Evaluation of two different biomarkers for use in the assessment of toxic chemical exposure in California sea lions (*Zalophus californianus*)

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Industrial pollution is a significant environmental problem that has the potential to cause serious health consequences in humans and wildlife. In 1998, over 248 million pounds of toxic chemicals were reportedly released into the surface water of rivers, bays and the oceans (US EPA Toxic Release Inventory (TRI), 1998). Examples of these toxicants, according to the TRI, include trichloroethylene, benzene, 1,3-butadiene, glycol ethers, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and mercury. Once toxicants enter the aquatic environment, they can accumulate in the benthic sediments, be distributed throughout the water column, be incorporated by organisms, and then bioaccumulate up the food chain. The benthic sediments often act as an almost limitless reservoir for environmental contaminants and continually change in response to both abiotic and biotic conditions (Malins and Ostrander, 1991). The sea-surface microlayer, the upper 50 μm of the water column, is an area of particular concern for some species, as some contaminant concentrations, such as aromatic hydrocarbons, are 10–1000 times higher in this layer than in the water column below (Hardy, 1982; Hardy et al., 1985).

Until recently, evidence of neoplasms in marine mammals has been scarce. There were only seven reports of neoplasms documented in pinnipeds (seals, sea lions, walruses) prior to the early 1970s (Mawdesley-Thomas, 1974). However, in the early 1980s, neoplastic lesions were found in 2.5% of 1500 marine mammals surveyed (Howard et al., 1983). In 1994, 40% of the small sub-population of beluga whales located in the St. Lawrence Estuary had confirmed tumors (De Guise et al., 1994). An environmental link was sought by comparing PCB levels and benzo[*a*]pyrene (B[*a*]P) adduct levels for St. Lawrence belugas with those of Arctic belugas. Levels of PCBs in St. Lawrence belugas were much higher than in Arctic belugas, and B[*a*]P adducts were detectable in liver and brain tissues from several St. Lawrence animals but not in tissues from Arctic belugas (Martineau et al., 1994). PCBs have been associated with immunosuppression in marine mammals, and B[*a*]P is known to be a potent carcinogen (Levin et al., 1982). De Guise et al. (1995) speculate that the high prevalence of tumors in St. Lawrence belugas could be explained by high levels of exposure to carcinogens in their environment and/or the related suppression of immunosurveillance against tumors. To date, the highest prevalence of neoplasms in marine mammals (18%) has been reported in adult California sea lions (*Zalophus californianus*) that stranded live along the central California coast (Gulland et al., 1996). A high prevalence of neoplasia in California sea lions has been epidemiologically associated with exposure to environmental contaminants (Ylitalo et al., 2005). However, the etiology and pathogenesis of these neoplasms in California sea lions are unknown. Certain types of chemical contaminants can directly induce carcinogenesis through DNA damage or by increasing cell proliferation (Faroon et al., 2001; Glauert et al., 2001; Ludewig, 2001).

Given the well-documented prevalence of neoplasms in California sea lions and their potential for exposure to environmental toxins, the goal of this pilot study was to start establishing California sea lions as a sentinel species by adapting genetic biomarker assays that are typically used with human lymphocytes for use with sea lion lymphocytes. Various sentinel species have been shown to elicit biological responses to environmental pollutants similar to responses expected in humans (LeBlanc and Bain, 1997). Humans living on or near polluted waters could receive high levels of exposure to the same toxic chemicals as sea lions are exposed to through ingestion of contaminated seafood, or by inhalation or absorption. Thus, sea lions may represent a relevant animal model for comparison to humans. Our interest was to compare responses of lymphocytes from California sea lions and humans to two different types of toxins. We selected benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) as a representative DNA adducting agent and hydrogen peroxide (H_2O_2) as an oxidizing agent. In recent years, various biomarkers have been used in wildlife populations to measure exposure and the potential effects of exposure to environmental contaminants (Gauthier et al., 1999). In our study, we used the comet assay to monitor DNA damage and the TUNEL assay to determine defects in apoptosis.

Blood samples were obtained from 25 California sea lions from 1998 to 2000 through 'The Marine Mammal

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Center' (TMMC) in Sausalito, California, which is an organization dedicated to the rescue, rehabilitation and release of stranded marine mammals. Thirteen of these animals were trauma animals that could not be rehabilitated due to severe injuries from fisheries interactions (i.e. net entanglement or fish hook injuries), gunshot wounds, or shark bites. The remaining 12 animals had metastatic carcinomas. The animals from both groups were euthanized and necropsied.

Human controls ($n = 6$) were selected and their lymphocytes were run in parallel with the California sea lion samples. All subjects signed a consent form, and to ensure the selection of healthy, unexposed subjects, information about possible exposures, use of tobacco, consumption of alcoholic beverages, general health, medications and recent exposures to X-rays was obtained.

Short-term lymphocyte cultures were established for each of the sea lion and human samples following standard methods (Hsu et al., 1989). Briefly, lymphocytes were washed twice with RPMI 1640 and were resuspended at a concentration of $1–3 \times 10^6$ cells/ml. Two cultures were prepared from each blood sample. Culture media consisted of RPMI 1640 with 25 mM HEPES, 4 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco-Invitogen). The medium was further supplemented with 20% fetal bovine serum (FBS, Hyclone) and 1% of the purified mitogen, phytohaemagglutinin (PHA-P, Murex, final concentration 1 μg/ml). Tubes were then incubated for 24 h at 37 °C. At 24 h after stimulation of cultures using the mitogen, cells were exposed in vitro to benzo(*a*)pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (+/−) (anti), (BPDE 2 mg; NCI# L0137), or hydrogen peroxide (H_2O_2; 30% w/v; Sigma) and reincubated for another 48 h.

BPDE stock solution was prepared according to a National Cancer Institute protocol, as published in the NCI online catalog (Midwest Research Institute, 2000). Briefly, the solvent used to dissolve the BPDE was anhydrous tetrahydrofuran, (THF; Aldrich). The THF was passed through a column of neutral alumina (Sigma) immediately prior to its use for dissolving the BPDE. A stock solution of 1 mM was prepared and aliquoted into 1.5 ml polypropylene tubes. Since BPDE is light, air, and temperature sensitive, the preparation was done very quickly in a darkened room. Argon was bubbled into the tubes before they were closed, to prevent oxidation. The tubes were wrapped in foil and stored at −80 °C until use. H_2O_2 was used as the positive control for DNA damage, and was prepared fresh, before the exposure, from a stock solution of 30% w/v H_2O_2 (Sigma) and diluted in RPMI 1640 to a concentration of 10 mM.

Initially, dose response experiments were performed to determine appropriate doses of BPDE and H_2O_2 for the experiments. Lymphocytes from two human subjects and two sea lions were cultured in duplicate using the standard conditions described above to test each toxin independently. The stock BPDE was removed from −80 °C storage and quickly added to the cultures 5 h before harvest, to achieve final concentrations of 0, 2, 4, 8 and 12 μM. For the H_2O_2 dose response cultures, 10 mM, was added to the cultures 40 min before harvest, to achieve final concentrations of 0, 10, 25, 50, 100 and 150 μM. After the chemicals were added, the cultures were returned to the incubator until harvest and fixation at 72 h.

The comet assay was performed using the Trevigen CometAssay™ kit, following the manufacturer's protocol. Briefly, after a 72-h culture of lymphocytes, a volume containing approximately 1×10^5 cells was used to prepare the Comet slide®. The slides were further processed following the manufacturer's recommendations. The slides were scored using the Komet© 4.0 imaging system (Kinetic Imaging, Ltd.). Fifty cells were scored for each dose per animal or subject. The mean tail extent moment (TEM) was used for statistical analysis since the TEM (which is a calculation of the tail length [extent] X% tail DNA/100) was reported to exhibit the greatest sensitivity of all parameters calculated by the Komet© software (Yusuf et al., 2000).

The TUNEL apoptosis assay was performed with the APO-BRDU™ kit following the manufacturer's recommendations. After the standard 72-h culture, the lymphocytes were harvested by centrifugation and washed twice with phosphate buffered saline (PBS). The supernatant was discarded and the cells were fixed by resuspension in 5 ml 1% paraformaldehyde for 20 min. The cells were then washed in PBS twice, resuspended in 5 ml ice-cold 70% ethanol, and stored at −20 °C until prepared for flow cytometry. Cells were then resuspended in 50 μl of the DNA labeling solution, consisting of TdT reaction buffer, TdT enzyme and BrdUTP, and incubated for 60 min at 37 °C. Cells were resuspended in 0.1 ml of the antibody solution for 30 min. Following the incubation, the cells were incubated for 30 min with propidium iodide/RNase A solution. Analysis was performed using a FACScan™. The outcome variable measured was the percentage of lymphocytes in apoptosis (defined as fluorescein-labeled cells) for each individual culture.

Marine mammals are likely to be metabolically similar to humans with regard to the biotransformation of xenobiotics, since homologous forms of cytochrome P450s 1A1, 2E and 2B have been isolated in Beluga, Minke and Pilot whales (Stegeman et al., 1995). In addition, some genetic sequences (in interleukin 2 and 6) are highly conserved between marine mammals and humans (King et al., 1995; Ness et al., 1995; Shoda et al., 1998). Marine mammals are also similar to humans in terms of their longevity, size, and position at the top of the food chain in their environment. In the present study, we compared the baseline and toxin-induced levels of DNA damage and apoptosis in lymphocytes from both California sea lions and humans. We chose appropriate doses for in vitro treatment with H_2O_2 and BPDE based on the responses of the human and sea lion lymphocytes in the comet assay. Initial dose-response results, as shown in Fig. 1 suggested that 8 μM BPDE would elicit the greatest response. However the 8 μM dose appeared to be slightly toxic to the lymphocytes, as

reflected by appreciable reduction of measurable comets on the slides. Therefore, the 4 μM BPDE dose was chosen as suitable for the species comparison study.

The results in the comet assay, as shown in Fig. 2, suggest a species difference in extent of DNA damage in response to H_2O_2. California sea lion lymphocytes showed little effect at 10 and 25 μM H_2O_2, and considerable damage at the 50 μM dose. The 100 μM dose began to have toxic effects on the lymphocytes (data not shown). Human lymphocytes, however, showed appreciable damage after exposure to both the 10 and 25 μM doses of H_2O_2. At 50 μM, one human sample had damage too extensive to measure; yet the other human sample responded best to this dose. Therefore, a dose of 60 μM H_2O_2 was selected. This dose was chosen based on the observation that reasonable responses in the comet assay could be elicited at this dose in both human and sea lion lymphocytes.

Fig. 3 shows the species comparisons for responses after treatment with 4 μM BPDE and 60 μM H_2O_2 for the apoptosis endpoints. Background rates of apoptosis were consistently more than 8-fold higher in the lymphocytes from sea lions compared to humans. The type of health problem in the sea lions was not an appreciable factor in the high background rate of apoptosis since the averages were 53.7% for the trauma animals and 64.7% for the cancer animals. Neither treatment with H_2O_2 nor BPDE resulted in an appreciable increase in apoptosis in the sea lions above the high background when all the sea lions were considered or when the subgroups of trauma and cancer sea lions were examined separately (mean of 55.3% and 68.9% versus 55.8% and 69.4% for H_2O_2 and BPDE in trauma and cancer sea lions, respectively).

Our observations, as shown in Fig. 3, of an average apoptosis background rate of $6.64 \pm 4.78\%$ in humans is similar to the baseline of $3.8 \pm 0.8\%$ that Fowke et al. (2000) reported in lymphocytes from healthy humans, using bis-benzamide nuclear staining followed by scoring of nuclear morphology. We could not find anything in the literature about rates of background cell death in lymphocytes from sea lions. Of the numerous toxins and environmental agents that California sea lions are exposed to, many are known to induce apoptosis. For example, the environmental agents DDT (Tebourbi et al., 1998), PCBs (Shin et al., 2000), PAHs (Salas and Burchiel, 1998), methyl mercury and cadmium (Shenker et al., 2000; Waalkes et al., 2000), and the biological toxin okadaic acid (Leira et al., 2001) are all well characterized as inducers of apoptosis in mammalian cells. In addition to xenobiotic induction of apoptosis, many physical factors could be involved as well. Another possible explanation for the high level of cell death in the lymphocytes from the sea lions in our study is in vivo induction of damage to the lymphocytes by trauma to, or stress on the animals. A study of humans who sustained acute traumatic injuries, due to vehicle collision or gunshot wounds, revealed a rapid increase in intestinal epithelial and tissue lymphocyte apoptotic cell death when compared with controls undergoing elective bowel resections (Hotchkiss

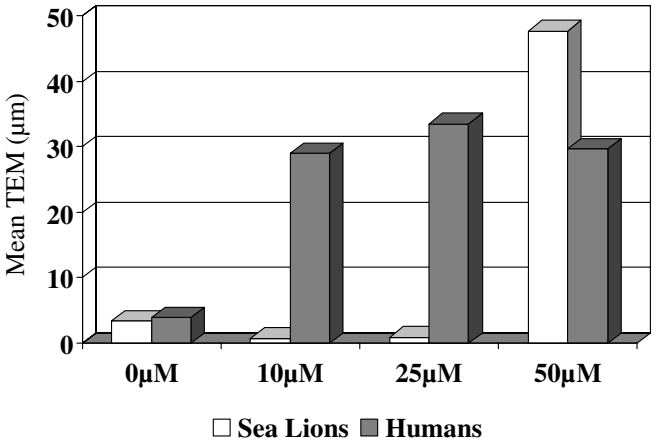

Fig. 2. Dose response to in vitro treatment with H_2O_2 in lymphocyte samples from two California sea lions and two humans. Data expressed in mean TEM as measured by the comet assay.

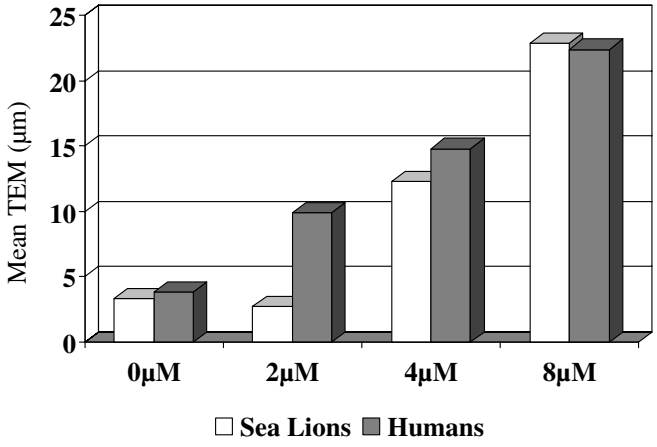

Fig. 1. Dose response to in vitro treatment with BPDE in lymphocyte samples from two California sea lions and two humans. Data expressed in mean TEM as measured by the comet assay.

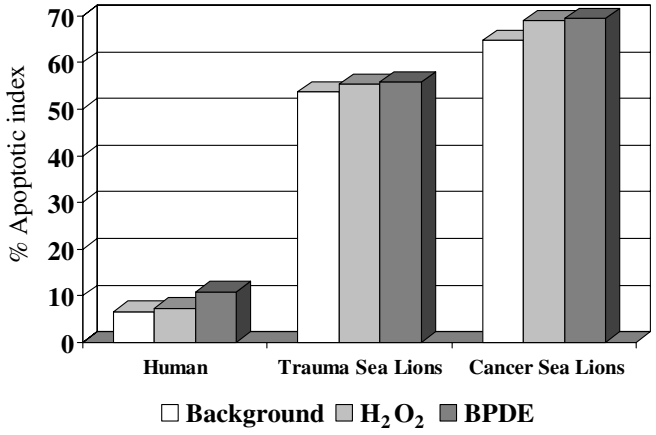

Fig. 3. Background apoptosis index versus treatment effects from in vitro exposure to 60 μM H_2O_2, or 4 μM BPDE of human lymphocytes ($n = 6$) and sea lion lymphocytes in the trauma ($n = 13$) and cancer ($n = 12$) subgroups.

et al., 2000). This increase was transient, and repeat intestinal samples from two patients revealed no apoptosis at time of the follow-up surgery. In addition, physical and psychological stress has long been associated with immune system modulation, whether by enhancement or suppression (Yin et al., 2000; Sainz et al., 2001). A physical stress study, in which rats were run to exhaustion on a treadmill, showed an induction of apoptosis in rat thymoctes (Concordet and Ferry, 1993). Another study, which involved the physical restraint of mice for 12-h time periods over 2 days, revealed an increase in apoptosis of splenic lymphocytes (Yin et al., 2000). Both groups of California sea lions in our study experienced physical and psychological stress while being handled by humans and hauled from the beaches to TMMC, and then during subsequent medical testing and captivity. In addition, we cannot rule out a species difference in response to the media required to maintain viable lymphocytes. The high baseline apoptosis in our sea lion population makes it very difficult to define an association with any disease or with environmental factors.

Fig. 4 shows the species comparison for DNA damage after exposure to BPDE and H_2O_2 using the comet assay for detecting DNA damage. Treatment with either BPDE or H_2O_2 resulted in statistically significant increases in DNA damage for both species. The results for the California sea lion trauma ($n = 7$) and cancer ($n = 8$) subgroups indicate that the type of health problem was not an appreciable factor for background rates of DNA damage or the treatment-associated increase in DNA damage (mean TEM of 21.48 ± 14.85 and 12.12 ± 5.65 versus 26.74 ± 14.48 and 15.51 ± 4.29 for H_2O_2 and BPDE in trauma and cancer sea lions, respectively).

Interestingly, each treatment caused similar degrees of DNA damage in the sea lions compared to the humans. The Comet Assay™ TEM is a measure of the amount of DNA damage from single- or double-strand breaks, thus our observations suggest that lymphocytes from humans and from this population of sea lions have similar background levels of DNA damage. Although the baseline TEM for the cancer sea lion group is nearly two times the TEM for the trauma group, the difference is not significant. The mean TEM for the trauma group of sea lions was very similar to the mean TEM for humans. In the future, a larger sampling of lymphocytes from healthy humans could support this similarity seen with the trauma animals, lending further strength to the use of California sea lion as a relevant sentinel species for monitoring the effects of pollution in the marine environment.

Some of the known environmental toxicants that California sea lions encounter have been reported to increase DNA strand breaks, as measured by the comet assay and expressed as an increase in TEM (Kannan et al., 2004; Kajiwara et al., 2001). For example, an increase of DNA damage by PAH exposures in lymphocytes is well documented using the comet assay (Venkatachalam et al., 1995; Wei et al., 1996; Wu et al., 1999; Yusuf et al., 2000). The only reports which we could find that evaluated

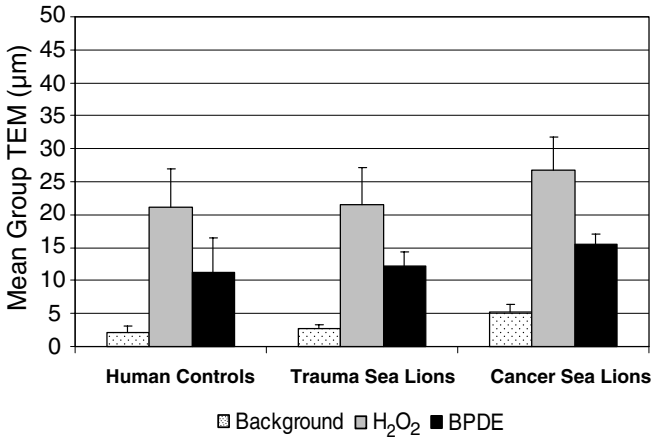

Fig. 4. Background Comet Assay™ TEM (μm) versus TEM (μm) for lymphocytes exposed in vitro to 60 μM H_2O_2 or 4 μM BPDE from human controls ($n = 3$), California sea lion trauma ($n = 7$) and cancer ($n = 8$) groups. Data represent means for each group.

toxin effects on marine mammal lymphocyte using the comet assay were by Betti and Nigro (1996) and Taddei et al. (2001). These investigators detected a dose dependent increase in DNA strand breaks when lymphocytes from bottlenose dolphins were exposed to methyl mercury. Therefore, our observations with in vitro exposures to representative adduct-forming and oxidizing agents indicate that comet assay may be a sensitive tool for monitoring trends in DNA damage among sea lions; however, this measurement of DNA damage is non-specific and must be accompanied by a more specific determination of exposure in order to associate the effect with a causative agent.

In summary, the use of biomarkers with marine mammal populations needs further evaluation. Many of the previous studies of marine mammals simply measure metabolizing enzyme levels or contaminant burdens, without looking at the effects of those contaminants on the animals. Marine mammals may be less vulnerable to, or have better repair mechanisms for damage caused from exposures. This capacity for repair can be studied as Taddei et al. (2001) have done using the comet assay to measure DNA damage in lymphocytes of bottlenose dolphin after exposure in vitro to methyl mercury, followed by an evaluation of the efficiency of DNA repair. Their study indicated that the dolphin lymphocytes have a higher efficiency for repair of methyl mercury damage when compared to human lymphocytes. Observations from our current study indicate that the comet assay appears to be a useful biomarker of effect for California sea lions.

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The effects of metal distribution and anthropogenic effluents on the benthic environment of Gwangyang Bay, Korea

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The sediment characteristics of coastal and bay regions are influenced by local geology and anthropogenic effluents from neighboring industrial and urban areas. Such sediments are likely to have higher concentrations of organic materials and metals (Förstner and Wittman, 1981; Salomons and Förstner, 1984). It is difficult, however, to determine the portions of total metal concentrations attributable to anthropogenic factors because of post-depositional remobilization of the metals (Spencer et al., 2003). One approach that may successfully distinguish among contamination inputs is the extensive and multidisciplinary examination of surface sediments. High heavy metal concentrations and their distribution in terms of pollution and environmental changes have been reported for many areas (Soto-Jimenes and Paez-Osuna, 2001; Soto-Jimenes et al., 2003; Spencer et al., 2003).

Gwangyang Bay, located at the extreme southern coast of Korea, is characterized by its semi-enclosed geographic surroundings and it borders one of the most concentrated industrial areas of Korea. The cities of Gwangyang and Sunchun are nearby, a representative industrial complex (steel manufacturing factory; SMF) and a container terminal are located in the northern part of the study area. The Yulchon Industrial Complex (YIC) and the Yeocheon Petrochemical Complex (YPC) are situated in the southern part of the study area (Fig. 1).

Numerous investigations have been conducted in the study area to examine environmental changes to the sedimentary environment and benthic community related to heavy metal concentrations (Cho et al., 1999; Choi et al., 2003; Hyun et al., 2003). However, synthesized studies of metal concentrations and anthropogenic effluents associated with benthic environmental changes are not enough. We therefore investigated metal concentrations and anthropogenic effluents of the surface sediments in Gwangyang Bay and conducted multidisciplinary analysis to examine the association between the metal pollution and the concentrations and its distributions.

Surface sediment samples were collected in Gwangyang Bay using a Van Veen grab sampler from 2001 to 2003 (Fig. 1). We analyzed the metal concentrations of 109 surface sediments to evaluate the anthropogenic effluents affecting the sediments using inductively coupled plasma (ICP) at the Korea Basic Science Institute (KBSI). Also, to examine more accurate associations among grain size variation, carbonate content, organic carbon content and hydrogen sulfide contents, total sediments were measured by the Sedigraph 5100, by a CHNS analyzer (EA 1112) and a H_2S gas detector, respectively. Duplicate analysis showed that the analytical errors were less than 5% for each element. Principal Component Analysis (PCA) of metal concentrations was conducted using the SIMCA-S program (Ver. 6.01 for PC, UMETRI AB).

The grain sizes of surface sediments were extremely variable from site to site. Coarse sandy sediments were distributed east of SMF and in the channel between Namhe-do and the YPC (Yeosu Bando), whereas mud-dominated sediments were found in most other sites. Based on grain-size parameters, the study area could be divided into four sedimentary facies: sandy mud (sM), mud (M), muddy sand (mS) and sand (S), as shown in Fig. 2a. The M and sM facies were the dominant types, occupying most of the study area. The inner part of Gwangyang Bay in particular was characterized by the M type sediment distribution (Hyun et al., 2003; Cho et al., 1999).

Total organic carbon (TOC) levels in the surface sediments were highly variable, ranging from 0.2 to 2.1%. Higher TOC levels were observed in fine sediments, whereas lower TOC levels were observed in coarse sediments (Fig. 2b). This grain-size dependent variation in organic carbon concentrations coincides well with previous studies

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