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Effects of methyl-, phenyl-, ethylmercury and mercurychlorid on immune cells of harbor seals (*Phoca vitulina*)

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Abstract

Mercury (Hg) is present in the marine environment as a natural metal often enhanced through human activities. Depending on its chemical form, Hg can cause a wide range of immunotoxic effects. In this study, the influence of methyl-, ethyl- and phenylmercury as well as mercurychloride on immune functions was evaluated. Two parameters of cellular immunity, proliferation and mRNA cytokine expression of interleukin-2, -4, and transforming growth factor β, were investigated in harbor seal lymphocytes after *in vitro* exposure to Hg compounds. While all Hg compounds had a suppressive effect on proliferation, differences between juvenile and adult seals were found. Lymphocytes from juveniles showed a higher susceptibility to the toxic effect compared to lymphocytes from adults. Furthermore, the degree of inhibition of proliferation varied among the four Hg compounds. The organic compounds seem to be more immunotoxic than the inorganic compound. Finally, for the cytokine expression of methylmercury-incubated lymphocytes, time-dependent changes were observed, but no dose-dependency was found. Marine mammals of the North Sea are burdened with Hg, and lymphocytes of harbor seals may be functionally impaired by this metal. The present *in vitro* study provides baseline information for future studies on the immunotoxic effects of Hg on cellular immunity of harbor seals.

Key words: harbor seal; mercury; lymphocyte proliferation; cytokine expression; North Sea
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Introduction

In the past the North Sea ecosystem was polluted with mercury (Hg), but current studies revealed a trend of reduced input of Hg into the ecosystem (Schmolke *et al*., 2005; Wängberg *et al*., 2007). However, for the German Bight the concentrations of selected metals, including Hg, in water and sediment are still elevated compared to the "Background Reference Concentrations" which the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) derived for the "Greater North Sea" (Schmolke *et al*., 2005). Furthermore, Hg contamination remains a problem for marine organisms due to bioaccumulation in marine biota and biomagnification through the food web. In the Southern and Eastern North Sea, several recent studies measured Hg concentrations in various species including mussel and fish (Baeyens *et al*., 2003; Bakker *et al*., 2005; Schmolke *et al*., 2005; Kakuschke and Prange, 2007).

As top predators marine mammals take up Hg by consumption of contaminated fish. In the Southern and Eastern North Sea, for example, high concentrations of Hg were found in livers of harbor porpoises *Phocoena phocoena* (Siebert *et al*., 1999; Das *et al*., 2004; Strand *et al*., 2005; Lahaye *et al*., 2007) and harbor seals *Phoca vitulina* (Reijnders, 1980; Skaare *et al*., 1990; Wenzel *et al*., 1993), the main species of mammals in the Wadden Sea as well. A recent study on seals of the Wadden Sea describes Hg concentrations in blood samples between 0.043 and 0.611 μg/mL (Das *et al*., 2008). It is well known that Hg can cause a wide range of adverse health effects in mammalian species (Rawson *et al*., 1993; Siebert *et al*., 1999; Bennett *et al*., 2001).

Various chemical forms of Hg can react differently. Organic Hg seems to be more toxic than inorganic mercury ($HgCl_2$) for animals of higher trophic level (Ullrich *et al*., 2001). Most studies have focused on methyl (Me)-Hg, while organic compounds like ethyl (Et)- and phenyl (Ph)-Hg have been largely ignored. All three forms are produced as industrial compounds, primarily as biocides (Goldmann and Shannon, 2001). Some studies showed the presence of Et- and Ph-Hg in the environment (Hintelmann *et al*., 1995; Cai *et al*., 1997). An input into the marine ecosystem was shown on the Danish west coast near a chemical factory producing fungicides; this caused higher Me- and Ph-Hg concentrations in mussels (Riisgard *et al*., 1985).

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The environmental Hg exposure results in concentrations bioavailable for immune cells and high enough to affect their function. An imbalance of the immune system caused by pollutants may play a significant role in the incidence of infectious diseases in marine mammals. Therefore, studies on grey seals (*Halichoerus grypus*), beluga whales (*Delphinapterus leucas*), and bottlenose dolphins (*Tursiops truncatus*) using *in vitro* tests to investigate the influence of Me-Hg on immune cells have been performed (Betti and Nigro, 1996; De Guise *et al*., 1996; Lalancette *et al*., 2003; Kakuschke *et al*., 2006). However, limited information is available for harbor seals. In our own studies on harbor seals considering metal-specific hypersensitivities, several metals including different mercury compounds were tested (Kakuschke *et al*., 2005, 2008a, 2008b).

In recent years, the effects of different Me-Hg concentrations on lymphocyte proliferation and protein synthesis in harbor seal peripheral blood mononuclear cells (PBMCs), as well as cytokine mRNA expression after a long-term Me-Hg exposure of 72 h were evaluated (Das *et al*., 2008). The present study provides further information on these research topics by evaluating the effects of different Hg compounds on lymphocyte proliferation as well as a dose-dependent cytokine mRNA expression after short-term Me-Hg exposure of 6, 24, and 48 h.

1 Materials and methods

1.1 Lymphocyte transformation tests

Metal-specific lymphocyte transformation tests (LTTs) according to the MELISA® (memory lymphocyte immuno-stimulation assay) modification were performed previously for several groups of harbor seals of the North Sea (Kakuschke *et al*., 2005, 2008a, 2008b). For this study, the mercury-specific results of these animals were considered in respect to the different mercury compounds including Me-Hg, Ph-Hg, Et-Hg and $HgCl_2$. Data from 21 harbor seals caught along the Wadden Sea or from animals of the Seal Center Friedrichskoog, Germany, were used. The animals were grouped into juveniles (n = 9, between 1–4 month old) and adults (n = 12, older than one year).

Blood was collected into monovettes with CPDA-solution (Citrate-Phosphate-Dextrose-Adenin) (Sarstedt AG & Co., Nümbrecht, Germany) and stored at room temperature until further processed (not longer than 15 h). The blood was diluted 1:1 with phosphate buffered saline (PBS). Lymphocytes were separated on a Ficoll-Histopaque gradient (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and washed twice in PBS. Cells were resuspended in 20% Medium (RPMI-1640 containing Hepes (Life Technologies GmbH, Karlsruhe, Germany) supplemented with 6.25 mmol/L L-glutamine (Biochrom AG Seromed, Berlin, Germany), 8 mg/L Gentamycin (Sigma-Aldrich Chemie GmbH) and 20% fetal calf serum) and incubated in tissue culture flasks for 30–45 min at 37°C in 5% CO_2 for depletion of monocytes. The lymphocytes were counted and resuspended in 10% Medium to a concentration of 1×10^6 lymphocytes/mL. One milliliter of cell suspension was pipetted into the wells of a 24-well plate (CM-Lab, Vordingborg, Denmark). The cells were cultured for 5 d at 37°C in 5% CO_2 with or without Et-Hg 2-$(C_2H_5HgS)C_6H_4CO_2Na$, Me-Hg ((CH_3)-HgCl), Ph-Hg ($CH_3COOHg(C_6H_5)$) and $HgCl_2$ at concentration of 0.25 and 0.5 μg/mL. The number of cell culture experiments to each Hg compound varied due to different blood sample quantities and numbers of isolated cells. After incubation 600 μL of each cell suspensions were transferred to a new plate and incubated for a further 4 hours with 3°C radioactive marked methyl-^{3}H-thymidine (Amersham Buchler GmbH & Co., KG, Braunschweig, Germany). The cells were then harvested onto filters and the radioactivity measured in a scintillation counter (1450 Microbeta Trilux Wallac Distribution GmbH, Freiburg, Germany).

Incorporation of methyl-^{3}H-thymidine (counts per minute) in the Hg-incubated wells compared to non-Hg-incubated control cultures defined the stimulation index (SI):

$$SI = \frac{\text{Proliferation of Hg – incubated cells}}{\text{Proliferation of non – Hg – incubated cells}}$$

where, SI = 1 reflected no influence of Hg compounds on lymphocyte proliferation compared to non-Hg-incubated (control) cells. SI < 0.1 was regarded as a strong immunosuppressive or cytotoxic influence. SI > 2 was interpreted as a metal-specific hypersensitivity reaction as described for seals (Kakuschke *et al*., 2005, 2006, 2008a, 2008b). Animals showing a Hg-specific hypersensitivity with a SI > 2 were not included in this study.

The Mann-Whitney U Test was used for investigating age and Hg compound correlated differences. Statistical significance was designated as $P \leqslant 0.05$.

1.2 Measurement of cytokine expression using RT-PCR

Primers for the detection of cytokines interleukin (IL)-2, IL-4, and transforming growth factor β (TGF β) were published sequences (Fonfara *et al*., 2008; NCBI GenBank database www.ncbi.nlm.nih.gov) and are shown in Table 1.

In this preliminary study, the cytokine expression of only Me-Hg-incubated lymphocytes from two adult seals (seal I and II) of the Seal Center Friedrichskoog was analyzed. Lymphocytes were separated on a Ficoll-Histopaque gradient and incubated in 96-well plates at 37°C. Each well contained 200000 cells in 100 μL MEME Eagle's Minimum Essential Medium with 10% fetal calf serum. Cells were incubated with 0 μg/mL, 0.05 μg/mL (0.2 μmol/L), 0.1 μg/mL (0.4 μmol/L), 0.5 μg/mL (2 μmol/L) Me-Hg. The total RNA isolation was performed after 6, 24, and 48 h using the Ambion Blood Kit (Ambion Europe, Huntingdon, UK) according to the manufacturers' protocol. RNA was reverse transcribed with murine reverse transcriptase (RT-PCR Core Kit, Applied Biosystems, Weiterstadt, Germany), and the resulting cDNA served as a template for real-time PCR using the Thermocycler MX4000™ (Stratagene Europe, Amsterdam, the Netherlands). The RT-PCR started with an initial step at 95°C for 10 min,

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Table 1 Primer sequences and annealing temperatures for cytokine transcripts for the RT-PCR

	Primer sequences (5′-3′)	Annealing temperature (°C)	Accession numbers (NCBI) and references
IL-2 s	CTTGCATCGCACTGACTCTT	55	AY919322 (Fonfara *et al*., 2008)
IL-2 as	GCTCCAACTGTTGCTGTGTT		
IL-4 s	CCTCCCAACTGATTCCAACT	50	AY919338 (Fonfara *et al*., 2008)
IL-4 as	GACAAAGGTGCTGGTGAGTG		
TGF β s	TTCCTGCTCCTCATGGCCAC	58	AY919328 (Fonfara *et al*., 2008)
TGF β as	GCAGGAGCGCACGATCATGT		

IL: interleukin; TGF β: transforming growth factor β; s: sense; as: antisense.

followed by 40 cycles with denaturation at 95°C for 1 min, annealing temperature for 30 s and elongation at 72°C for 1 min. Annealing temperatures are shown in Table 1. All reactions were performed in duplicates.

2 Results

2.1 Suppressive effect of Hg compounds on lymphocyte proliferation

A higher immunotoxic influence on lymphocytes of the juvenile seals compared to adults was determined. Significant differences were found for Et-Hg (0.5 μg/mL) incubated cells (Mann-Whitney U Test; $N_{juvenils} = 7$; $N_{adults} = 10$; $U = 55$; $P = 0.05$). Furthermore, in juveniles, both concentrations of Me-Hg and the higher concentration of Et-Hg (0.5 μg/mL) reduced the cell proliferation more than 10-fold (SI median < 0.1) compared to control cells (SI = 1) (Figs. 1a and 1c). For adults, none of the four Hg compounds significantly suppressed lymphocyte proliferation to SI median < 0.1 (Figs. 1b and 1d).

The degree of Hg-induced inhibition of lymphocyte proliferation varied among the Hg compounds (Fig. 1). For the concentration 0.50 μg/mL, the three organic Hg-compounds were more immunotoxic than the inorganic compound. For juveniles, the measured lymphocyte proliferation was significantly different between $HgCl_2$- and Et-Hg-incubation ($N_{HgCl_2} = 9$; $N_{Et\text{-}Hg} = 7$; $U = 52$; $P = 0.03$), as well as between $HgCl_2$- and Me-Hg-incubation ($N_{HgCl_2} = 9$; $N_{Me\text{-}Hg} = 9$; $U = 67$; $P = 0.02$), but not between Et-Hg and Me-Hg incubated cells. For the LTTs with cells from adult seals, as well as with lower concentrations of Hg compounds, no significant difference was observed.

As only two concentrations were tested in the LTT, dose-dependency was not considered in detail in this study.

Thus, this study showed that all Hg compounds tested had a suppressive effect on lymphocyte proliferation compared to control cells (all SI median < 1), but the extent of

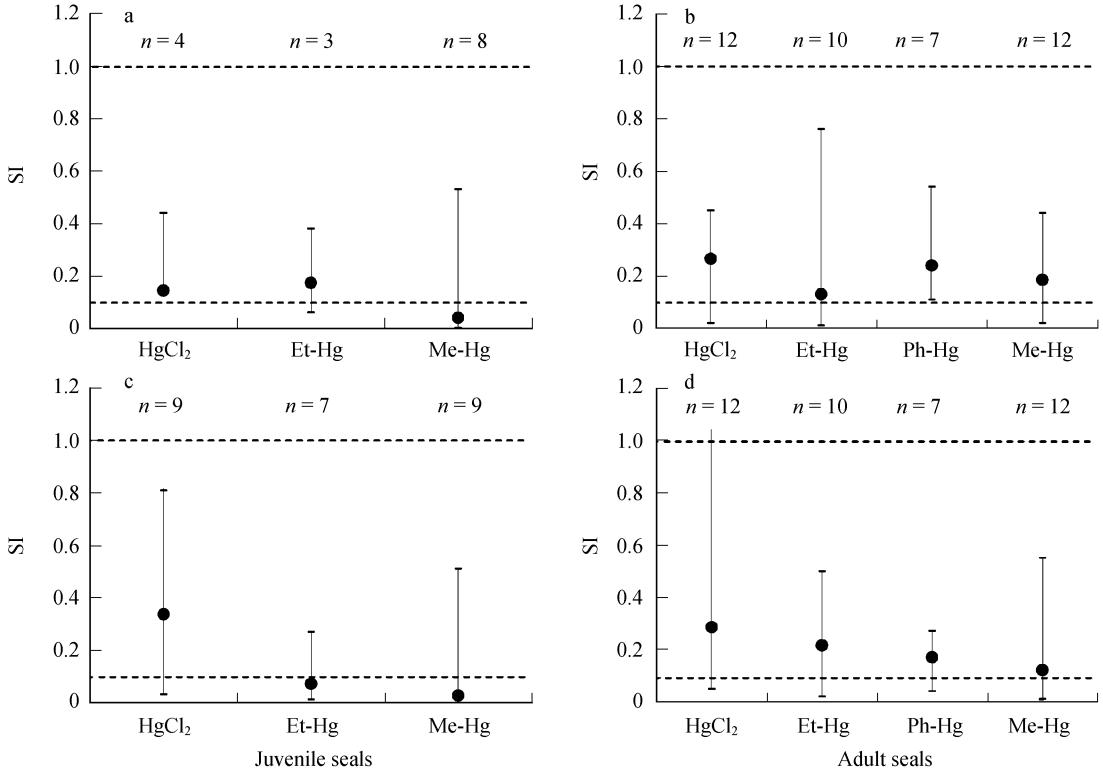

Fig. 1 Lymphocyte proliferation of juvenile (a, c) and adult (b, d) harbor seals after incubation with different Hg compounds at concentrations of 0.25 (a, b) and 0.50 (c, d) μg/mL (stimulation index (SI), medians, min-, max-values, *n* = number of animals). Dotted lines: SI = 1, i.e., the proliferation of Hg-incubated cells was comparable to non-Hg-incubated cells, SI = 0.1, i.e., the proliferation of Hg-incubated cells was 10-fold lower than the proliferation of non-Hg-incubated cells.

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the effect varied between juveniles and adults, and among the Hg compounds.

2.2 Influences of Me-Hg on lymphocyte cytokine mRNA expression

The IL-2, IL-4, and TGF β expression of Me-Hg incubated lymphocytes was investigated after 6, 24, and 48 h. After 6 hours, the expression of IL-2 and IL-4 increased in lymphocytes incubated with Me-Hg in all concentrations tested compared to the non-Hg-incubated control cells. In most cell cultures, the expression of both cytokines decreased after 24 and 48 h (Fig. 2).

In contrast, the expression of TGF β showed no increase after 6 h in all experiments with different Me-Hg concentrations. The expression was significantly decreased after 24 and 48 h in most cell cultures. The TGF β expression appeared to be more strongly influenced by duration of Hg-incubation than IL-2 and IL-4.

Thus, in this study no significant difference between the cytokine mRNA expressions after incubation with various Me-Hg concentrations was found. But time-dependent changes were observed, which were variable between the cytokines investigated.

3 Discussion

The present study on harbor seals of the North Sea showed that both parameters of the cellular immune system – lymphocyte proliferation and cytokine expression – were influenced by Hg *in vitro*. Differences were found depending on animal age, chemical form of Hg, and duration of exposure.

A higher immunotoxic influence on lymphocytes of juvenile seals compared to adults was found. Silva *et al*. (2005) investigated the influence of $HgCl_2$ on cells of the immune system (cells from lymph nodes, spleen, and

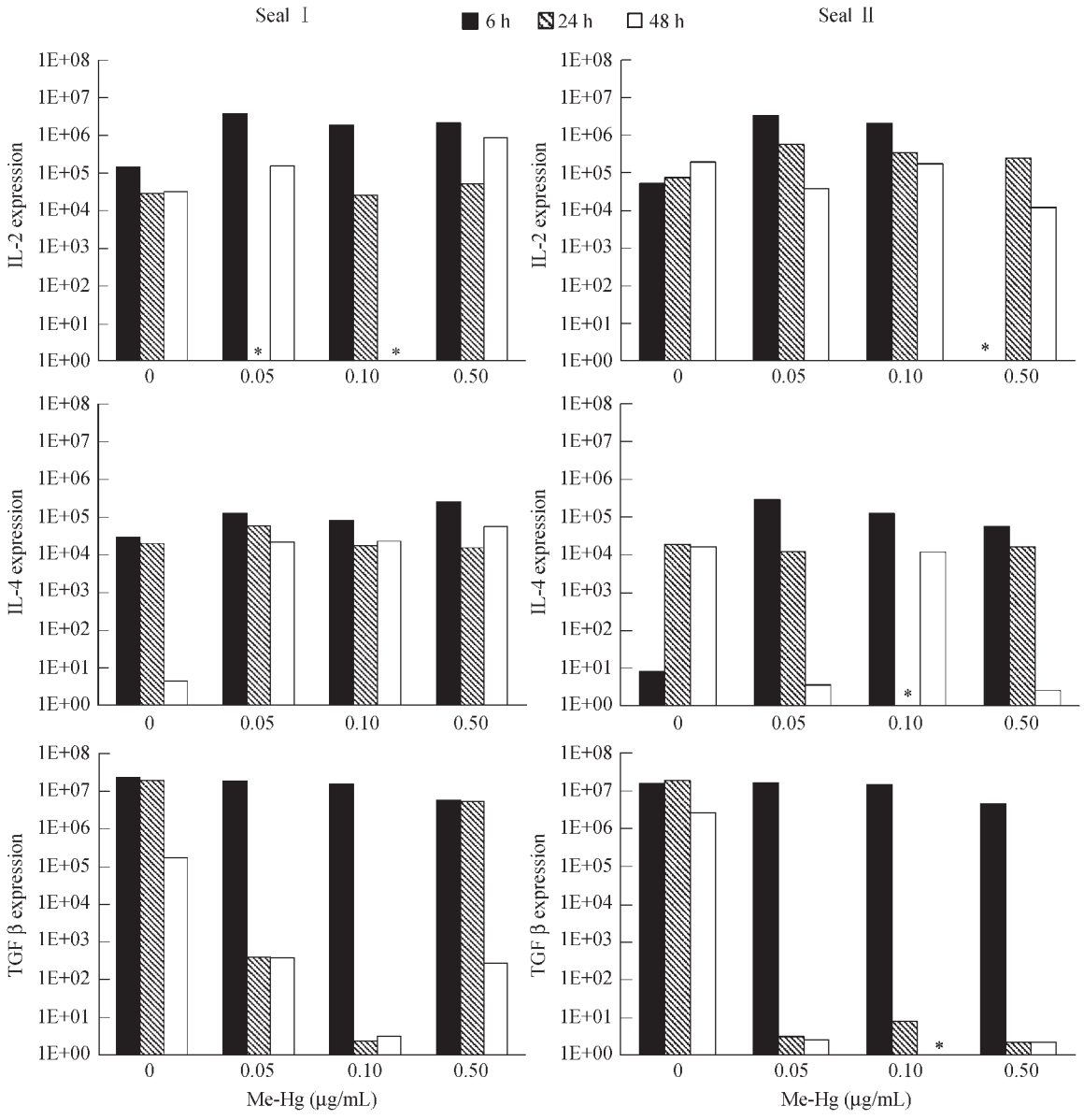

Fig. 2 Lymphocyte cytokine expression of IL-2, IL-4 and TGF β after incubation with different concentrations of Me-Hg measured after 6, 24, and 48 h. * Data not available.

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thymus) of 7-, 10-day old and adult mice. $HgCl_2$ decreased proliferative responses, with significant effects observed in lymph nodes cells from 7-day-old pups, and in thymus cells from 7 and 10-day old pups. In a study on harbor seal pups of varying ages (7–10, 50–53, and 73–76 day old seals) we analyzed metal effects on lymphocyte proliferation (Kakuschke *et al*., 2008a). For seals tested repeatedly, the suppressive effect was detected in newborns but was not found in the same animals when they were older and immunological competent. These studies confirmed the hypothesis that inherent properties of immune cells at different stages of development may influence the response to immunotoxins.

Furthermore, the chemical structure of Hg is important for its immunotoxic property. In the present study, the organic Hg-compounds were more immunotoxic than the inorganic compound. Similar results were found in a cell culture study with beluga whale splenocytes, where cells exposed to Me-Hg showed a 10-fold lower proliferation than cells exposed to $HgCl_2$ (De Guise *et al*., 1996).. Equally, in *in vitro* cultured beluga skin fibroblasts, the methylated form of Hg was one order of magnitude more potent in inducing micronuclei and inhibiting cell proliferation than $HgCl_2$ (Gauthier *et al*., 1998).

In addition, a dose-dependent response of Hg was shown in several studies on marine mammals (De Guise *et al*., 1996; Lalancette *et al*., 2003; Gauthier *et al*., 1998; Pellisso *et al*., 2008). In particular for harbor seals of the North Sea, the influence of Me-Hg concentration on the number of PBMCs, viability, metabolic activity, as well as DNA and RNA synthesis was shown (Das *et al*., 2008). In our study dose-dependent effects of different Hg compounds on cell proliferation were not considered due to the limited number of lymphocytes available.

Due to the high immunotoxic properties of Me-Hg, the lymphocyte mRNA cytokine expression of IL-2, IL-4, and TGF β was investigated in a preliminary substudy on two seals. However, the mRNA cytokine expression showed no significant difference with various Me-Hg concentrations in this study.

Nevertheless, the mRNA cytokine expression seemed to be influenced by duration of Me-Hg exposure. Interestingly, the expression of IL-2 and IL-4 increased after 6 h of incubation with all concentrations of Me-Hg compared to the non-Hg-incubated control cells. Several studies have shown an activation of the immune system by Hg, with both antigen-specific (allergy, autoimmune disease) and non-specific (mitogenic) effects. In both cases, the Hg-induced effect was associated with a modification of the cytokine production (Stejskal and Stejskal, 1999; Bagenstose *et al*., 1999). After the increase, the expression of both cytokines decreased after 24 and 48 h in most cell cultures.

The mRNA expression of TGF β showed no increase after 6 h, and was significantly decreased after 24 and 48 h in most cell cultures. The TGF β expression appeared to be more strongly influenced by the duration of Hg-incubation than IL-2 and IL-4. Other human and murine studies showed a Hg-induced inhibition of cytokine expression (Warbrick *et al*., 1995; Silva *et al*., 2005). In accordance with our results, Das *et al*. (2008) described a decrease of IL-2 and TGF β mRNA expression in lymphocytes after a long-term Me-Hg exposure (72 h). Further studies with a larger number of seals should be performed to clarify the effects of Hg on cytokine expression.

4 Conclusions

Both parameters of the cellular immune system investigated in this study, lymphocyte proliferation and cytokine expression, were influenced by Hg *in vitro*. The influences depended on different factors such as animal age, chemical form of Hg, and duration of exposure. Seals of the North Sea are burdened with Hg, and lymphocytes may be functionally impaired by Hg *in vivo* as well.

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