Final Draft
of the original manuscript:

Kakuschke, A.; Valentine-Thon, E.; Griesel, S.; Gandreass, J.; Perez Luzardo, O.; Dominguez Boada, L.; Zumbado Pena, M.; Almeida Gonzalez, M.; Grebe, M.; Proefrock, D.; Erbsloeh, H.-B.; Kramer, K.; Fonfara, S.; Prange, A.:

First health and pollution study on harbor seals (Phoca vitulina) living in the German Elbe estuary

In: Marine Pollution Bulletin (2010) Elsevier

DOI: 10.1016/j.marpolbul.2010.07.011

FIRST HEALTH AND POLLUTION STUDY ON HARBOR SEALS (*PHOCA VITULINA*) LIVING IN THE GERMAN ELBE ESTUARY

Antje Kakuschke[a,*], *Elizabeth Valentine-Thon*[b], *Simone Griesel*[a], *Juergen Gandrass*[a], *Octavio Perez Luzardo*[c], *Luis Dominguez Boada*[c], *Manuel Zumbado Peña*[c], *Maira Almeida González*[c], *Mechthild Grebe*[a], *Daniel Pröfrock*[a], *Hans-Burkhard Erbsloeh*[a], *Katharina Kramer*[a], *Sonja Fonfara*[d], *Andreas Prange*[a]

a GKSS Research Center Geesthacht, Marine Bioanalytical Chemistry, Institute for Coastal Research, Max-Planck-Strasse 1, 21502 Geesthacht, Germany

b Laboratory Center Bremen, Department of Immunology, Friedrich-Karl-Strasse 22, 28205 Bremen, Germany

c University of Las Palmas de Gran Canaria, Toxicology Unit, Department of Clinical Sciences, and Instituto Canario de Investigación del Cáncer, P.O. Box 550, 35080 Las Palmas de Gran Canaria, Spain

d Small Animal Teaching Hospital, University of Liverpool, Leahurst, Chester High Road, Neston CH64 7TE, United Kingdom

[*] Corresponding author:

Antje Kakuschke

GKSS Research Center Geesthacht, Marine Bioanalytical Chemistry

Institute for Coastal Research, 21502 Geesthacht, Germany

E-mail address: antjekakuschke@web.de

Tel: +49 40 42913833

Abstract

The Elbe is one of the major rivers releasing pollutants into the coastal areas of the German North Sea. Its estuary represents the habitat of a small population of harbor seals (*Phoca vitulina*). Only little is known about the health status and contamination levels of these seals. Therefore, a first-ever seal catch was organized next to the islands of Neuwerk and Scharhörn in the region of the Hamburg Wadden Sea National Park. The investigations included a broad set of health parameters and the analysis of metals and organic pollutants in blood samples. Compared to animals of other Wadden Sea areas, the seals showed higher γ-globulin levels, suggesting higher concentrations of pathogens in this near urban area, elevated concentrations for several metals in particular for V, Sn, Pb, and Sr, and comparable ranges for chlorinated organic contaminants, except for elevated levels of hexachlorobenzene, which indicates characteristic inputs from the Elbe.

Keywords: harbor seal; *Phoca vitulina*; Elbe estuary; North Sea; health; pollution;

1. Introduction

Due to their role as top predators within the marine food web, marine mammals such as harbor seals (*Phoca vitulina*) can be used as indicators for ecosystem change (Trilateral Monitoring and Assessment Program, TMAP). Increasing commercial use, e.g. fisheries and offshore wind parks, as well as ongoing inputs of pollutants strongly influence the North and Baltic Sea ecosystems. The German states Schleswig-Holstein, Hamburg and Lower Saxony have declared their Wadden Sea areas as National Parks. The Hamburg Wadden Sea area includes also parts of the Elbe estuary, where harbor seals play an important role for the regional tourism.

The harbor seal population in the Elbe estuary is relatively small in comparison to other populations that can be found along the Wadden Sea coast line. In one of the latest aerial surveys conducted in 2008, on an average 427 animals were counted in the area of the Hamburg Wadden Sea (Hellwig and Krüger-Hellwig, 2008). Most animals (371) were present on the western haul out sites “Robbenplate” and “Wittsandloch”. Fifty-six animals were counted on the eastern haul out site “Hundebalje”. Beside regular aerial surveys since 2002, no further investigations, e.g. of the health status of these animals, have been carried out.

In 2002, the phocine distemper virus (PDV) epizootic reduced the harbor seal population to 50 percent in this and other areas of the Wadden Sea (Reijnders et al., 2005). Since the epidemic impact, the seal population of the Hamburg Wadden Sea area has grown continuously. However, the size of the population has not yet reached its original size before the virus outbreak (Hellwig and Krüger-Hellwig, 2008).

Whether environmental pollution-related immunosuppression might have contributed to the severity and extent of morbillivirus-caused mass mortalities among marine mammals is still under discussion (Härkönen et al., 2006; Ross, 2002). However, several studies have shown a

relationship between contaminant body burdens and immunological dysfunctions (Beckmen, 1999; De Guise et al., 2006; De Swart et al., 1994; Kakuschke et al., 2007). Despite partly decreasing inputs of contaminants into the North Sea, the Elbe River is still the primary contributor to the contamination of its estuary and of the German Bight (Loewe et al., 2006). Several studies concerning the health status (Hasselmeier et al., 2008; Kakuschke et al., 2010; Siebert et al., 2007) and/or contaminant body burdens (Ahrens et al., 2009; Griesel et al., 2008; Weijs et al., 2009) of harbor seals were conducted in the Wadden Sea. To our knowledge, we report for the first time results for seals of the Elbe estuary. Our investigation included a common set of health parameters and pollutants, applied in the studies mentioned above. In addition, a new method for the determination of transferrin (Tf) isoforms (established markers for specific disorders in humans) as a potential new biomarker for seals was applied.

2. Material and methods

2.1. Animals

The seal catch was carried out in the estuary of the river Elbe next to the islands of Neuwerk and Scharhörn in the area of the Hamburg Wadden Sea National Park (Germany) in October 2008 (Figure 1).

FIGURE 1. Sampling location in the estuary of the river Elbe.

The seal catch was coordinated from on board the GKSS research vessel “Ludwig Prandtl” and carried out with two Zodiac boats. Harbor seals were captured using a 120 m x 8 m net with a

mesh size of 10 cm x 10 cm, adapted from a method described by Jeffries et al. (1993). Briefly, the net was spread out slowly between both Zodiac boats in a distance of around 100 m to the animals. Due to the low water depth, the net reached to the ground and the animals were not able to dive below the net. Both boats moved simultaneously towards the beach, trapping the seals within the net. After the landing of the two boats, the net was moved manually onto the shore line. The caught animals were removed from the net, transferred into tube nets, and restrained manually to assess length, weight, sex and age and to collect anal smears and blood samples. The handling for measurements and blood collection took 10 - 15 minutes for each seal. During the procedure the animals were continuously under observation of two veterinarians. After completing the investigations, the animals were released back into the wildlife. The time span between transferring all animals in tube nets and releasing back into the wildlife took one hour.

Blood was collected into monovettes after puncture of the epidural vertebral vein using a 20 mL syringe and a 12 mm x 100 mm needle (TSK-Supra, TSK Laboratory, Japan). The tubes were carefully agitated and kept at room temperature until further sample processing. Most blood samples were processed within 1 to 12 h. Swabs taken from the anus were used for microbiological investigations.

During this catch five animals were caught and coded sequentially (Table 1). The age was estimated based on length and weight and the animals were grouped into seals < 1 year, between 1 - 2 year, and > 2 years.

TABLE 1. Details of the harbor seals caught in the Elbe estuary in 2008.

2.2. Hematology

For hematology, EDTA monovettes (Sarstedt AG & Co, Nümbrecht, Germany) were used. A basic hematology profile (white blood cells [WBC], red blood cells [RBC], hemoglobin [HGB], hematocrit [HCT], mean cellular volume [MCV], mean cellular hemoglobin [MCH], mean cellular hemoglobin concentration [MCHC], thrombocytes, and reticulocytes), was analyzed at Synlab.vet Hamburg in Geesthacht, Germany, using a Sysmex XT – 2000 analyser (Sysmex Deutschland GmbH, Norderstedt, Deutschland). The leukocyte subgroups (neutrophiles, eosinophiles, lymphocytes, and monocytes) were counted manually.

2.3. Lymphocyte proliferation assay

The MELISA® (Memory Lymphocyte Immunostimulation Assay), a modification of the lymphocyte transformation test (LTT), was performed as previously described in the Laboratory Center Bremen, Germany (Kakuschke et al., 2005, 2006, 2008a,b) and briefly described in the Supporting Information S1. The mitogen- and non-stimulated lymphocyte proliferation was tested as well as the metal-specific proliferation of following metals/metal species: Al, Be, Cd, ethylmercury (EtHg), mercurychloride (HgCl), methylmercury (MeHg), phenylmercury (PhHg), Mo, Ni, Pb, Sn, and Ti. The metals were tested at two concentration levels. Level II is the 1:1 dilution of level I. The concentrations of level I are given in µg/well: Al (40), Be (50), Cd (6), EtHg (0.5), HgCl (0.5), MeHg (0.5), PhHg (0.5), Mo (25), Ni (5), Pb (25), Sn (25) and Ti (50). The stimulation index (SI) was calculated as followed:

SI = metal-stimulated proliferation (cpm) / non-stimulated proliferation (cpm).

SI ≥ 3 was regarded as a positive hypersensitivity response.

2.4. Serum protein electrophoresis, investigations on acute phase proteins, and serology

Serum protein electrophoresis was done at the Synlab.vet Hamburg with an automated analyzer (Olympus Hite 320, Olympus Deutschland GmbH, Hamburg, Germany).

C-reactive protein (CRP) was measured at the Synlab.vet Hamburg using turbidometry (Olympus AU 2700, Olympus Deutschland GmbH).

For the measurement of haptoglobin (Hp), a multispecies Hp assay from Tridelta Development Limited (Maynooth, Kildare, Ireland) was used. The Hp concentrations were quantified in EDTA plasma samples collected by using EDTA monovettes according to the manufacturer's instructions. Colorimetric measurements were performed using a photometer (Multilabel Counter WALLAC 1420, Perkin Elmer). All samples were analyzed in duplicate at the GKSS, Geesthacht.

The serology included the analysis of *Brucella* spp. and distemper virus antibodies and was performed at Synlab.vet using an immunofluorescence antibody test (IFAT).

2.5. Determination of transferrin isoforms

Tf isoforms were analyzed in serum at the GKSS as described recently (Grebe et al., 2010). Briefly, the procedure utilizes a strong anion-exchange (SAX) chromatography hyphenated with inductive-coupled plasma mass spectrometry (ICP-MS). The setup consisted of a high performance liquid chromatograph (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) and an ICP-MS (Agilent 7500cs, Agilent Technologies, Tokyo, Japan).

Seal blood was sampled in Serum Gel S monovettes (Sarstedt AG & Co.). Tf in blood samples was saturated with iron by incubation with $FeCl_3$ solution. After the precipitation of lipoproteins the samples were centrifuged and the resulting supernatant was diluted with starting buffer (20 mM Bis-Tris, pH 6.5). After the separation with a linear gradient of

ammonium acetate on a SAX column (Poros HQ 2.1 x 100 mm, 10 µm particles, Applied Biosystems, Foster City, USA), Tf isoforms were measured using element-specific detection of ^{56}Fe . Interferences were reduced by using the collision cell with 5 mL min^{-1} H_2.

The evidence for being Tf isoforms with differing degrees of sialination was provided by specific enzymatic digestions and partial mass spectrometric determination of the amino acid sequence of seal Tf found in the SAX fractions (Grebe et al., 2010).

2.6. Clinical chemistry and bacteriology

Clinical chemistry and bacteriology were performed at the Synlab.vet Hamburg. The enzyme activities of alkaline phosphatase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (γ-GT), cholinesterase, glutamate dehydrogenase (GLDH), lactate dehydrogenase (LDH), alpha-amylase, lipase, creatine kinase (CK) as well as the amount of total bilirubin, cholesterol, creatinine, bile acid, urea, uric acid, triglyceride, glucose, and inorganic phosphate were analyzed using photometry (Olympus AU 2700). Chloride was quantified by potentiometry. Cortisol and thyroxin were analyzed using a chemiluminescence immunoassay (CLIA, Immulite 2000, Siemens AG, Erlangen, Germany), and folic acid and vitamin B12 using an electrochemiluminescence immunoassay (ECLIA, Immulite 2000). Swabs (Heinz Herenz Medizinal Bedarf GmbH, Hamburg) from the anus were investigated microbiologically by Synlab.vet Hamburg.

2.7. Element analysis of whole blood

For the element analysis blood samples were collected in special Lithium Heparin (LH) monovettes for metal analysis (Sarstedt AG & Co) and stored at –80°C. Twenty-five elements

209 were analyzed in whole blood samples following the procedure described in our previous study
210 at the GKSS (Griesel et al., 2008).
211 The elements were determined with two different analytical methods. Al, Be, Bi, Cd, Co, Cr,
212 Cs, Li, Mg, Mn, Mo, Na, Ni, Pb, Sn, and V were analyzed using an ICP-MS equipped with a
213 collision cell (Agilent 7500c ICP-MS, Agilent Technologies). The standard mode was used for
214 Al, Be, Cs, Li, Na, Pb, Sn, and V. For the other elements, better results were obtained using He
215 as collision gas (flow rate 3.0 mL min^{-1}). Measurements of As, Ca, Cu, Fe, K, Rb, Se, Sr, and
216 Zn were performed by total-X-ray-fluorescence spectrometry (TXRF) (Atomika TXRF 8030 C,
217 FEI Company, Oberschleissheim, Germany).
218 For internal quality control, the reliability of the analytical procedures was checked with the
219 human reference material Seronorm™ Trace Elements Whole Blood L-2 (SERO AS,
220 Billingstad, Norway) and/or Clin Check® Whole Blood Control Level II (Recipe,
221 Chemicals+Instruments, Munich, Germany). In addition the laboratory successfully completed
222 the NIST/NOAA 2005 and 2007 Interlaboratory Comparison Exercise for Trace Elements in
223 Marine Mammals (Christopher et al., 2007).
224
225 **2.8. Chlorinated Pesticides and PCBs in plasma**
226 Aliquots of LH plasma were subjected to solid-phase extraction (SPE) and analyzed by gas
227 chromatography-mass spectrometry (GC-MS). Twenty chlorinated pesticides and metabolites
228 as well as 19 polychlorinated biphenyl congeners (PCBs) were included in this study (Table 4).
229 Standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Riedel-de Haën
230 (Seelze, Germany), and Sigma-Aldrich Laborchemikalien GmbH (Steinheim, Germany). The
231 measurements were performed at the University of Las Palmas de Gran Canaria, Spain.

Two-ml aliquots of plasma were applied to 60 mg (3 mL) Oasis® HLB cartridges (Waters Corporation, Milford, USA) mounted in a vacuum manifold (Waters Corporation). Before the application of the plasma samples, the HLB cartridges were cleaned and conditioned as indicated by the manufacturer. Samples were then passed through the cartridge by gravity flow. The adsorbed pesticides and PCBs were eluted with 1 mL of methylene chloride. After a gentle nitrogen blow down and immediate resolubilization in 200 µl n-hexane, the resulting final extracts were subsequently analyzed by GC-MS.

GC-MS was performed with a TRACE DSQ (Thermo-Finnigan) instrument. The GC column was a fused silica capillary column BPX5 (crosslinked 5% phenyl methylpolysiloxane, SGE Inc.,Austin, USA) with a length of 30 m, 0.25 mm i.d. and a film thickness of 0.25 µm. Helium at a flow rate of 2.1 ml min^{-1} was used as carrier gas. Temperatures were programmed as follows: Initial oven temperature of 80°C held for 1 min, ramped at 10°C/min to 300°C and held for 9 min. Injector and transfer line were set at 200°C and 310°C, respectively. Standards and samples were injected (2 µl) in the splitless mode.

Two chromatographic runs were performed for each sample to obtain mass spectra in two different ionization modes. DDT and metabolites, methoxychlor, and PCB congeners 28, 52, 101, and 118 were ionized in electron impact mode at 70 eV with an ion source temperature of 200°C. For the rest of analytes included in this study, negative chemical ionization was applied using methane as reactant gas at a flow rate of 2.5 ml min^{-1}. The MS was operated in selected ion monitoring mode.

For the quantification of target analytes, six-level calibrations were generated from standard solutions. PCB 202 was used as internal and tetrachloro-m-xylene as surrogate standard.

Limits of quantification (LOQs) were determined as 10-fold standard deviations of blanks. LOQs for DDT and metabolites, methoxychlor, and PCB congeners 28, 52, 101, 118 and 138

were 10 pg mL^{-1} and for PCB congeners 153 and 180 5 pg mL^{-1}. LOQs for the rest of analytes were 1 pg mL^{-1}. The recovery rates were higher than 85 % for all the chlorinated pesticides and between 58-67 % for the PCB congeners.

3. Results and Discussion

The aim of the present study was to investigate the health status in combination with measuring body burdens of harbor seals living in the German Elbe estuary, an area where seals have not been previously investigated. However, this area is strongly influenced by anthropogenic activities such as shipping or dredging and shows a high pollution level compared to offshore regions of the North Sea.

3.1. Hematology profile

The hematology profile of the animal W 01/08 Pv showed an elevated number of WBC in general, and neutrophiles and monocytes in particular, compared to the other animals of this study (Table 2) and other investigations on harbor seals (De Swart et al., 1995; Engelhardt, 1979; Hasselmeier et al., 2008). Interestingly, this animal revealed also increased levels for cortisol (Table S2), CRP and Hp (Table 2). As other measured parameters did not differ markedly to results of the other seals and no obvious impairment was present on physical examination (data not shown), this result is most likely consistent with a stress-leukogram (Jackson, 2010).

TABLE 2. Immunological investigations of seals of the Elbe estuary.

3.2. Lymphocyte proliferation

280 The lymphocyte proliferation was similar to the range measured previously in other seals of the
281 North Sea (Kakuschke et al., 2005). However, W 04/08 Pv and W 05/08 Pv showed higher
282 stimulation indices compared to the older seals W 02/08 Pv and W 03/08 Pv (Table 2). Further
283 parameters indicate no differences between both age groups.
284 Additionally, seals of the Elbe estuary were investigated for metal-specific hypersensitivity
285 reactions as described for Wadden Sea seals and different groups of animals living in the Seal
286 Station Friedrichskoog (Schleswig Holstein, Germany) (Kakuschke et al., 2005, 2006,
287 2008a,b). For one seal (W 03/08 Pv) Sn- and Ti-specific hypersensitivity reactions were found
288 (Figure S1). As shown below, the Sn concentrations in blood of the Elbe seals were elevated
289 and might induce hypersensitivities. However, this result was not present in other seals of this
290 study, and investigations of a larger number of animals from this geographical area are
291 necessary to confirm this relationship and to evaluate the influence of metal pollutants on the
292 immune system.
293
294 **3.3. Serum proteins**
295 The total protein, albumin, and albumin/globulin ratio were comparable to other investigations
296 on harbor seals (Table 2) (Engelhardt, 1979; Hasselmeier, 2006). Interestingly, α-, β-, and γ -
297 globulins showed differences compared to other studies on harbor seals: α- and β-globulins
298 were lower, and, in particular, γ-globulins were higher in our study on harbor seals of the Elbe
299 estuary compared to animals of other regions of the Wadden Sea (Engelhardt, 1979;
300 Hasselmeier, 2006). Gamma-globulins are the group of immunoglobulins consisting of
301 different antibodies and are elevated in various inflammatory, infectious, and neoplastic
302 conditions. This result suggests that seals sampled at near-urban sites might have an activated
303 humoral immune system caused by higher exposure to pathogens. The role of the biological

304 pollution on the immune system was also shown in a study on harbor seals captured from
305 remote and near-urban sites in British Columbia, Canada, and Washington State, USA (Mos et
306 al., 2006).
307
308 **3.4. Transferrin isoforms**
309 Isoforms of Tf, an iron-transport glycoprotein in mammals, has been investigated by us, to our
310 knowledge, for the first time in seals. It is well known that human Tf can be separated into
311 several isoforms based on differences in their carbohydrate moities and particularly their
312 number of negatively-charged terminal sialic acid residues (Del Castillo Busto et al., 2009;
313 Helander et al., 2001). Altered distributions of Tf isoforms, in particular Carbohydrate
314 Deficient Transferrin (CDT, defined as the sum of α-, mono- and disialotransferrin) and their
315 elevated concentrations in serum are used in human medicine as biomarkers, e.g. for damage to
316 the liver and liver diseases (Arndt, 2001; Helander et al., 2001; Murawaki et al., 1997).
317 The patterns of eight isoforms found in the seal serum samples are depicted in Figure 2.
318
319 **FIGURE 2.** Anion-exchange chromatograms of the separated Tf isoforms (1-8) from seals of
320 the Elbe estuary measured by ICP-MS (^{56}Fe), one typical chromatogram for each group: group
321 I (W01/08 Pv, W03/08 Pv, W05/08 Pv); group II (W02/08 Pv, W04/08 Pv).
322
323 With an increasing degree of sialination, the isoforms elute at higher retention times from the
324 anion-exchange column (Grebe et al., 2010). Supporting information on retention times and
325 relative peak areas is given in Table S1 and all five chromatograms in Figure S2.
326 Despite the small set of samples, two distinctly different sets of Tf isoform patterns were
327 observed (Figure 2). For two animals (W 02/08 Pv and W 04/08 Pv), the relative amounts of

328 lower sialinated isoforms 1 and 2 (CDT) added up to more than 30% and 23%, respectively,
329 while for the other three animals CDT was below 1%. The two animals with high CDT levels
330 also exhibited higher levels of creatine kinase whereas the other diagnostic clinical parameters
331 showed no notable differences.
332 Due to the small set of samples, our case study does not allow an interpretation of the different
333 Tf isoform patterns. However, in analogy to their application as biomarkers in human
334 medicine, Tf isoforms could be a potential biomarker as well for seals.
335
336 **3.5. Clinical chemistry and bacteriology**
337 Most of the results of the clinical chemistry measured in this study were within the ranges
338 described in other studies on harbor seals (Table S2) (Bossart et al., 2001; Trumble, 2002). For
339 several enzyme activities the animal W 05/08 Pv showed elevated values compared to the other
340 four animals of this study. However, most diagnostic parameters showed no remarkable
341 differences.
342
343 **3.6. Element profile in whole blood samples**
344 Essential and non-essential/toxic elements were analyzed in whole blood samples.
345 Firstly, interesting results for the essential trace elements were found (Table 3). For the seals W
346 01/08 Pv, W 02/08 Pv, W 03/08 Pv, and W 05/08 Pv, the values for Fe and Zn in whole blood
347 were comparable to results of our previous studies on Wadden Sea seals living on the sandbank
348 Lorenzenplate (Schleswig-Holstein, Germany) and on Römö (Denmark), whilst the
349 concentrations of K and Cu were higher (Griesel et al., 2008). Contrarily, animal W 04/08 Pv
350 showed normal K and Cu concentrations, lower values for Fe and Zn compared to published
351 values and lower concentrations of essential trace elements such as Mg, Mn, and Se in

352 comparison to the other seals of this study. Furthermore, several Ca concentrations measured in
353 this study were higher compared to our previous studies on free ranging seals (Griesel et al.,
354 2008). However, the concentrations were comparable to those measured in harbor seal pups
355 (Kakuschke et al., 2009).
356
357 **TABLE 3.** Element profile in whole blood samples (concentrations are given in μg L^{-1}) of seals
358 caught in the Elbe estuary compared to our previous study on seals of the German Bight
359 (Griesel et al., 2008).
360
361 Secondly, among the toxic metals, interesting differences in comparison to other Wadden Sea
362 areas of the North Sea and further inshore areas in the world were found.
363 The concentrations of V and Sn in blood samples were significantly higher in the Elbe seals
364 compared to our previous study on animals of other Wadden Sea areas: The levels of V were
365 more than two times higher than those from seals living on the sandbank Lorenzenplate and on
366 Römö (Griesel et al., 2008). Compared to marine mammals of other inshore areas, e.g. to
367 manatees (*Tricheus manatus latirostris*) of the upper Crystal River, Florida, the V blood
368 concentrations for the Elbe animals were also elevated (Stavros et al., 2008). However, blood
369 samples of northern fur seals (*Callorhinus ursinus*) from northeast Japan revealed higher V
370 concentrations than our results (Saeki et al., 1999). Furthermore, elevated Sn concentrations
371 were measured in blood of Elbe seals compared to seals caught at the Lorenzenplate and Römö
372 (Griesel et al., 2008). However, in blood samples of Florida manatees of the upper Crystal
373 River up to 3 μg Sn kg^{-1} ww blood were measured (Stavros et al., 2008). Similar higher Sn
374 concentrations were found in the liver of cetaceans from Japanese coastal water compared to
375 animals from offshore northwest North Pacific (Takahashi et al., 2000). Furthermore, elevated

376 Sn concentrations were found in liver samples of harbor porpoises (*Phocoena phocoena*) from
377 the river Elbe in comparison to samples taken from North Sea porpoises (Fahrenholtz et al.,
378 2009). These results suggest that Sn levels may be correlated to the high shipping traffic in
379 estuaries or inshore areas. Despite its ban in 2003, most ships are still covered with antifouling
380 paint containing tributyltin (TBT). Parts of these biocides are incorporated into marine
381 organisms. Terlizzi *et al.* describe the impact of antifouling technologies on the marine
382 environment (Terlizzi et al., 2001).
383 Furthermore, the concentrations of Pb and Sr also showed differences between samples of Elbe
384 seals and animals of other Wadden Sea areas. Pb concentrations in blood of Elbe seals were
385 similar to concentrations measured in seal pups found along the coasts of Schleswig-Holstein
386 and seals from the island Römö, whereas seals caught on the Lorenzenplate revealed lower Pb
387 concentrations (Griesel et al., 2008; Kakuschke et al., 2009). Stavros et al. (2008) suggested
388 that Pb concentrations in blood of Florida manatees may be caused by increased Pb
389 concentrations transported via rivers.
390 Al concentrations were likewise higher in Elbe and Römö animals compared to animals of the
391 Lorenzenplate (Griesel et al., 2008). For most animals caught on the Lorenzenplate and Römö,
392 the Be concentrations were below the detection limit, whereas all five seals of this study
393 revealed concentrations > 1 $\mu g\ L^{-1}$.
394 Additionally, while the As concentrations in blood of the Elbe seals were within the range
395 measured in seals from Lorenzenplate and Römö, the values were higher than the median levels
396 calculated for these seals (Griesel et al., 2008).
397 Despite the small number of seals investigated, the results of this study suggest that animals
398 living in estuaries and inshore habitats with industrial emissions and sewage, shipping traffic

399 and dredging tasks are exposed to higher levels of contaminants compared to animals living
400 offshore.
401
402 **3.7. Chlorinated Pesticides and PCBs in plasma**
403 Plasma concentrations of the investigated chlorinated pesticides (including some common
404 metabolites) and PCBs are given in Table 4. As the sampling area of this study lies in the Elbe
405 estuary and is supposed to be influenced by riverine inputs, a comparison with results for seals
406 in bordering coastal areas is of special interest. Weijs et al. (2009) reported serum
407 concentrations of hexachlorobenzene (HCB), 4,4'-DDT and metabolites, and PCBs: medians
408 and ranges (minimum – maximum) in ng L^{-1} for 47 harbor seals from Helgoland,
409 Lorenzenplate, and Römö were < 20 for HCB, 2750 (722 – 8440) for 4,4'-DDE, and 7670
410 (1700 – 34,200) for PCB 138.
411 In comparison, the corresponding plasma concentrations for the investigated seals in the Elbe
412 estuary are slightly lower for PCBs, in the same range for 4,4'-DDT and metabolites, and 10 to
413 100-fold higher for HCB.
414 The increased HCB levels of the investigated seals are possibly caused by inputs of the Elbe
415 River. In suspended particulate matter of the lower Elbe River, HCB is dominating over PCBs
416 and DDT metabolites. In addition, in sediments of the German North Sea, variable
417 concentration patterns are observed. Further away from the Elbe estuary, HCB concentrations
418 decrease relative to concentrations of PCBs and DDT metabolites (Loewe et al., 2006).
419
420 **TABLE 4.** Chlorinated pesticides and PCBs in plasma in ng L^{-1}.
421
422 **4. Conclusion**

The Elbe River is one of the major rivers releasing organic and inorganic pollutants into the coastal areas of the German North Sea, and distinctive toxic effects on biota in bordering coastal areas may be expected. This investigation represents the first health and pollution study of seals living in the Elbe estuary. It indicates significant differences in comparison to the results obtained during the investigations of animals from other Wadden Sea areas. The seals in the Elbe estuary show higher γ-globulin levels suggesting higher concentrations of pathogens in this near-urban area, elevated blood concentrations for several metals in particular for V, Sn, Pb, and Sr, and elevated levels of HCB, which indicates characteristic inputs from the River Elbe.

Acknowledgements

The authors like to thank all the participants who supported the seal catch: Peter Körber (National Park Hamburg Wadden Sea), Bastian Tiemann (University Medical Center Hamburg-Eppendorf), Lene Kämper, Tim Fetting and Sebastian Teske (Seal Center Norddeich), Bernd Peters, Michael Janik and Horst Garbe (GKSS Research Center) as well as the crew of the ship "Ludwig Prandl" Helmut Bornhöft and Jan Marx and Heiko Gerbatsch. The wild catch was supported by the Free and Hanseatic City of Hamburg, Ministry of Urban Development and Environment and Ministry of Social, Family Affairs, Health and Consumer Protection. The authors are also grateful to all the helpers who participated in preparing excursions: Thomas Borchardt (National Park Service Schleswig-Holstein), Nicolas Fitz and Heike Helmholtz (GKSS Research Center), Kai Abt, and Gabriele Müller.

445

446 **References**

447 Ahrens, L., Siebert, U., Ebinghaus, R., 2009. Total body burden and tissue distribution of
448 polyfluorinated compounds in harbor seals (*Phoca vitulina*) from the German Bight.
449 Mar. Pollut. Bull., 520-525.

450 Arndt, T., 2001. Carbohydrate-deficient transferrin as a marker of chronic alcohol abuse: a
451 critical review of preanalysis, analysis, and interpretation. Clin. Chem. 47, 13-27.

452 Beckmen, K., 1999. Blood organochlorines, immune function and health of free-ranging
453 northern fur seal pups (*Callorhinus ursinus*). University of Alaska Fairbanks, Fairbanks.

454 Bossart, G. D., Reidarson, T. H., Dierauf, L. A., Duffield, D. A., 2001. Clinical Pathology. In
455 *Marine Mammal Medicine*, Dierauf, L. A., Gulland, F. M. D., Eds. CRC Press LLC:
456 Boca Raton, Florida.

457 Christopher, S. J., Pugh, R. S., Ellisor, M. B., Mackey, E. A., Spatz, R. O., Porter, B. J., Bealer,
458 K. J., Kucklick, J. R., Rowles, T. K., Becker, P. R., 2007. Description and results of the
459 NIST/NOAA 2005 Interlaboratory comparison exercise for trace elements in marine
460 mammals. Accred. Qual. Assur. 12, 175-187.

461 De Guise, S., Levin, M. J., Mori, C., Morsey, B., Nambiar, P., 2006. Immunotoxicity of PCBs
462 in marine mammals, mouse and humans upon in vitro exposure. Mar. Environ. Res. 62,
463 S370-S371.

464 Del Castillo Busto, M. E., Montes-Bayon, M., Sanz-Medel, A., 2009. The potential of mass
465 spectrometry to study iron-containing proteins used in clinical diagnosis. Anal. Chim.
466 Acta 634, 1-14.

467 De Swart, R. L., Ross, P. S., Vedder, L. J., Boink, P. B. T. J., Reijnders, P. J. H., Mulder, P. G.
468 H., Osterhaus, A. D. M. E., 1995. Haematology and clinical chemistry values for

harbour seals (*Phoca vitulina*) fed environmentally contaminated herring remain within normal ranges. Can. J. Zool.-Rev. Can. Zool. 73, 2035-2043.

De Swart, R. L., Ross, P. S., Vedder, L. J., Timmerman, H. H., Heisterkamp, S., Vanloveren, H., Vos, J. G., Reijnders, P. J. H., Osterhaus, A. D. M. E., 1994. Impairment of immune function in harbor seals (*Phoca vitulina*) feeding on fish from polluted waters. Ambio 23, 155-159.

Engelhardt, F. R., 1979. Haematology and plasma chemistry of captive pinnipeds and cetaceans. Aquatic Mammals 7, 11-20.

Fahrenholtz, S., Griesel, S., Pröfrock, D., Kakuschke, A., 2009. Essential and non-essential elements in tissues of harbour porpoises (*Phocoena phocoena*) stranded on the coasts of the North and Baltic Seas between 2004–2006. J. Environ. Monit. 11, 1107-1113.

Grebe, M., Pröfrock, D., Kakuschke, A., Broekaert, J.A.C., Prange, A., 2010. Metallomics approach for the identification of the iron transport protein transferrin in the blood of harbour seals (*Phoca vitulina*). Metallomics 2(10), 683-693.

Griesel, S., Kakuschke, A., Siebert, U., Prange, A., 2008. Trace element concentrations in blood of harbor seals (*Phoca vitulina*) from the Wadden Sea. Sci. Total Environ. 392, 313-323.

Härkönen, L., Dietz, R., Reijnders, P., Teilmann, J., Harding, K., Hall, A., Brasseur, S., Siebert, U., Goodman, S. J., Jepson, P. D., Rasmussen, T. D., Thompson, P., 2006. A review of the 1988 and 2002 phocine distemper virus epidemics in European harbour seals. Dis. Aquat. Org. 68, 115-130.

Hasselmeier, I. 2006. Evaluation of blood tests to assess the health status of harbor seals (*Phoca vitulina*) of the German North Sea. Christian-Albrechts-Universität zu Kiel, Kiel.

493 Hasselmeier, I., Fonfara, S., Driver, J., Siebert, U., 2008. Differential hematology profiles of
494 free-ranging, rehabilitated, and captive harbor seals (*Phoca vitulina*) of the German
495 North Sea. Aquatic Mammals 34, 149-156.
496 Helander, A., Eriksson, G., Stibler, H. and Jeppsson, J.-O., 2001. Interference of transferrin
497 isoform types with carbohydrate-deficient transferrin quantification in the identification
498 of alcohol abuse. Clin. Chem. 47, 1225-1233.
499 Hellwig, U., Krüger-Hellwig, L., 2008. Programm zur ökologischen Dauerbeobachtung im
500 Nationalpark Hamburgisches Wattenmeer. Jahresbericht 2008. Freie und Hansestadt
501 Hamburg, Behörde für Stadtentwicklung und Umwelt, Hamburg.
502 Jackson, M.L., 2010. Leukocytes in health and disease, in: Ettinger, S.J., Feldman, E.D. (Eds.),
503 Textbook of veterinary internal medicine. Seventh edition. Saunders Elsevier, Missouri,
504 pp. 801-809.
505 Jeffries, S. J., Brown, R. F., Harvey, J. T., 1993. Techniques for capturing, handling and
506 marking harbour seals. Aquatic Mammals 19, 21-25.
507 Kakuschke, A., Erbsloeh, H.-B., Griesel, S., Prange, A., 2010. Acute phase protein haptoglobin
508 in blood plasma samples of harbour seals (*Phoca vitulina*) of the Wadden Sea and of the
509 isle Helgoland. Comp. Biochem. Physiol. B-Biochem. Mol. Biol. 155, 67-71.
510 Kakuschke, A., Griesel, S., Fonfara, S., Rosenberger, T., Prange, A., 2009. Concentrations of
511 selected essential and non-essential elements in blood of harbor seal (*Phoca vitulina*)
512 pups of the German North Sea. Biol. Trace Elem. Res. 127, 28-36.
513 Kakuschke, A., Prange, A., 2007. The Influence of Metal Pollution on the Immune System A
514 Potential Stressor for Marine Mammals in the North Sea. Int. J. Comp. Psychol. 20,
515 179-193.

516 Kakuschke, A., Valentine-Thon, E., Fonfara, S., Griesel, S., Rosenberger, T., Siebert, U.,
517 Prange, A., 2008. Metal-induced impairment of the cellular immunity of newborn
518 harbor seals (*Phoca vitulina*). Arch. Environ. Contam. Toxicol. 55, 129-136.
519 Kakuschke, A., Valentine-Thon, E., Fonfara, S., Griesel, S., Siebert, U., Prange, A., 2006.
520 Metal sensitivity of marine mammals: A case study of a gray seal (*Halichoerus grypus*).
521 Mar. Mamm. Sci. 22, 985-996.
522 Kakuschke, A., Valentine-Thon, E., Griesel, S., Fonfara, S., Siebert, U., Prange, A., 2005.
523 Immunological impact of metals in harbor seals (*Phoca vitulina*) of the North Sea.
524 Environ. Sci. Technol. 39, 7568-7575.
525 Kakuschke, A., Valentine-Thon, E., Griesel, S., Rosenberger, T., Mundry, R., Siebert, U.,
526 Prange, A., 2008. Blood metal levels and metal-influenced immune functions of
527 harbour seals in captivity. Mar. Pollut. Bull. 56, 764-769.
528 Loewe, P., Becker, G., Brockmann, U., Dick, S., Frohse, A., Herrmann, J., Klein, B., Klein, H.,
529 Nies, H., Schmolke, S., Schrader, D., Schulz, A., Theobald, N., Weigelt, S., 2006.
530 *Nordseezustand 2004. Berichte des Bundesamtes für Seeschifffahrt und Hydrographie.*
531 *Nr.40.* Bundesamt für Seeschifffahrt und Hydrographie (BSH): Hamburg, Rostock.
532 Mos, L., Morsey, B., Jeffries, S. J., Yunker, M. B., Raverty, S., De Guise, S., Ross, P. S., 2006.
533 Chemical and biological pollution contribute to the immunological profiles of free-
534 ranging harbor seals. Environ. Toxicol. Chem. 25, 3110-3117.
535 Murawaki, Y., Sugisaki, H., Yuasa, I., Kawasaki, H., 1997. Serum carbohydrate-deficient
536 transferrin in patients with nonalcoholic liver disease and with hepatocellular
537 carcinoma. Clin. Chim. Acta 259, 97-108.
538 Reijnders, P. J. H., Reineking, B., Abt, K. F., Brasseur, S., Camphuysen, K. C. J., Scheidat, M.,
539 Siebert, U., Stede, M., Tougaard, J., Tougaard, S., 2005. Marine mammals. In *Wadden*

Sea Quality Status Report 2004. Wadden Sea Ecosystem No. 19. Trilateral Monitoring and Assessment Group., Essink, K., Dettmann, C., Farke, H., Laursen, K., Lüerßen, G., Marencic, H. and Wiersinga, W., Ed. Wilhelmshaven, Germany: Common Wadden Sea Secretariat, Nr.19, pp 317-330.

Ross, P. S., 2002. The role of immunotoxic environmental contaminants in facilitating the emergence of infectious diseases in marine mammals. Hum. Ecol. Risk Assess. 8, 277-292.

Saeki, K., Nakajima, M., Noda, K., Loughlin, T. R., Baba, N., Kiyota, M., Tatsukawa, R., Calkins, D. G., 1999. Vanadium accumulation in pinnipeds. Arch. Environ. Contam. Toxicol. 36, 81-86.

Siebert, U., Wohlsein, P., Lehnert, K., Baumgärtner, W., 2007. Pathological findings in harbour seals (*Phoca vitulina*): 1996-2005. J. Comp. Pathol. 137, 47-58.

Stavros, H. C. W., Bonde, R. K., Fair, P. A., 2008. Concentrations of trace elements in blood and skin of Florida manatees (*Trichechus manatus latirostris*). Mar. Pollut. Bull. 56, 1215-1233.

Takahashi, S., Le, L. T. H., Saeki, H., Nakatani, N., Tanabe, S., Miyazaki, N., Fujise, Y., 2000. Accumulation of butyltin compounds and total tin in marine mammals. Water Sci. Technol. 42, 97-108.

The Trilateral Wadden Sea Cooperation, 2010. The Trilateral Monitoring and Assessment Program (TMAP). Wadden Sea Plan 2010. Common Wadden Sea Secretariat, Wilhelmshaven, Germany..

Terlizzi, A., Fraschetti, S., Gianguzza, P., Faimali, M., Boero, F., 2001. Environmental impact of antifouling technologies: state of the art and perspectives. Aquatic Conserv: Mar. Freshw. Ecosyst. 11, 311-317.

564 Trumble, S. J., Castellini, M. A., 2002. Blood chemistry, hematology, and morphology of wild
565 harbor seal pups in Alaska. J. Wildl. Manage. 66, 1197-1207.
566 Weijs, L., Das, K., Siebert, U., van Elk, N., Jauniaux, T., Neels, H., Blust, R., Covaci, A., 2009.
567 Concentrations of chlorinated and brominated contaminants and their metabolites in
568 serum of harbour seals and harbour porpoises. Environ. Int. 35, 842-850.

569 **Figures and tables**

570

571 **TABLE 1.** Details of the harbor seals of this study caught in the Elbe estuary in 2008.

572

Seal code	Date of blood sampling	Sex	Age (year)	Total length (cm)	Reduced length (cm)	Weight (kg)
W 01/08 Pv	10.10.2008	male	< 1	96	51	25
W 02/08 Pv	10.10.2008	male	> 2	130	85	48
W 03/08 Pv	10.10.2008	male	> 2	147	89	49
W 04/08 Pv	10.10.2008	male	< 1	112	56	24
W 05/08 Pv	10.10.2008	male	1 - 2	119	78	39

573

574 **TABLE 2.** Immunological investigations of seals of the Elbe estuary.

575

	W 01/08 Pv	W 02/08 Pv	W 03/08 Pv	W 04/08 Pv	W 05/08 Pv
Hematology profile					
White blood cells (WBC, $x10^9$ L^{-1})	19.9	10.4	7.0	11.5	9.4
Red blood cells (RBC, $x10^{12}$ L^{-1})	5.01	5.08	6.40	5.68	5.07
Hemoglobin (HGB, g L^{-1})	193	221	261	217	195
Hematocrit (HCT, L L^{-1})	0.56	0.60	0.71	0.62	0.56
Mean cellular volume (MCV, μm^3)	112.2	118.7	110.2	109.2	109.5
Mean cellular hemoglobin (MCH, pg)	38.5	43.5	40.8	38.2	38.5
Mean cellular hemoglobin concentration (MCHC, g dL^{-1})	34.3	36.7	37.0	35.0	35.1
Thrombocytes ($x10^9$ L^{-1})	333	301	178	115	110
Reticulocytes (μL^{-1})	35070	81280	32000	39760	86190
Neutrophiles (μL^{-1})	14726	3432	4060	7360	5076
Lymphocytes (μL^{-1})	2587	5928	2030	3220	2914
Monocytes (μL^{-1})	796	312	210	460	nd
Eosinophiles (μL^{-1})	1791	728	700	460	1410
Lymphocyte proliferation					
Non-stimulated proliferation (cpm)	-	2936	1801	801	1022
PWM-stimulated proliferation (cpm)	-	139121	120072	198268	229449
Stimulation index	-	41	43	283	225
Serum protein electrophoresis					
Albumin absolute (g L^{-1})	27.8	32.3	32.8	30.2	31.6
α-Globulin absolute (g L^{-1})	13.7	8.9	9.0	14.1	7.6
β-Globulin absolute (g L^{-1})	6.0	12.5	12.0	5.1	12.1
γ-Globulin absolute (g L^{-1})	26.5	21.3	22.1	29.6	26.7
Ratio albumin / globulin	0.60	0.75	0.76	0.62	0.68
Total protein (g L^{-1})	74	75	76	79	78
Acute phase proteins					
C-reactive protein (mg L^{-1})	100	35	30	62	57
Haptoglobin (g L^{-1})	0.93	0.71	0.67	0.56	0.13
Serology					
Antibodies against *Brucella spp.*	< 1:50	< 1:50	< 1:50	< 1:50	< 1:50
Antibodies against distemper virus	< 1:50	< 1:50	< 1:50	< 1:50	< 1:50

576 nd = not detected

577 **TABLE 3.** Element profile in whole blood samples (concentrations are given in µg L^{-1}) of seals
578 caught in the Elbe estuary compared to our previous study on seals of the German Bight
579 (Griesel et al, 2008).
580

	W 01/08 Pv	W 02/08 Pv	W 03/08 Pv	W 04/08 Pv	W 05/08 Pv	Seals German Bight
Al	17.2	29.9	16.2	14.5	13.1	<0.17 – 499
As	564	283	190	459	190	42.0 – 592
Be	1.28	1.39	1.20	1.18	1.04	< 0.08 – 1.80
Bi	2.20	2.50	1.86	1.86	1.65	
Ca	$59.1x10^3$	$66.5x10^3$	$45.3x10^3$	$74.3x10^3$	$48.2x10^3$	$29.8 – 55.0x10^3$
Cd	0.90	1.05	0.85	0.84	0.87	<0.12 – 3.10
Co	0.58	0.89	0.72	0.80	0.65	<0.02 – 7.56
Cr	6.36	7.56	5.96	4.24	5.02	1.52 – 84.9
Cs	0.74	1.98	1.23	0.68	1.15	
Cu	$1.09x10^3$	$1.54x10^3$	$1.06x10^3$	$0.70x10^3$	$1.13x10^3$	$0.53 – 1.40x10^3$
Fe	$670x10^3$	$993x10^3$	$810x10^3$	$244x10^3$	$797x10^3$	$520 – 1137x10^3$
K	$249x10^3$	$323x10^3$	$236x10^3$	$194x10^3$	$244x10^3$	$131 – 197x10^3$
Li	$4.52x10^3$	$3.90x10^3$	$4.78x10^3$	$8.20x10^3$	$3.93x10^3$	
Mg	$57.2x10^3$	$72.1x10^3$	$52.8x10^3$	$31.6x10^3$	$61.1x10^3$	
Mn	88.6	127	146	23.2	144	67 – 151
Mo	7.82	8.88	6.26	6.30	8.58	1.27 – 22.8
Na	$3.31x10^6$	$3.86x10^6$	$3.09x10^6$	$3.31x10^6$	$3.13x10^6$	
Ni	3.78	5.92	4.61	3.34	3.60	<0.38 – 25.7
Pb	11.4	8.88	3.63	3.80	7.81	<0.02 – 4.52
Rb	77.0	115	80.7	65.6	83.4	52 – 149
Se	$0.97x10^3$	$1.85x10^3$	$1.57x10^3$	$0.58x10^3$	$1.05x10^3$	$0.52 – 2.26x10^3$
Sn	1.81	1.66	1.01	0.81	0.93	<0.06 – 0.47
Sr	77.9	73.2	43.4	125.4	70.4	25 – 70
V	4.94	7.38	4.70	4.44	4.98	<0.05 – 1.30
Zn	$3.46x10^3$	$4.98x10^3$	$4.21x10^3$	$1.36x10^3$	$3.97x10^3$	$2.73 – 4.57x10^3$

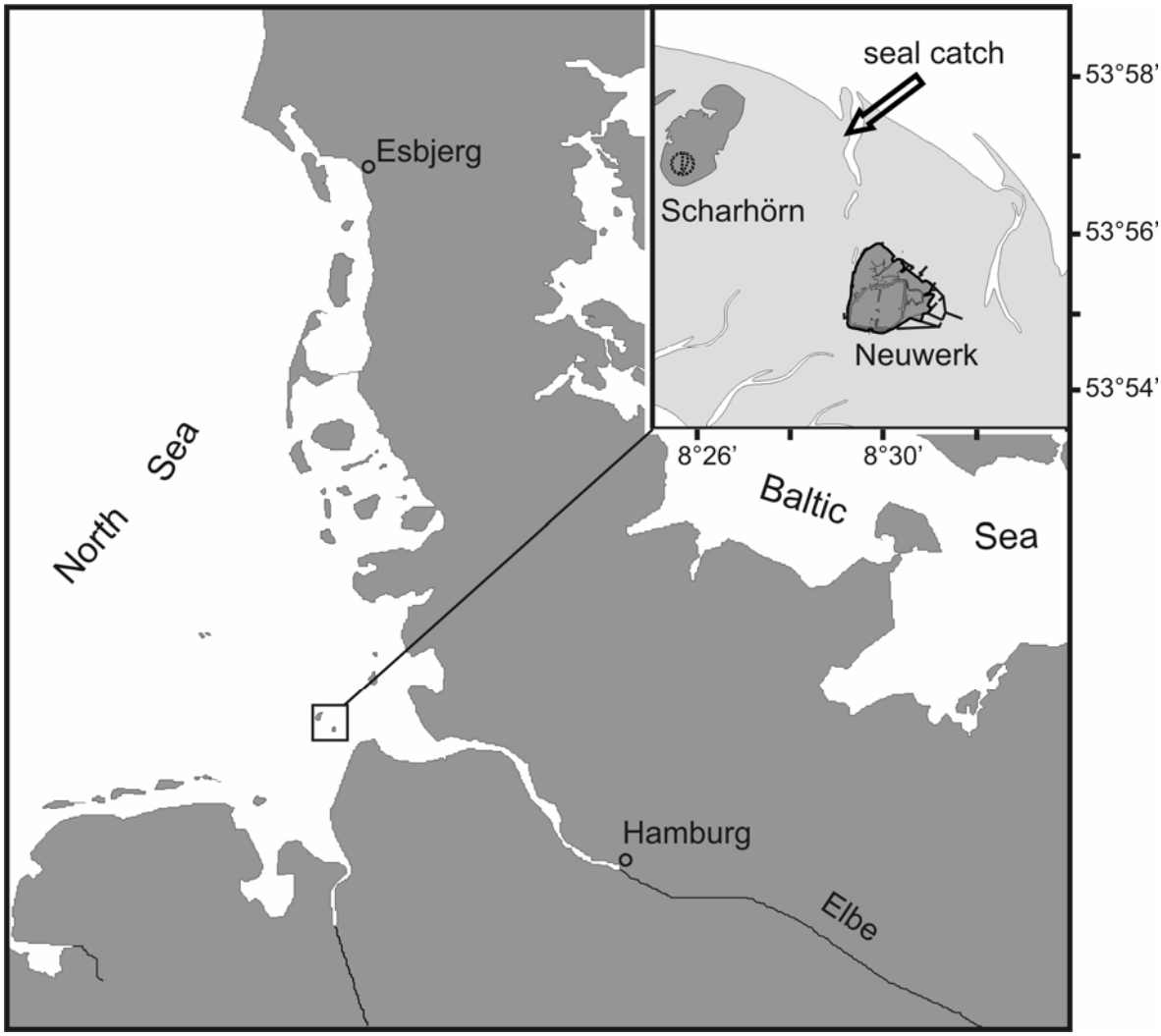
581 **TABLE 4.** Chlorinated pesticides and PCBs in plasma in ng L^{-1}.

582

	W 01/08 Pv	W 02/08 Pv	W 03/08 Pv	W 04/08 Pv	W 05/08 Pv
α-HCH	nd	9	2	2	3
HCB	271	2730	1860	1160	1400
β-HCH	5	12	4	5	6
γ-HCH	nd	9	2	3	3
δ-HCH	nd	6	4	nd	4
heptachlor	nd	8	2	3	4
aldrin	nd	9	2	3	4
heptachlor epoxide	nd	4	1	2	1
trans-chlordane	nd	2	< 1	< 1	nd
cis-chlordane	nd	2	nd	nd	nd
dieldrin	nd	9	3	2	3
endrin	nd	2	nd	nd	nd
endosulfan	nd	3	1	1	1
2,4'-DDE	nd	< 10	< 10	nd	nd
4,4'-DDE	1770	12000	6000	2030	3800
2,4'-DDT	nd	nd	nd	nd	nd
4,4'-DDT	nd	430	121	nd	nd
2,4'-DDD	nd	nd	nd	nd	nd
4,4'-DDD	nd	nd	nd	nd	nd
methoxychlor	nd	nd	nd	nd	nd
PCB 28	nd	92	73	21	66
PCB 52	11	39	37	30	73
PCB 77	nd	nd	10	nd	nd
PCB 81	nd	nd	nd	nd	nd
PCB 101	19	320	433	89	125
PCB 105	nd	59	21	5	15
PCB 114	nd	nd	nd	nd	nd
PCB 118	6	115	83	31	39
PCB 123	nd	nd	nd	nd	nd
PCB 126	nd	nd	nd	nd	nd
PCB 138	299	1000	1720	464	1040
PCB 153	216	3280	4950	1010	2890
PCB 156	nd	18	24	8	21
PCB 157	nd	6	7	nd	6
PCB 167	nd	nd	5	nd	5
PCB 169	nd	nd	nd	nd	nd
PCB 170	13	153	271	38	148
PCB 180	< 50	738	635	105	387
PCB 189	nd	nd	nd	nd	nd

583 nd = not detected

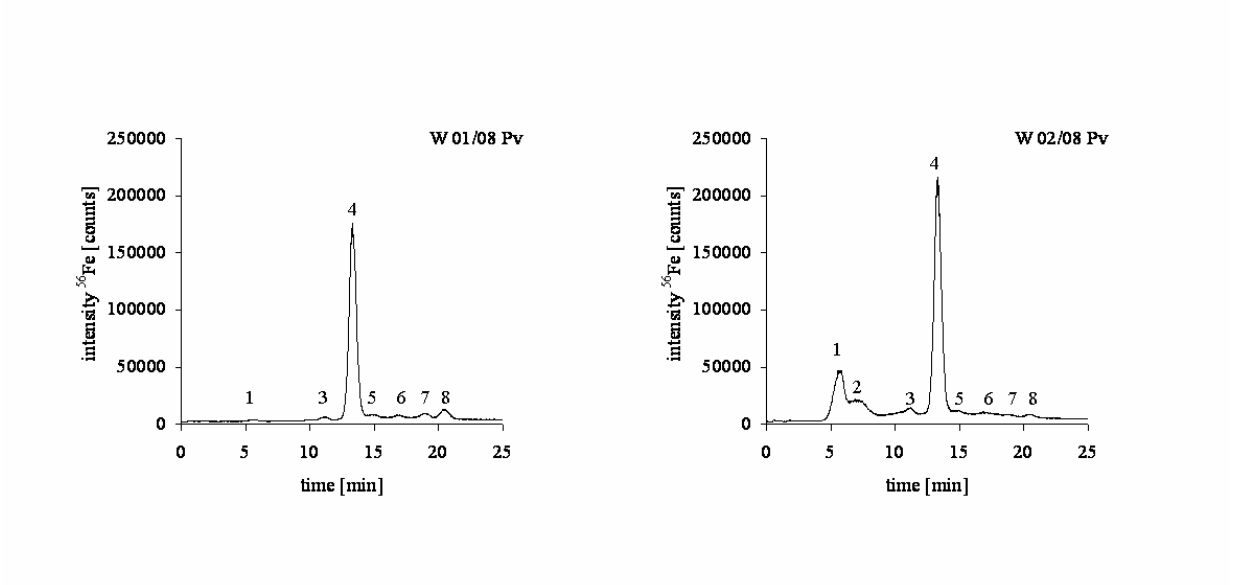
584



585

586

587 **FIGURE 1.** Sampling location in the estuary of the river Elbe.



588

589 **FIGURE 2.** Anion-exchange chromatograms of the separated Tf isoforms (1-8) from seals of
590 the Elbe estuary measured by ICP-MS (^{56}Fe), one typical chromatogram for each group: group
591 I (W01/08 Pv, W03/08 Pv, W05/08 Pv); group II (W02/08 Pv, W04/08 Pv).

592

593

594

595

596

597

598

599

- Supporting Information -

FIRST HEALTH AND POLLUTION STUDY ON HARBOR SEALS (*PHOCA VITULINA*) LIVING IN THE GERMAN ELBE ESTUARY

Antje Kakuschke[a,], Elizabeth Valentine-Thon[b], Simone Griesel[a], Juergen Gandrass[a], Octavio Perez Luzardo[c], Luis Dominguez Boada[c], Manuel Zumbado Peña[c], Maira Almeida González[c], Mechthild Grebe[a], Daniel Pröfrock[a], Hans-Burkhard Erbsloeh[a], Katharina Kramer[a], Sonja Fonfara[d], Andreas Prange[a]*

a GKSS Research Center Geesthacht, Marine Bioanalytical Chemistry, Institute for Coastal Research, Max-Planck-Strasse 1, 21502 Geesthacht, Germany

b Laboratory Center Bremen, Department of Immunology, Friedrich-Karl-Strasse 22, 28205 Bremen, Germany

c University of Las Palmas de Gran Canaria, Toxicology Unit, Department of Clinical Sciences, and Instituto Canario de Investigación del Cáncer, P.O. Box 550, 35080 Las Palmas de Gran Canaria, Spain

d Small Animal Teaching Hospital, University of Liverpool, Leahurst, Chester High Road, Neston CH64 7TE, United Kingdom

[*] Corresponding author:

Antje Kakuschke

GKSS Research Center Geesthacht, Marine Bioanalytical Chemistry

Institute for Coastal Research, 21502 Geesthacht, Germany

E-mail address: antjekakuschke@web.de

Tel: +49 40 42913833

- **6 pages (including cover page)**
- **2 Tables**
- **2 Figures**

Supporting information S1

Experimental Section

Lymphocyte proliferation assay. Blood was collected into CPDA monovettes (Citrate-Phosphate-Dextrose-Adenin) and stored at room temperature until further analysis (no longer than 15 hours). Lymphocytes were separated on a Ficoll-Histopaque gradient, and $1x10^6$ cells were pipetted into the wells of a 24-well plate according to the planned cell culture design. Cells were cultured with 2 µg/mL Poke Weed Mitogen (PWM) to investigate the mitogen-stimulated proliferation, as well as without a mitogen to investigate the non-stimulated cell proliferation. After 5 days of incubation at 37 °C in a 5 % CO_2 environment, cells were incubated for further 4 hours with 3 µC methyl-^{3}H-thymidine. The cells were then harvested onto filters and the radioactivity measured in a scintillation counter. The incorporation of thymidine was expressed as counts per minute (cpm). The stimulation index (SI) was calculated as followed:

SI = mitogen-stimulated proliferation (cpm) / non-stimulated proliferation (cpm).

The metal-specific lymphocyte proliferation was investigated in the same way as described for the mitogen- and non-stimulated lymphocyte proliferation.

TABLE S1. Retention time (RT) and peak areas of the different Tf isoforms separated by using anion-exchange chromatography and ICP-MS detection (^{56}Fe) (see also Figure 2).

	W01/08Pv			W03/08Pv			W05/08Pv		
	RT [min]	area	area [%]	RT [min]	area	area [%]	RT [min]	area	area [%]
1	5.64	761980	0.95%	5.76	294793	0.39%	5.49	250870	0.25%
2									
3	11.14	1440292	1.80%	11.23	2423224	3.20%	11.19	3594180	3.52%
4	13.30	69439070	86.86%	13.34	68864186	90.88%	13.36	91079159	89.16%
5	14.96	1808274	2.26%	14.89	1217463	1.61%	14.83	2027508	1.99%
6	16.80	997190	1.25%	16.81	820935	1.08%	16.93	923994	0.91%
7	18.88	1888518	2.36%	18.98	733823	0.97%	18.92	1321894	1.29%
8	20.48	3610448	4.52%	20.36	1420827	1.88%	20.59	2952922	2.89%
sum		79945772	100.00%		75775251	100.00%		102150527	100.00%
sum 1+2			0.95%			0.39%			0.25%

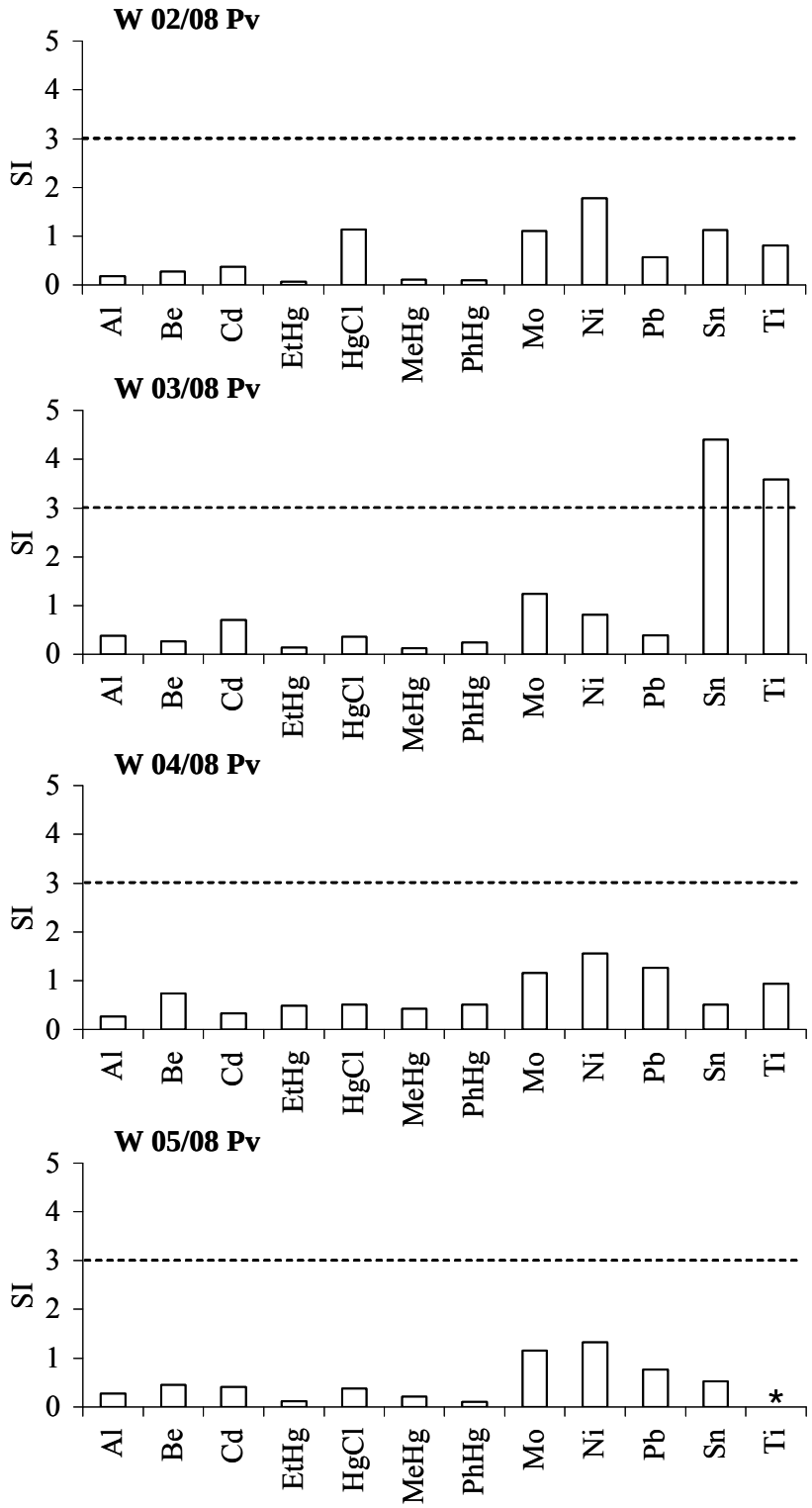
	W02/08Pv			W04/08Pv		
	RT [min]	area	area [%]	RT [min]	area	area [%]
1	5.59	27023632	20.08%	5.66	20500322	17.00%
2	6.88	15069021	11.20%	7.02	7580251	6.29%
3	11.22	3684830	2.74%	11.14	2413238	2.00%
4	13.27	84483871	62.78%	13.27	82892816	68.73%
5	14.93	1274499	0.95%	15.02	1941601	1.61%
6	16.78	1164307	0.87%	16.93	1295482	1.07%
7	18.68	562146	0.42%	18.97	1328465	1.10%
8	20.43	1299533	0.97%	20.36	2646871	2.20%
sum		134561839	100.00%		120599046	100.00%
sum 1+2			31.28%			23.28%

649 **TABLE S2.** Clinical chemistry and bacteriology of seals of the Elbe estuary.

	W 01/08 Pv	**W 02/08 Pv**	**W 03/08 Pv**	**W 04/08 Pv**	**W 05/08 Pv**
Clinical chemistry					
Alkaline phosphatase (U L^{-1})	25	70	46	75	31
Aspartate aminotransferase (AST, U L^{-1})	66	109	63	101	176
Alanine aminotransferase (ALT, U L^{-1})	40	82	45	46	176
Gamma-glutamyl transferase (gamma-GT, U L^{-1})	14	10	11	9	12
Cholinesterase (KU $L^{-1)}$)	2197	1585	1598	2092	2427
Glutamate dehydrogenase (GLDH, U L^{-1})	5.57	21.05	9.47	8.43	28.12
Lactate dehydrogenase (LDH, U L^{-1})	681	854	538	859	984
Alpha-Amylase (U L^{-1})	325	317	533	323	410
Lipase (U L^{-1})	71	65	48	109	72
Creatine kinase (CK, U L^{-1})	555	1681	214	2366	323
Bilirubin total (µmol L^{-1})	3.08	2.05	3.59	2.39	5.30
Cholesterol (mmol L^{-1})	5.59	6.66	5.96	5.46	4.97
Triglyzeride (mmol L^{-1})	0.90	5.72	1.32	0.72	0.91
Creatinine (µmol L^{-1})	44.2	62.8	59.2	51.3	62.8
Bile acid (µmol L^{-1})	28.6	65	3	20.7	10.5
Urea (mmol L^{-1})	21.15	27.14	22.64	20.65	16.48
Uric acid (µmol L^{-1})	113	268	173	143	n.d.
Glucose (mmol L^{-1})	9.05	5.33	6.77	6.33	9.16
Chloride (mmol L^{-1})	106	101	n.d.	103	103
Phosphate (mmol L^{-1})	1.68	2.83	1.77	2.01	2.13
Free Thyroxine (T4, ng dL^{-1})	1.10	n.d.	1.29	1.09	1.41
Cortisol (ng mL^{-1})	205	56	155	133	164
Folic acid (Vitamin B 9, ng mL^{-1})	6.3	8.5	7.7	5.7	6.8
Vitamin B 12 (pg mL^{-1})	1570	1073	1255	3619	1473
Bacteriological investigations					
Swab (anus)	*hemolytic E. coli*, abundant	*E. coli*, moderate	*Edwardsiella tarda*, moderate	*hemolytic E. coli*, moderate	*E. coli*, moderate

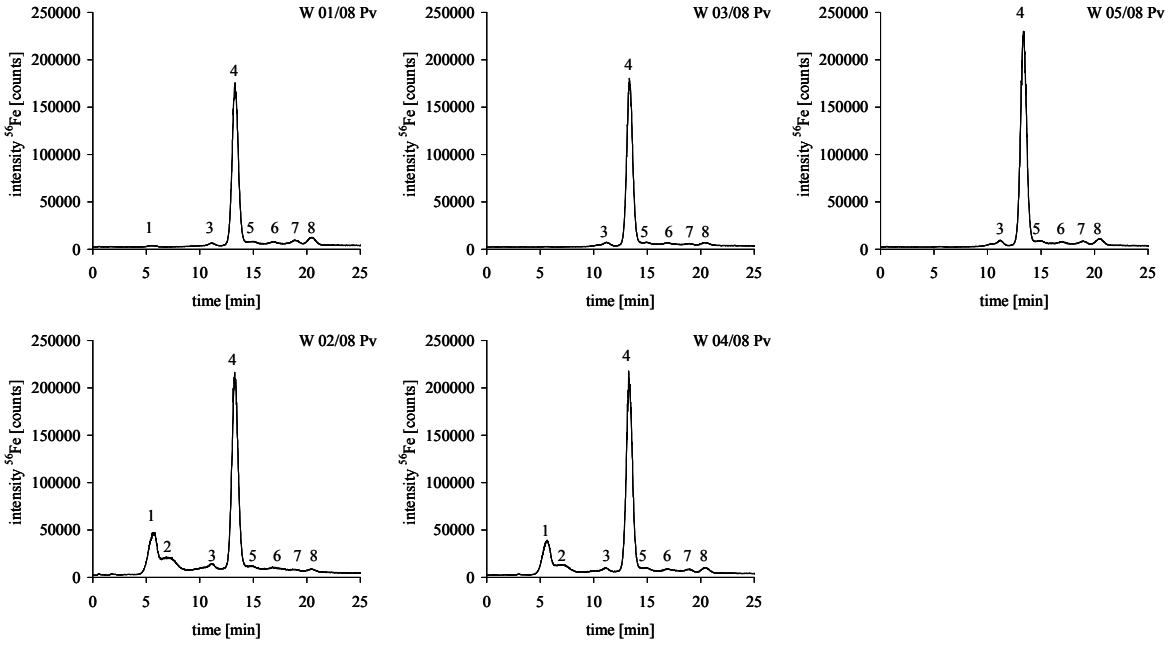
650

651



652

653 **FIGURE S1:** Metal-induced proliferation of lymphocytes (SI = stimulation index) of four seals
654 of the Elbe estuary (dotted line: SI = 3, SI ≥ 3 was regarded as a positive hypersensitivity
655 response; * metal not tested).



656

657 **FIGURE S2.** Anion-exchange chromatograms of the separated Tf isoforms (1-8) from all five

658 seals investigated measured by ICP-MS (^{56}Fe).

659

660

661