C. and Gilewics, M. (1984). Induction of cytochrome P-450 by hydrocarbons in a mollusk bivalve: *Mytilus galloprovincialis*. In : Proceeding of the 5th Congress of the European Society for Comparative Physiology and Biochemistry. Springer-Verlag, Berlin.
13. Hames, B.D. (1990). One-dimensional polyacrylamide gel electrophoresis. In: Gel Electrophoresis of Proteins. A Practical Approach. 2nd ed. Hames, B.D. and D. Rickwood (Eds.), IRL Press at Oxford Univ. Press, Oxford, UK.
14. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 : 680-685.

15. LEMIGAS – CNEXO (1984). Occurrence of tar pollution along some shores in Indonesia. Paper of the Seminar on Marine Sciences Research in Indonesia, 1980-1984 of the Indonesian- French Joint Cooperative Research on Oceanology, organized by CNEXO, French Ministry of External Relation and BPPT in Jakarta, 18-19 Sept. 1984.

16. Lindquist, S. (1986). The heat shock response. Ann. Rev. Biochem., 55, 1151-1191.

17. Livingstone, D.R. (1985). Determination of the taurine : glycine ratio and the sum of threonine and serine. In: The Effects of Stress and Pollution on Marine Animals. B.L. Bayne, *et al* (Eds.), Praeger Publisher, New York. pp. 215-220.

18. Mizzen, L.A., Chang, C., Garrels, J.G. and Welch, W.J. (1989). Identification, characterization and purification of two mammalian stress proteins present in mitochondria: one related to hasp 70, the other to GroEL. J. Biol. Chem., 264, 20664 – 70.

19. Moore, M.N. and D.M. Lowe (1985). Cytological and cytochemical measurements. In : The Effects of Stress and Pollution on Marine Animals. B.L. Bayne, *et al.*, (Eds.). Praeger Publisher, New York, pp. 215-220.

20. Neff, J.M., B.A. Cox, D. Dixit, and J.W. Anderson (1976). Accumulation and release of petroleum-derived aromatic hydrocarbons by four species of marine animals. Mar. Biol., 38, 279-289.

21. Neff, J.M. and J.W. Anderson (1981). Response of Marine Animals to Petroleum and Specific Petroleum Hydrocarbons. Applied Science Publishers Ltd., London. P. 93 – 130.

22. Sanders, B.M. (1990). Stress proteins: potential as multitiered biomarkers. In : Biological Markers of Environmental Contamination. J.McCrthy and L. Shugart (Eds.), CRC Publishers, Boca Raton, Florida. Pp. 135-161.

23. Sanders, B.M., Martin, L.S., Nelson, W.G., Phelps, D.K. and Welch, W. (1991). Relationship between accumulation of a 60 KDa stress protein and scope for growth in *Mytilus edulis* exposed to a range of copper concentrations. Mar. Environ. Res. 31, 81-97.

24. Schlesinger, M.J., Ashburner, M. and Tissiers, A. (Eds). (1982). Heat Shock from Bacteria to Man. Cold Spring Harbor, New York. 440 pp.

25. Stegeman, J.J. and J.M. Teal (1973). Accumulation, release and retention of petroleum hydrocarbon by the oysters, *Crassostrea virginica*. Mar. Biol. 22, 37-44.

26. Stegeman, J.J. (1980). Mixed-function oxygenase studies in monitoring for effects of organic pollution. Rapp., P.v.Reun Conc.int.Explor.Mer. 179, 33-38.

27. Stegeman, J.J. (1981 a). Polynuclear aromatic hydrocarbons and their metabolism in the marine environment. In: Polycyclic Hydrocarbon and Cancer. Vol. 3. H.V. Gelboin & POP.Ts'O (Eds.), Acad. Press, New York. pp. 1-60.

28. Stegeman, J.J. (1981 b). Metabolism of aromatic hydrocarbons by bivalve mollusks, *Mytilus edulis*. In: 1980-81 Annual Sea Grant Report. Woods Hole Oceanographic Institution, 15 p.

29. Syukri, A. (1991). Molecular indicators of stress in temperate and tropical anthozoans. MSc Thesis. Dept. of Marine Sciences and Coastal Management, University of Newcastle Upon Tyne, U.K.

30. Wibisono, S. (1992). Stress proteins as a molecular indicator of oil pollution in mussels *Mytilus edulis*. Post Grad.Diploma in Tropical Coastal Management Dissertation. Dept. of Marine Sciences and Coastal Management, University of Newcastle Upon Tyne, U.K. •