IET Project No. 16c

Metabolic studies in the coral reef: Cellular-level biomarkers

Principal Investigators: Avigdor Abelson and Vladimir Bresler (IUI)

Date of final submission: 28/06/04

1. Objectives

The scope of the project was to assess stress symptoms in corals, symptoms which are likely to be induced by various anthropogenic stressors, notably pollutants, along the coasts of Eilat. Our specific goal was to compare the responses of stony corals in 14 stations along the Eilat coast, to environmental conditions, by assessing cytologic/ genotoxic parameters (i.e. Micronucleus frequency, MNT; DNA breakage DNA-br; Esterase Activity, EA), which are known to respond to environmental stressors. Should the examined parameters prove to significantly differ between the study sites, they are expected to serve as sensitive biomonitoring tools indicating environmental stressors that can be related to human impact on marine organisms.

2. Methods

Background of the used methodologies.

MNT. In the early 1970s, Heddle (1973) and Schmid (1975) proposed the micronucleus test (MNT; also named MN assay) to quantitatively assess signs of clastogenic and/or aneugenic effects on organismal cells. Later on, during the three following decades this test was, and still is, a major component of modern toxicogenetic screening programs throughout the world (Jensen and Leary, 1990; Torous et al., 1998; UNEP/RAMOGE, 1999; Kirsch-Volders et al., 2003; Dailianis et al., 2003). MNT shows the frequencies of the chromosomal/mitotic spindle damages induced by various chemical or physical agents. Clastogenic agents induce in mitotic cells chromosome breaks and produce free acentric chromosome fragments, while aneugenic agents induce spindle apparatus breaks and produce lagged whole chromosome(s). These lagged fragments and/or whole chromosomes form after telophase small single or multiple nuclear-like structure(s) named micronucleus/micronuclei, since they generally contain only 1-10 % of diploid DNA. Manual microscopic counting of micronuclei-containing cell frequency (usually in ‰) demonstrates objectively the level of clastogenic/aneugenic action under given conditions. For example, in the bone marrow of mice treated with known clastogenic agents, micronuclei-containing reticulocytes occur at a frequency of approximately 10-100‰, whereas in the bone marrows of unexpected mice the frequency is typically 1-5‰ (Jensen and Leary, 1990). Different staining procedures such as Giemsa stain, Feulgen reaction for DNA, or fluorescent probes for DNA (acridine orange, Hoechst, DAPI, and ethidium bromide) are used to unmask micronuclei-containing cells and count their frequency. Specific fluorescent probes for DNA have made manual counting easier and more objective (Jensen and Leary, 1990;). Special pan-centromeric fluorescent probes allow distinguishing clastogenic and aneugenic actions (Nutley et al., 1996; Torous et al., 1998; Palus et al., 2003).

Since micronuclei formation is the result of mitotic apparatus damage, the MNT has been applied to all eukaryotic cells. It was adapted to plant cells (Grover and Kaur, 1999; Isidori et al., 2003; Monarca et al., 2003; Gong et al., 2003), various cell lines *in vitro* (Kirsch-Volders et al., 2003), cells of invertebrate species (Bolognesi et al., 1999; Dailianis et al., 2003), and vertebrate cells (Cavas and Ergene-Gozukara, 2003**,** Stoncius and Lazutka, 2003**,** Torres-Bulgarin et al., 2003; Iavicoli et al., 2003).

DNA-br. Single-stranded DNA breaks (DNA-br) represent the most frequent primary DNA lesion by genotoxic agents in eukaryotic cells. Determination of the DNA-br frequencies, therefore, is widely used to assess action of given genotoxic agents or to monitor environmental genotoxicity (Shugart, 1988; Meyers-Schone et al., 1993; Sugg et al., 1995; Schroder et al., 1998; Batel et al., 1999; Bresler et al., 1999, 2002, 2003; UNEP/RAMOGE. 1999; Bihari et al., 2002; Klobucar et al., 2003).

The most common indirect method for determination of DNA-br is alkaline DNA-unwinding assay. Treatment of DNA with high pH produces DNA denaturation (unwinding) that expresses sites of single-stranded DNA breaks. Determination of double-stranded and single-stranded DNA allows calculation of the relative level of DNA damage. To separate double-stranded and single-stranded DNA, hydroxyl apatite columns (Herbert and Hansen, 1998), alkaline filter elution (UNEP/RAMOGE. 1999), or electrophoresis are used. Single-cell agarose gel electrophoresis under alkaline conditions, the so-called comet assay, is a well-established semi-quantitative assay to assess the amount of DNA-br and their repair (Tice et al., 2000). Hydroxyl apatite and alkaline filter elution techniques are expensive, time-consuming, labor-intensive and non-precise; whereas, Alkali treatment and DNA elution may produce artificial single-stranded DNA.

The methods that determine double-stranded and single-stranded DNA without the need of separating them, but rather keeping them in the same homogenate solve the above noted problems (Haugland, 1999). In our study, we used metachromic staining with acridine orange (AO) to determine Single-stranded DNA breaks *in situ* in cell nuclei, by using acidic DNA unwinding assay and the fluorescent probe, AO, and two-wavelength microfluorometry. For this we used a Nikon epi-fluorescent microscope with stabilized super high pressure mercury lamp, equipped with a measuring head which include mirror measuring diaphragms, changeable barrier interference filters, a highly sensitive photomultiplier, current pre-amplifier and the readout (Oriel Co, Stratford, ST, USA). Excitation of AO was made by blue light (450-490 nm). The intensity of the dsDNA-associated green fluorescence (520-540 nm) and red fluorescence (590-620 nm) of AO bound with ssDNA was determined in each nucleus by using two-wavelength microfluorometry with contact objective Fluor 43x1.0. The microfluorometer was calibrated using microcuvettes and Multispectral Fluorescence Microscopy Standard Kit (MultiSpeck^{TM}, Molecular Probes, Inc, Eugene, OR, USA). Acidic DNA unwinding in whole nuclei produce less artifact then alkali unwinding. Fraction of dsDNA (Fds) after unwinding was calculated as ratio of green fluorescence of dsDNA to fluorescence of total DNA (green + red fluorescence). The negative log of Fds (-log) is proportional to the relative number of DNA-unwinding points The relative number of SSBs per unwinding units (n) was calculated as

$n = -\log Fds.in / -\log Fds.ref$,

where the index *in* indicates the suspected disturbed site and the index *ref* indicates reference site.

It should be noted that there are new specific probes for double-stranded DNA (PicoGreen®) and for single-stranded DNA (OliGreen®). However, the method we used is highly accurate and enables the discrimination of single- from double stranded DNA. Unlike measurements of UV absorbance, these assays are not affected by the presence of proteins, free nucleotides or short oligonucleotides, making quantitation of intact oligonucleotides and nucleic acids much more accurate - in complex mixtures such as serum, whole blood, and tissue homogenates (Schroder et al., 1998; Bihari et al., 2002).

EA. Determination of various enzymes activities, and in particular cholinesterase, non-specific esterase, and detoxifying enzymes, play an important role in biological monitoring (Galgani etal., 1992; Sturm et al., 1998; Nacci et al., 1998; Bresler et al., 1999, 2001, 2003). The activities of these enzymes are studied *in vitro* using homogenates or isolated fractions of tissue, or *in situ* in living cells using fluorogenic substrates (Van Noorden and Frederiks, 1992; Nacci et al., 1998; Haugland RP. 1999;. Bresler et al., 1999, 2001,2003; Dailianis et al., 2003). However, there are some general enzymes, like esterase, the activity of which can be measure even after fixation. The ability of using this enzyme in fixed specimens is very important for monitoring of sensitive species like reef corals, the sampling of which is limited and transportation is problematic due to low survival.

Detailed description of the used methods.

To assess and quantify the three cytological parameters we made use of contact microscopy, contact fluorescent microscopy and microfluorometry. To address our objectives, we sampled two species of stony corals, *Stylophora pistillata* and *Pocillopra damicornis*. The sampling sites of corals were chosen to fit the sites of recruitment (IET recommendation #18) and to cover the whole coastal range of Eilat (14 stations, 6 sites; See Fig. 1). Six specimens (three of each species) were sampled from each of the 14 stations. In each specimen 3'000 cells were examined for MNT, 1'000 for DNA-br, and 200 cells for EA. Three of the samples were fixed in Methanol for the SSBs test. The other three samples were fixed in 4% Formaldehyde (in filtered sea water) for the MN and esterase activity tests.

Soft tissues of fixed corals were separated from the skeleton in water under dissecting microscope by shaking and using aquarelle brush. The obtained suspension of cells and small pieces of tissue were used for the cytological tests. For MN test, nuclei were stained with DNA-specific fluorochrome ethidium bromide (Haugland, 1999). From each colony 3,000 cells were examined under Nikon epifluorescent microscope (Excitation filter 510-560 nm, dichroic mirror 580 nm, barrier filter 580 nm and objective Fluor 40x0.85) to calculate frequency of micronuclei-containing cells.

Cell treatment for unmasking single-strand DNA breaks (SSBs) was made according to Darzynkiewicz (1993, 1994) and Bresler et al. (1999, 2003) with small modifications. That is, cells were treated by RNAse A (100 units) at 370 C for 1h in buffered salt solution, incubated in phosphate-citric buffer at pH 2.6 for 80 sec and stained in 20 μM Acridine Orange (AO) solution in the same buffer for 5 min and used for two-wavelength microfluorometry. Excitation of AO was made with interference filter 450-490 nm, fluorescence of AO-ds DNA complex with barrier filter 520-540 nm, fluorescence of AO-ss DNA complex with barrier filter 590-620 nm, and objective Fluor 40x0.85.

Non-specific esterase activity was determined with fluorogenic substrate fluorescein diacetate (FDA; Haugland, 1999; Bresler et al., 1999, 2003). Cells were incubated with final concentration of 10 μM of FDA for 10 min. Liberated fluorescein was determined by microfluorometry (excitation filter 450-490 nm, dichroic mirror 510 nm, barrier filter >520 nm and objective Fluor 40x0.85).

3. Results

The results obtained by the three different methods of assessment of stress symptoms (MNT, Fig. 2; DNA breakage, Fig. 3; EA, Fig. 4) revealed the same general pattern where the strongest stress symptoms were found in the north beach (Peace Lagoon) and the lightest symptoms in the southernmost site, the Lighthouse (Figs. 2-4). In addition to the Peace Lagoon, high values were found for specimens sampled in the ports area (i.e. Dekel Beach, Eilat's civil Port; stations 7-10 in Figs. 2-4). It should be noted that the obtained pattern of MNT was the same for both coral species, *S. pistillata* and *P. damicornis*. These high values of stress-indicating parameters, which were consistent for the three parameters and the two species, indicate two major stress hot-spots along the Eilat coast - the North Beach and the Port area.

4. Justification of the used methods.

During the summarizing meeting there were some claims which challenged the validity of our MNT and DNA-br methods. Below we answer these claims and provide evidence for the reliability of the methods used.

MNT. At present, it seems that the MN test is the most objective and indispensable direct method for *in vitro* and *in vivo* monitoring of clastogenic/aneugenic effects that are produced by environmental stressors. Due to its universality, high sensitivity and reliability, MNT *in vitro* was recommended by the 3rd International Workshop on Genotoxicity Testing, for use in medicine and pharmaceutical industry (Moore et al., 1996; Fench et al., 1997; Pavanello and Clonfero, 2000; Kirsch-Volders et al., 2003). Likewise, the wide use of MNT can be seen by the numerous scientific reports which either use, or mention this important parameter. For example, the Internet site PubMed revealed, in 21.10.03, 3229 publications containing the term “micronucleus test”.

However, as claimed during the summarizing meeting, it is true that the studied species in our project do not have analytical baselines which provide dose-response curves. It should be stressed, however, that such baselines are required when there is attempt to create a standard monitoring procedure, of a given species, cell type and assessment protocol, for wide-scale comparisons between different regions, periods and labs. In our case, we do not compare data of different species, different regions, or time periods. We claim, therefore, that for our comparative studies of within species and samplings events, any set of data that shows significant differences between sites, is valid.

Another claim raised during the meeting was that the differences we show, even if significant they are not enough to point out a problem, since the MNT values of the 'stressed cells' should be above 10 micronucleated cells per 1000 cells. Celik et al. (2003) indicate significant differences in MNT values of smokers versus non-smokers and petrol-station workers and non-workers control. The MNT values in all the treatment

groups were dramatically lower then 10, and the significant differences were of factor 2 to 3. This study contradicts the claim raised during the summarizing meeting, according to which the values found in our study are meaningless. The study of Celik et al. (2003) and similar other studies on various cells of human and mice (e.g. Garaj-Vrhovac et al. 1997, Karahalil et al. 1999, Soares et al. 2003) clearly show that the "contradicting facts" raised in this regard have no scientific basis.

DNA-br. A major claim that has been raised against our method of DNA breakage (i.e. acid denaturation), was that this method less reliable then the alkaline unwinding methods. The major answer to this claim can be provided by a comparison we made of the 'AO-acid denaturation' with the alkaline DNA unwinding method. This comparison has been done during the German-Israeli MARS project, and the results of analyses of mollusc and fish showed higher sensitivity of the AO-acid denaturation method (Bresler et al., 1999). Moreover, recently we compared results of acidic unwinding in whole nuclei and the new 'fast micromethod®', which uses alkaline unwinding method, in tissues of molluscs and corals. The 'fast Micromethod®' was developed on the base of PicoGreen® as an assay applicable both for vertebrate and invertebrate tissues (Batel et al., 1999). The results indicate a very high correlation between the two methods (r^2= 0.97, $p<0.001$; Ben-Tzvi et al, in prep), indicating that the acidic unwinding method can be used as a sensitive and reliable method for determination of single-stranded DNA.

Finally, the use of AO and acid denaturation for detection of DNA damage has been reported during recent years in diverse journals (e.g. Dobrinski and Darzynkiewicz 2001, Watanabe 2001, Erenpreiss et al. 2002, Sanchez-Pena et al. 2004). These studies justify the use of our method and support the validity of our obtained data. Furthermore, these studies, as well as our comparison with other, common methods, show that the claims raised during the summarizing meeting have no sound basis.

5. Discussion

In the present study we have used three parameters which are used as environmental biomarkers; that is, they describe the response of organisms to their environment. The three parameters demonstrate the same spatial pattern, according to which the problematic sites, among the studied sites, are the North Beach (Peace Lagoon) and the Ports area (Civil Port and Dekel Beach). Providing that each one of the parameters and the methods to measure it are independent in indicating stress responses of organisms, and the three parameters show the same pattern for two distinct species, we explicitly conclude that the two most disturbed sites along the Eilat coasts (among the studied sites), are the North Beach and the Ports area. Our results suggest that in these sites exist stronger agents (most likely contaminants), which disrupt cell functions (i.e. enzyme activities) and cause genotoxic responses (i.e. MNT and DNAbr) in organisms.

At present, we cannot pinpoint the exact pollution hotspots. In the ports there are various potential agent which can cause stress responses in marine organisms. As for the north beach, it should be noted, that similar studies of the same methods, which have been conducted in some more sites in Eilat and on other taxons, show the highest stress symptoms in the vicinity of the fish cages (Bresler et al. 1999, 2003). For instance, specimens of the limpet species *Cellana rota* sampled from the fish cages have found to possess significantly higher MXRtr and SATOA activities, cholinesterase activities and levels of DNA breaks (determined by both alkaline unwinding and AO), as compared

with specimens from the coastal site across the fish cages ("Ardag-stones"). These findings suggest that the fish cages are, at least one of the major pollution hot-spots of the north beach area. Moreover, the results of Zvoluni et al. (submitted) which suggest some role of selection in the Eilat populations of Stylophora pistillata, show the largest decline of genotypic diversity in the fish cages vicinity (further described in the 'coral recruitment' chapter of the report).

5. Conclusions

I. Based on our results, we explicitly conclude that the two most disturbed sites along the Eilat coasts (among the studied sites), are the North Beach and the Ports area. Our results suggest that in these sites exist stronger agents, which disrupt cell functions (i.e. enzyme activities) and cause genotoxic responses in organisms.

II. A compilation of various studies strongly suggests that the major pollution hotspot in the north beach is the fish-cage area.

III. During the summarizing meeting there was some criticism regarding the validity of our used parameters and/or the methods. The literature-based evidence and our own comparisons strongly support the use of our methods. Therefore, we conclude that the parameters we studied, and the methods we used, are efficient and reliable in indicating stress symptoms in marine organisms.

6. Open Questions

Our results, although indicate sites of environmental stressors, do not show causality and cannot pinpoint the exact agent(s) responsible for the observed stress symptoms. Further investigation is required to identify the exact stress agents (i.e. pollutants) along the Eilat coasts, notably the North Beach and the Ports areas.

7. References

Abramsson-Zetterberg L, Zetterberg G, and Bergquist M. 2000. Human cytogenetic biomonitoring using flow-cytometric analysis of micronuclei in transferrin-positive immature peripheral blood reticulocytes. – *Environ Mol Mutagen* 36:22-31.

Al-Sabti K, Hardig J.1990. Micronucleus test in fish for monitoring the genotoxic effects of industrial waste products in the Baltic Sea, Sweden. – *Compar Biochem Physiol* 97C:179-182.

Azevedo L, Gomes JC, Stringheta PC, Gontijo AM, Padovani CR, Ribeiro LR, and Salvadori DM. 2003. Black bean (*Phaseolus vulgaris* L.) as protective agents against DNA damage in mice. –*Food Chem Toxicol* 41:1671-1676.

Batel R, Jaksic Z, Bihari N, Hamer B, Fafandel M, Chauvin C, Schröder HC, Müller WEG, and Zahn RK. 1999. A microplate assay for DNA damage determination (Fast Micromethod) in cell suspension and solid tissues. – *Anal Biochem* 270:195-200.

Barale R, Marrazzini A, Bacci E, Di Sibio A, et al. 1998a. Sister chromatid exchange and micronucleus frequency in human lymphocytes of 1,650 subjects in an Itlian population. I. Contribution of methodological factors. – *Environ Mol Mutagen* 31:218-227.

Barale R, Chelotti L, Davini T, Del Ry S et al.1998b. Sister chromatid exchange and micronucleus frequency in human lymphocytes of 1,650 subjects in an Itlian population. II. Contribution of sex, age, and lifestyle. – *Environ Mol Mutagen* 31228-242.

Bihari N, Hamer B, Jaksic Z, Fafandel M, Micic M, and Batel R. 2002. Application of alkaline elution, Fast Micromethod and flow cytometry in detection of marine contamination. – *Cell Mol Biol* 48:374-377.

Bloching M, Hofmann A, Lautenschlager C, Berghaus A, and Grumt T. 2000. Exfoliative cytology of normal buccal mucosa to predict the relative risk of cancer in the upper aerodigestive tract using the MN-assay. – *Oral Oncol* 36:550-555.

Bolognesi C, Landini E, Roggieri P, Fabbri R, Viarengo A. 1999. Genotoxicity biomarkers in the assessment of heavy metal effects in mussels: experimental studies. – *Environ Mol Mutagen* 33:287-292.

Bresler V, Bissinger V, Abelson A, Dizer H, Sturm H, Kratke R, Fishelson L, Hansen P-D. 1999. Marine molluscs and fishes as biomarker of pollution stress in littoral regions of the Red Sea, Mediterranean Sea and North Sea. – *Helgol Mar Res* 53:219-243.

Bresler V, Fishelson L, and Abelson A. 2001. Determination of primary and secondary responses to environmental stressors and biota health. In: Linkov I and Palma Olivera JM, editors, *Assessment and Management of Environmental Risk*. Kluwer, Dodrecht, pp 57-70.

Bresler V, Abelson A, Fishelson L, Feldstein T, Rosenfeld M, and Mokady O. 2003. Marine molluscs in environmental monitoring. I. Cellular and molecular responses. – Helgol Mar Res. Accepted: 15 April 2003. Published online before print: 26 June 2003.

Cabrera GL, Rodriguez DM, Maruri AB. 1999. Genotoxicity of the extracts from the compost of the organic and the total municipal garbage using three plant bioassays. – Mutat Res 426:201-206.

Celik A, Cavas T, and Ergene-Gozukara S. 2003. Cytogenetic biomonitoring in petrol station attendants: micronucleus test in exfoliated buccal cells. – *Mutagenesis* 18:417-421.

Celik A, Cavas T, and Ergene-Gözükara S. 2003. Cytogenetic biomonitoring in petrol station attendants: micronucleus test in exfoliated buccal cell. – *Mutagenesis*, 18:417-421.

Cavas T and Ergene-Gozukara S. 2003. Micronuclei, nuclear lesions and interphase silver-stained nucleolar organizer regions (AgNORs) as cyto-genotoxicity indicators in *Oreochromis niloticus* exposed to textile mill effluent. – *Mutat Res* 538:81-91.

Clark G, editor. 1981. Staining Procedures. Fourth edition. Williams and Wilkins, 512 pp.

Criswell KA, Krishna G, Zielinski D, Urda GA, Juneau P, Bulera S, and Bleavins MR. 2003. Validation of a flow cytometric acridine orange micronuclei methodology in rats. – *Mutat Res* 528:1-18.

Dailianis S, Pomouhtsidou GP, Raftopoulou E, Kaloyianni M, and Dmitriadis VK, 2003. Evaluation of neutral red retention assay, micronucleus test, acethylcholinesterase activity and signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. – *Mar Environ Res* 56:443-470.

Darzynkiewicz, Z. 1993. Mammalian cell-cycle analysis. - In: The Cell Cycle: A Practical Approach. Fantes, P. & Brooks, R., eds., Oxford University Press, Oxford. pp. 45-68.

Darzynkiewicz, Z. 1994. Simultaneous analysis of cellular RNA and DNA content. - Meth. Cell. Biol. *41*:401-424. De Flora S, Vigano L, D'Agostini F, Camoirano A et al. 1993. Multiple genotoxicity biomarkers in fish exposed *in situ* to polluted river water. – *Mutat Res* 319:167-177.

Dobrinski J. and Darzynkiewicz Z. 2001. Chromatin condensation and sensitivity of DNA in situ to denaturation during cell cycle and apoptosis – a congocal microscopy study. – Micron, 32:645-652.

Erenpreiss J., Hlevicka S, Zalkalnis A., and Erenpreisa J. 2002. Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. – *J Androl*., 22:717-723.

Fench M, Perpetskaya G, and Mikhalevich L. 1887. A more comprehensive application of the micronucleus technique for biomonitoring of genetic damage rates in human populations – experiences from the Chernobyl catastrophe. –Environ Mol Mutagen 30:112-118.

Galgani F, Bocquene G, and Cadiou Y. 1992. Evidence of variation in cholesterase activity in fish along a pollution gradient in the North Sea. – *Mar Ecol Progr Ser* 19:17-82.

Garaj-Vrhovac V, Fucuc A, Kubelka D, and Hebrang A. 1997. Assessment of genome damage in occupational exposure to ionizing radiation and ultrasound. – *Mutat Res* 395:101-105.

Gong P, Kuperman RG, and Sunahara GI. 2003. Genotoxicity of 2,4- and 2,6-dinitrotoluene as measured by the Tradescantia micronuclei (Trad-MCN) bioassay. – *Mutat Res* 538:13-18.

Grover IS, Kaur S. 1999. Genotoxicity of wastewater samples from sewage and industrial effluent detected by the *Allium* root anaphase aberration and micronucleus assays. – *Mutat Res* 436:183-188.

Haugland RP. 1999. Handbook of Fluorescent Probes and Research Chemicals. Seventh Edition. Molecular Probes, Eugene.

Hayashi M, Ueda T, Uyeno K, Wada K, Kinae N, Saotome K, Tanaka N, Takai A, Sasaki YF, Asano N, Sofuni T, Ojima Y. 1998. Development of genotoxicity assay systems that use aquatic organisms. – *Mutat Res* 399:125-133.

Hedle JA. 1973. A rapid *in vivo* test for chromosomal damage. – *Mutat Res* 18:307-317.

Herbert A and Hansen PD. 1998. Genotoxicity in fish embryos. In: *Microscale Testing in Aquatic Toxicology.* CRC Press. Boca Raton, pp. 491-505.

Jensen RH and Leary JF. 1990. Mutagenesis as measured by flow cytometry and cell sorting. In: Melamed MR, Lindmo T, and Mendelsohn ML, editors, *Flow Cytometry and Sorting.* Willey-Liss, New York, pp. 553-562.

Iavicoli S, Marinaccio A, Perniconi B, Palmi S, and Carvallo D. 2003. Study of the genotoxic effects of exposure to cosmic radiation in flight personel using cytogenetic and molecular techniques. – *Med Lav* 94:192-199.

Ilyinskikh NN, Ilyinskikh EN, and Ilyinskikh IN. 1998. Micronucleated erythrocytes frequency and radiocesium bioconcentration in pikes (*Esox lucius*) caught in the Tom river near nuclear facilities of the Sibirian Chemical Complex (Tomsk-7). – *Mutat Res* 42:197-203.

Isidori M, Ferrara M, Lavorgna M, Nardelli A, and Parrella A. 2003. In situ monitoring of urban air in Southern Italy with the *Tradescantia* micronucleus bioassay and semipermeable membrane device (SPMDs). – *Chemosphere* 52:121-126.

Karahalil B, Karakaya AE, Burgaz S. 1999. The micronucleus assay in exfoliated buccal cells: application to occupational exposure to polycyclic aromatic hydrocarbons. – *Mutat. Res* 442:29-35.

Kirsch-Volders M, Sofuni T, Aardema M et al. 2003. Report from the in vitro micronucleus assay working group. – *Mutat Res* 540:153-163.

Klobucar GI, Pavlica M, Erben R, and Papes D. 2003. Application of the micronucleus and comet assay to mussel *Dreissena polymorpha* haemocytes for genotoxicity monitoring of freshwater environment. – *Aquat Toxicol* 64:15-23.

Krauter PW. 1883. Micronucleus incidence and hematological effects in bullfrog tadpoles (Rana catesbeiana) exposed to 2-acetylaminofluorene and 2-aminofluorene – *Arch Environ Contam Toxicol* 24:487-493.

Maimulov VG, Kitaeva LV, Vereshchagina TV, Mikheeva EA, and Sholomova LF. 1998. Cytogenetic aberrations in somatic cells of children living in areas with various level of environmental pollution. – *Tsitologia* 40:686-689.

Marty J, Djomo JE, Bekaert C, Pfohol-Leszkowicz A. 1998. Relationships between formation of micronuclei and DNA-adducts and EROD activity in newts following exposure to benzo[a]pyrene. – *Environ Mol Mutagen* 32:397-405.

Meyers-Schöne L, Shugart LR, Beauchamp JJ, and Walton BT. 1993. Comparison of two freshwater turtle species as monitors of radionuclide and chemical contamination: DNA damage and residue analysis. – *Environ Toxicol Chem* 12:1487-1496.

Migliore L, Parrini M, Sbrana I, Biagini C, Battaglia A, and Loprieno N. 1991. Micronucleated lymphocytes in people occupationally exposed to potential environmental contaminants: the age effect. – *Mutat Res* 256:13-20.

Minissi S, Ciccatti E, and Rizzoni M. 1996. Micronucleus test in erythrocytes of *Barbus plebejus* (Teleostei, Pisces) from two natural environments: a bioassay for the in situ detection of mutagenesis in freshwater. – *Mutat Res* 367:245-251.

Monarca S, Rizzoni M, Gustavino B, Zani C, Alberti A, Feretti D, and Zerbini I. 2003. Genotoxicity of surface water treated with different disinfectants using *in situ* plant cells. –*Environ Mol Mutagen* 41:353-359.

Moore LE, Warner ML, Smith AH, Kalman D, and Smith MT. 1996. Use of the fluorescent micronucleiassay to detect the genotoxic effects of radiation and arsenic exposure in ex-foliated human epithelial cells. – *Environ Mol Mutagen* 27:176-184.

Nacci D, Coiro L, Kuhn A, Champlin D, Munn W, Specker J, and Cooper K. 1998. Nondestructive indicator of ethoxyresorufin-O-deethylase activity in embryonic fish. – *Environ Toxicol Chem* 17:2481-2486.

Nakamura J, Asakura S, Hester SD, de Murcia G, Caldecott KW, and Swenberg JA. 2003. Quantitation of intracellular NAD(P)H can monitor an imbalance of DNA single strand break repair in base excision repair deficient cells in real time. – *Nucleic Acid Res* 31:1-7.

Nishikava T, Haresaku M, Adachi K, Masuda M, Hayashi M. 1999. Study of a rat skin in vivo micronucleus test: data generated by mitomycin C and methyl methanesulfonate. – *Mutat Res* 444:159-166.

Nutley EV, Tcheong AC, Allen JW, Collins BW, Mo M, Lowe XR, Bishop JB, Moore DH, Wyrobeck AJ. 1996. Micronuclei induced in round spermatids of mice after stem-cell treatment with chloral hydrate: evaluation with centromeric DNA probes and kinetochore antibodies. – *Environ Mol Mutagen* 28:80-89.

Palus J, Rydzynski K., Dziubaltowska E, Wyszynska K, Natarajan AT, and Nilsson R. 2003. Genotoxic effects of occupational exposure to lead and cadmium. – *Mutat Res* 540:19-28.

Parton JW, Garriott ML. 1997. An evaluation of micronucleus induction in bone marrow and hepatocytes isolated from collagenase perfused liver of from formalin-fixed liver using four-week-old rat treated with known clastogens. – *Environ Mol Mutagen* 29:379-385.

Pavanello S, and Clonfero E. 2000. Biological indicators of genotoxic risk and metabolic polymorphism. – *Mutat Res* 463:285-308.

Robjohns S, Marshall R, Fellows M, and Kowalczyk G. 2003. *In vivo* genotoxicity studies with 3-monochloropropan-1,2-diol. – *Mutagenesis* 18:401-404.

Saito, H., Iwami, S. & Shigeoka, T., 1991. *In vitro* cytotoxicity of 45 pesticides to goldfish cells. - Chemosphere *23*:525-537.

Sanches-Galan S, Linde AR, and Garcia-Vazquez E. 1999. Brown trout and European minnow as target species for genotoxicity tests: differential sensitivity to heavy metals. – *Ecotoxicol Environ Saf* 43:301-304.

Sanchez-Pena LC, Reyes BE, Carrillo L., Recio R, Moran-Martinez J, Cebrian ME, and Quintanilla-Vega B. 2004. Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers. – *Toxicol Appl Pharmacol*., 196:108-113.

Schmid W. 1975. The micronucleus test. – *Mutat Res* 31:9-15.

Schröder HC, ChauvinC, Lauenroth S, Müller WEG, Elmendorff-Dreikorn K, Kutzner J, and Batel R. 1998. Application of Fast Micromethod for determination of DNA damage and repair in cell and tissue samples. – In: SchroderHC and Muller WEG, editors, *Development of Methods for Determination of Radiosensitivity*. Mathematisch-Naturwissenschaftliche Klasse Sitzungsberichte 10:15-24.

Shugart LR. 1988. Quantification of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. – *Aquat Toxicol* 13:42-52.

Smolenski P, Ruan Q, Vellon L, and Darzynliewicz Z. 2001. Micronucleus assay by laser scanning cytometry. – *Cytometry* 45:10-26.

Soares SR, Bueno-Guimaraes HM, Ferreira CM, Rivero DH, De Castro I, GarciaML, and Saldiva PH. 2003. Urban air pollution induces micronuclei in peripheral erythrocytes of mice in vivo. – *Environ Res* 92:191-196.

Steinkellner H, Mun-Sic K, Helma C, Ecker S et al. 1998. Genotoxic effects of heavy metals: comparative investigation with plant bioassays. – *Environ Mol Mutagen* 31:183-191.

Stoncius D and Lazutka JR. 2003. Spontaneous and benzo[a]pyrene-induced micronuclei in the embryos of the black-headed gull (*Larus ridibundus L*.). – *Mutat Res* 538:31-39.

Sturm A, Hansen PD, and Liess M. 1998. Potential use of cholinesterase in monitoring low levels of organophosphates in small streams: natural variability in three-spined stickleback (*Gasteroceus aculeatus*) and relation to pollution. – *Environ Toxicol Chem 14:5-9.*

Sugg DW, Chesser RK, Brooks JA, and Grasman BT. 1995. The association of DNA damage to concentration of mercury and radiocesium in large mouth bass. – *Environ Toxicol Chem* 14:661-668.

Taylor JL, Elgorashi EE, Maes A, Van Gorp U, De Kimpe N, Van Staden J, and Verschaeve L. 2003. Investigating the safety of plants used in South African traditional medicine: testing for genotoxicity in the micronucleus and alkaline comet assay. –*Environ Mol Mutagen* 42:144-154.

Torous DK, Dertinger SD, Hall NE, and Tometsko CR. 1998. An automated method for discriminating aneugen- vs clastogen-induced micronuclei. – *Environ Mol Mutgen* 31:340-344.

Torres-Bugarin O, Ventura-Aguilar A, Zamora-Rerez A, Gomez-Meda BC, Ramos-Ibarra ML, Morgan- Villela G, Gutierrez-Franco A, and Zuniga-Gonzalez G. 2003. Evaluation of cisplatin + 5-FU, carboplatin + 5-FU, and ifosfamide + epirubicine regiments using the micronucleus test and nuclear abnormalities in the buccal mucosa. – *Mutat Res* 539:177-186.

UNEP/RAMOGE. 1999. Manual on the biomarkers recommended for the MED POL biomonitoring programme, UNEP, Athens.

Van Noorden CJF and Frederiks WM. 1992. Enzyme Histochemistry: A Laboratory Manual Of Current Methods. Oxford University Press. Royal Microscopy Society. New York.

Watanabe N, Miura S, Zeki S, and Ishi H. 2001. Hepatocellular oxidative DNA injury produced by macrophage-derived nitric oxide. – *Free Radical Biol. Med*., 30:1019-1028.

Williams RC and Metcalfe CD.1992. Development of an *in vivo* hepatic micronucleus assay with rainbow trout. – *Aquat Toxicol* 23:193-202.

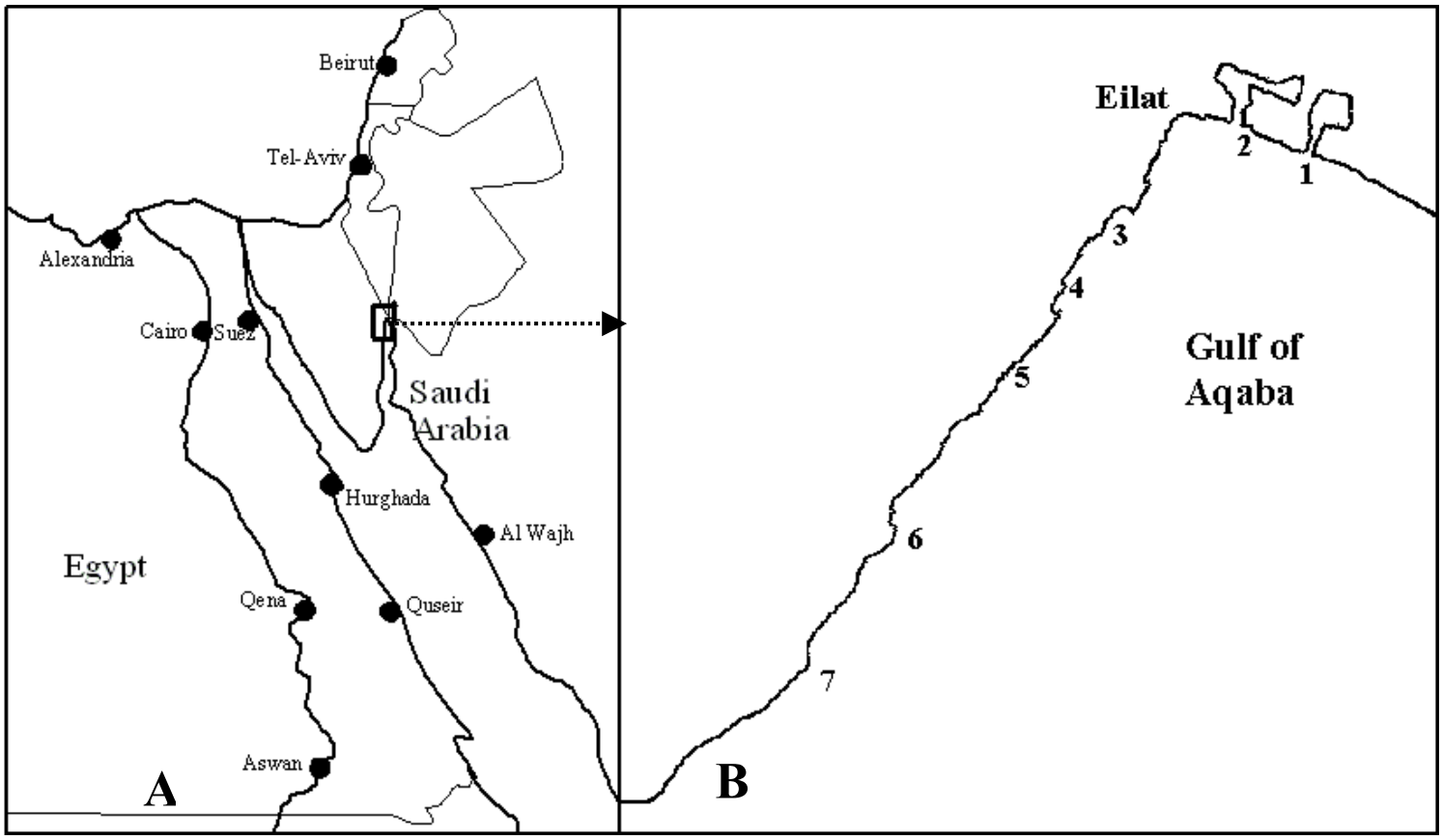

Fig. 1: The regional map (A; the Gulf of Aqaba, Red Sea) and the study sites along the Eilat coast (B). The sites are: 1) Peace Lagoon (PL); 2) Yachts Marina (MS); 3) Naval Base (NB); 4) Dekel Beach (DB); 5) Eilat's Port (P); 6) Tur-Yam (old marina; TY); 7) Lighthouse beach (LH).

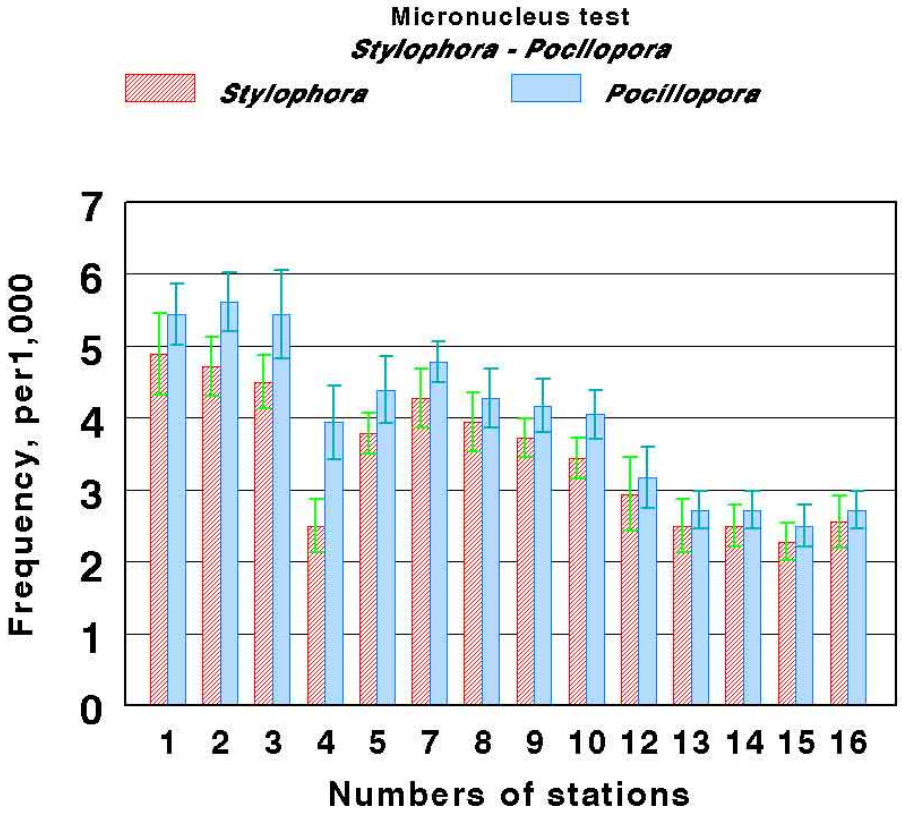

Fig. 2: Frequency (per 1000 cells) of micronuclei (MNT) in cells of two species of stony corals, *Pocillopora damicornis*, and *Stylophora pistillata*, sampled from 14 stations along the coast of Eilat. Peace Lagoon (PL), stations 1-3; Marina (MS), stations 4-5; Dekel Beach (DB), stations 7-9; Eilat's Port (P) station 10; Tur-Yam (old marina; TY), station 12; Lighthouse beach (LH), stations 13-16.

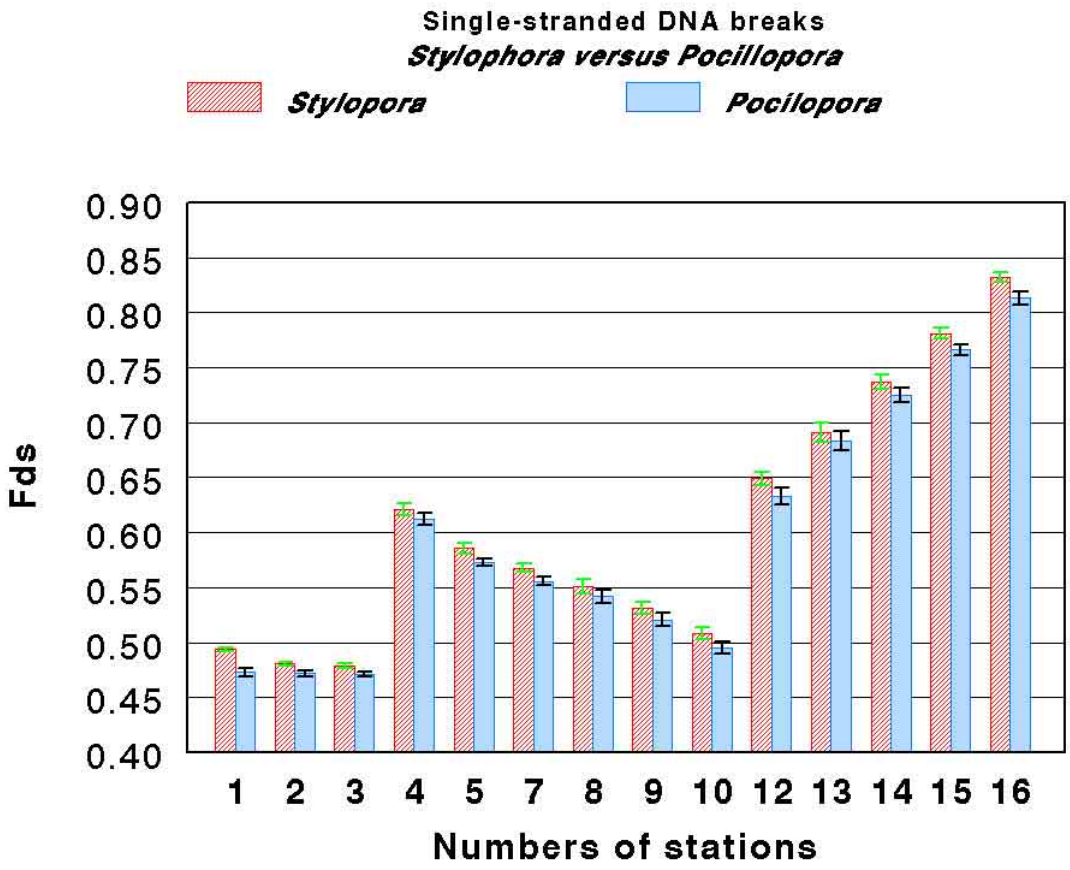

Fig. 3: Single-strand DNA breaks in cells of two species of stony corals, *Pocillopora damicornis*, and *Stylophora pistillata*, sampled from 14 stations along the coast of Eilat. Peace Lagoon (PL), stations 1-3; Marina (MS), stations 4-5; Dekel Beach (DB), stations 7-9; Eilat's Port (P) station 10; Tur-Yam (old marina; TY), station 12; Lighthouse beach (LH), stations 13-16.

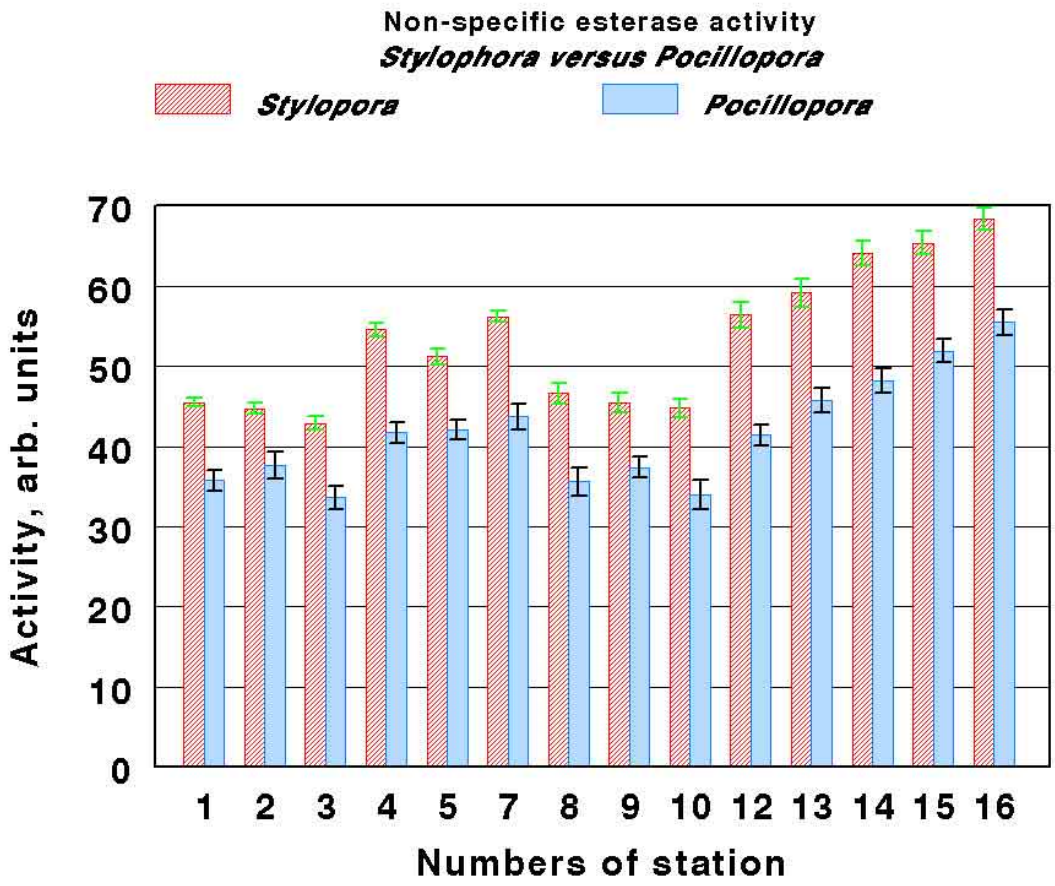

Fig. 4: Esterase Activity (EA) in cells of two species of stony corals, *Pocillopora damicornis*, and *Stylophora pistillata*, sampled from 14 stations along the coast of Eilat. Peace Lagoon (PL), stations 1-3; Marina (MS), stations 4-5; Dekel Beach (DB), stations 7-9; Eilat's Port (P) station 10; Tur-Yam (old marina; TY), station 12; Lighthouse beach (LH), stations 13-16.