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Oxidative stress biomarkers in the copepod *Limnocalanus macrurus* from the northern Baltic Sea: effects of hydrographic factors and chemical contamination

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ABSTRACT: Zooplankton channels energy and various inorganic and organic substances from primary production to consumers at higher trophic levels; thus, its nutritional value as well as content of harmful substances may have profound effects on marine food webs. Indicators of environmental stressors such as oxidative stress biomarkers are highly useful in estimating how environmental factors affect lower levels of the food web. These biomarkers were determined in field-collected brackish water calanoid copepods *Limnocalanus macrurus* to analyze possible spatial variations in specimens collected from open-sea areas of the northern Baltic Sea. *L. macrurus* from the Bothnian Sea showed elevated levels of glutathione metabolism-associated enzyme activities and total glutathione (totGSH), whereas samples from the Gulf of Finland showed higher levels of superoxide dismutase activity (SOD) and lipid peroxidation (LPX), and a lower ratio of reduced to oxidized glutathione (GSH/GSSG). Hydrographic factors and selected indirect proxies describing general contaminant loads in the study areas partly explained the observed differences in biomarker values. The higher levels of SOD, LPX and lower GSH/GSSG together with high concentrations of polychlorinated dibenzodioxins/furans (PCDD/Fs) and polychlorinated biphenyls (PCBs) measured in the *L. macrurus* samples suggest the presence of multiple environmental stressors in the Gulf of Finland compared to the Bothnian Sea. The novel miniature biomarker measurement methods used and results obtained can be further applied in studies on the effects of environmental stressors in zooplankton species and communities in conjunction with environmental quality assessments.

KEY WORDS: *Limnocalanus macrurus* · Zooplankton · Oxidative stress biomarkers · Baltic Sea

INTRODUCTION

The Baltic Sea is semi-enclosed large brackish water basin in northern Europe surrounded by 9 countries. Its position and special characteristics make it especially vulnerable to both human impacts and natural changes. Animals and plants inhabiting the Baltic Sea are subjected to multiple stressors, from considerable spatial, seasonal and vertical variability in hydrography to human-generated loads of harmful substances and nutrients (Voipio 1981). The Baltic Sea has one of the longest recorded histories of contamination and is often described as the most polluted sea in the world. According to the integrated classification of hazardous substances produced by the Baltic Marine Environment Protection Commis-

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sion (HELCOM), all open sea units of the Baltic Sea are currently considered as 'areas disturbed by hazardous substances' (HELCOM 2010). In a recent assessment, the toxicity of sediment samples collected from the northern Baltic Sea indicated markedly larger problems in the Gulf of Finland in comparison to the Gulf of Bothnia, mainly related to the hypoxic/anoxic conditions prevailing in the deep areas of the former region (Berezina et al. 2013). Large areas of the sea are highly eutrophic and suffer from excess loads of nutrients causing harmful algal blooms and adverse effects at the ecosystem level (HELCOM 2009). Over the past century, the Baltic Sea has also been subjected to a mean temperature increase greater than that reported on a global scale (BACC Author Team 2008): between 1982 and 2006, the sea surface temperature in the Baltic Sea increased by 1.35°C, more than in any other of the 63 large marine ecosystems of the world (Belkin 2009). The impacts of the various environmental stressors on different groups of organisms in the Baltic Sea vary spatially and temporally and affect the marine ecosystem in ways that are not yet fully understood. Research efforts targeting the effects of hazardous substances on organisms (and thus on the entire Baltic Sea ecosystem) have been dwarfed by the abundant number of studies on eutrophication caused by the excess of nutrients (Lehtonen & Schiedek 2006a,b). However, a true understanding of the malfunctioning of the Baltic Sea marine system should encompass both elements. This is especially important since eutrophication initially increases overall biological productivity, whereas the toxic effects of different chemicals decrease it.

Zooplankton are an important part of marine food webs, channeling energy and essential nutrients from primary production as well as key biochemical constituents (e.g. vitamins) to consumers at higher trophic levels. Thus, the species composition of the zooplankton, its nutritional value and the accumulation of beneficial as well as harmful substances (e.g. anthropogenic contaminants and algal toxins) may have consequences further up the food web. Copepods (which belong to Crustacea) are commonly the dominant members of zooplankton communities, and are major food organisms for small fish, seabirds and larger crustaceans, both in marine and freshwater pelagic and coastal systems. The calanoid copepod *Limnocalanus macrurus* is a brackish water, cold-water stenoterm species with a wide geographical distribution (Vanderploeg et al. 1998 and references therein). In the northern Baltic Sea it is the dominant zooplankton species in the Bothnian Bay and the Bothnian Sea during most of the year (Dahlgren et al. 2010), and is also abundant in the Gulf of Finland (Ojaveer et al. 1998, Peltonen et al. 2014). *L. macrurus* can therefore be considered an important component of the marine food web of the northern Baltic Sea.

Alterations in various abiotic and biotic factors may affect an organism's redox balance and cause a physiological state called oxidative stress. For marine organisms, some of these key environmental factors include temperature, oxygen concentration, chemical contaminants and changes in the composition and transfer of non-enzymatic antioxidants in the food web (Lesser 2006, Häubner 2010). Oxidative stress is essentially an imbalance between the production of reactive oxygen species (ROS) and the detoxification of reactive intermediates, which may result from increased ROS production or reduced oxidative defence capacity. ROS are involved in normal cellular signalling, but excessive amounts can cause damage to macromolecules such as lipids, protein and DNA, inhibiting their normal function (Halliwell & Gutteridge 2007, Monaghan et al. 2009). However, it should be noted that high levels of ROS do not necessarily result in oxidative stress if the ROS can be effectively handled by increased activities of antioxidant enzymes or buffered by non-enzymatic antioxidant molecules; nor does it follow that individuals having relatively high levels of non-enzymatic antioxidants and/or high activities of antioxidant enzymes are necessarily in a less oxidized state than those with lower levels (Monaghan et al. 2009).

In copepods, environmental stressors such as contaminants as well as changes in temperature and salinity have been found to increase the transcription levels of oxidative stress defence related mRNAs (Seo et al. 2006, Hansen et al. 2007, 2008, 2010, Lee et al. 2008). However, functional studies regarding oxidative stress responses in marine zooplankton to environmental stressors are still scarce. Cailleaud et al. (2007, 2009b) and Wang & Wang (2009) showed that in copepods, changes in salinity or temperature and exposure to hydrophobic contaminants or cadmium affect oxidative stress biomarkers. Apart from effects on individual fitness, oxidative stress may have consequences for the life-history traits of organisms (Monaghan et al. 2009, Rodriguez-Grana et al. 2010).

In the present study, oxidative stress biomarkers were determined in field-collected *L. macrurus* from different areas of the northern Baltic Sea. The geographical variability of biomarker values was investigated in association with selected environmental

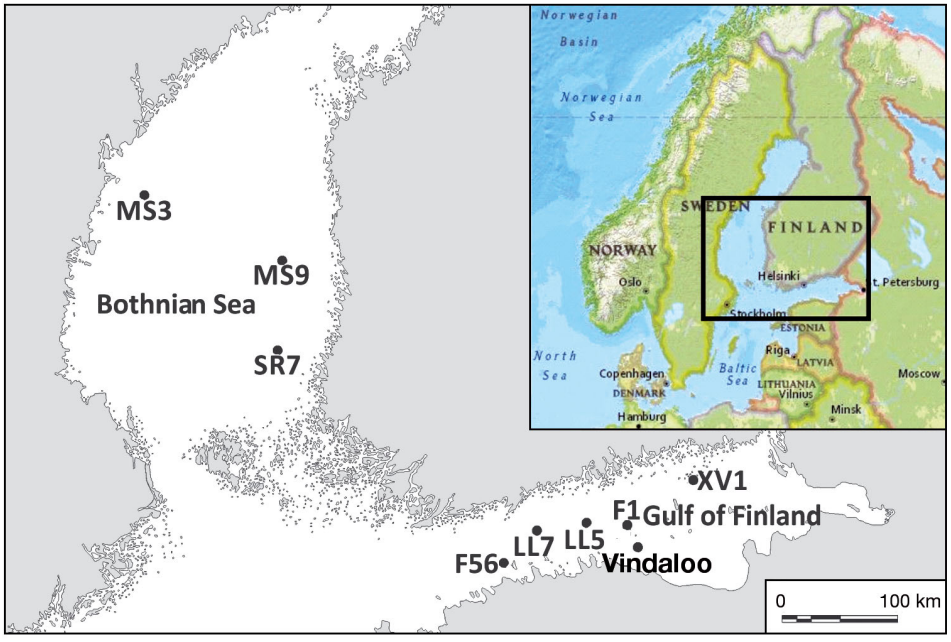

Fig. 1. Sampling locations in the Gulf of Finland and Bothnian Sea in the Baltic Sea

variables. Data on hydrography measured at the time of sampling as well as indirect proxies of environmental contamination in the study areas such as whole sediment bioassays, sediment trace metal concentrations and dioxin and polychlorinated biphenyl (PCB) levels in zooplankton communities were used as factors associated with the observed differences. The main rationale behind this research was the concept that functional indicators of environmental stresses such as oxidative stress biomarkers can be highly useful in estimating how environmental changes affect the lower levels of the food web. These issues have been too seldom investigated considering their undeniable significance for the correct functioning of the whole ecosystem, and also for directing proper actions for the protection and management of the marine environment.

MATERIALS AND METHODS

Sampling of zooplankton

Samples of *Limnocalanus macrurus* were collected at 9 offshore sites located in the Bothnian Sea (BS) and the Gulf of Finland (GoF) in the northern Baltic Sea (Fig. 1). The samplings were carried aboard the expeditions of the R/V 'Aranda' of the Finnish Environment Institute (SYKE) during the BONUS+ programme BEAST project (Biological Effects of Anthropogenic Chemical Stress: Tools for the Assessment of Ecosystem Health; Lehtonen et al. 2014) in August and September 2009 in the GoF, and August and September 2010 in the BS. Zooplankton samples were taken with a WP2 plankton net (mesh size 500 µm) with a closing mechanism that enabling sampling from the near-bottom water layer up to the thermocline at each location (approximately 30 m).

Individual *L. macrurus* are less than 2 mm in size. Thus for the measurement of biomarkers, a total of 30 individuals were pooled in each sample in order to yield a sufficient sample volume for enzyme activity and glutathione (GSH) assays. A pool of 15 individuals was sufficient for the determination of lipid hydroperoxide (LPX). The pools of *L. macrurus* were transferred manually with a pipette from a vial kept at 4°C to a collection vial containing water at the same temperature. To concentrate organisms, they were then quickly lifted out of the water on a special removable sieve. The specimens were subsequently transferred to an Eppendorf tube, frozen immediately in liquid nitrogen, and stored at −80°C until further processing.

Determination of enzyme activities, concentrations of GSH and levels of LPX

The samples were homogenized using a TissueLyser II bead mill (Qiagen) in 100 µl of 0.1 M K_2HPO_4 + 0.15 M KCl buffer (pH 7.4) for the protein homogenate, and in 125 µl of methanol for LPX determination. The protein homogenate was centrifuged for 15 min at 10 000 × *g* at 4°C. The resulting supernatant

was divided into several aliquots; each one was used for the preparation of a sample for the determination of GSH and the rest were frozen in liquid nitrogen and stored at −80°C until further measurements. Samples for the GSH determination were made from the protein homogenate via deproteinization by adding a 1:8 volume of 5% sulfosalicylic acid (SSA). The sample was incubated with SSA on ice for 10 min and centrifuged for 10 min at 10000 × *g* at 4°C. The supernatant was frozen in liquid nitrogen and stored at −80°C. The methanol homogenate was centrifuged at 5000 × *g* at room temperature for 10 min, and the supernatant was divided into 2 aliquots and stored at −80°C.

Glutathione reductase (GR; Enzyme Commission number [EC] 1.8.1.7) activity was measured according to Smith et al. (1988), which is based on the reduction of oxidized glutathione (GSSG) by GR. The product, GSH, reacts spontaneously with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) forming 5-thio(2-nitrobenzoic acid) (TNB), which can be measured with a spectrophotometer. Glutathione *S*-transferase (GST; EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to Habig et al. (1974), with the exception of using 2 mM GSH instead of 1 mM. Glutathione peroxidase (GP; EC 1.11.1.9) activity was measured using a commercial kit (Sigma Chemicals) with 2 mM H_2O_2 as the substrate. The inhibition rate of superoxide dismutase (SOD; EC 1.15.1.1) was measured using a commercial kit (Fluka). The reduced and oxidized glutathione species (i.e. GSH and GSSG, respectively) were measured with ThioStar glutathione detection reagent (Arbor Assays) using GSH as the standard (Sigma Chemicals). The sample (containing 5% SSA) was diluted 1:5 with 100 mM Na-phosphate buffer + 5 mM EDTA (pH 7.5) to obtain 1% SSA. Standards were diluted with 100 mM Na-phosphate + 5 mM EDTA + 1% SSA. A total of 12.5 µl of sample, standard or blank was pipetted on a 384-well microplate, and 6.5 µl of ThioStar reagent was added. The plate was then incubated for 15 min in the dark and fluorescence was measured (excitation 405 nm, emission 510 nm) to determine the free (reduced) glutathione concentration. After the first measurement, 6.5 µl of 4 mM NADPH + 8 U ml^{-1} GR in 100 mM Na-phosphate buffer + 5 mM EDTA (pH 7.5) was added to the wells, following a 15 min incubation in the dark, and fluorescence was measurement (excitation 405 nm, emission 510 nm) to determine the total glutathione concentration (totGSH). The enzyme activities and totGSH were normalised to the protein content of the samples, which was determined with the Bradford method using a Bio-Rad protein assay (BioRad) with bovine serum albumin (Sigma) as the standard. LPX levels were measured using the FOXII assay modified from the protocols described by Eymard & Genot (2003) and Bou et al. (2008) using 45 µl of sample, 5 µl of either 10 mM triphenylphosphine (TPP) or methanol, and 950 µl of the FOX reagent. After the addition of TPP, the samples were incubated for 0.5 h, and after adding the FOX reagent the samples were incubated for 2 h before measurement.

The analyses were conducted with an EnSpire™ plate reader (Wallac, PerkinElmer Life Sciences) using 96-well (protein and FOX assays) and 384-well plates (enzyme activities and glutathione, respectively). In order to perform all the projected measurements using the small sample volumes, GST, GR, GP, SOD and glutathione assays were miniaturised to total assay volumes of 25 to 50 µl. The measurements were performed for each sample in 3 replicates. Mean coefficient of variation percentages (CV%) of the triplicate measurements in the assays ranged from 4.0 to 6.6.

Five of the full measurement protocols described above were conducted aboard the R/V 'Aranda' during the 2009 expedition and 3 during the 2010 expedition; the rest were performed later in a land-based laboratory. The preparation and analyses of the evaluated biomarkers aboard a marine research vessel is a major technical step forward, and the subsequent rapid availability of results is a highly useful asset for outreach purposes.

Hydrographical observations and sampling of sediments

Onboard measurements of common hydrographical variables were routinely carried out at each study site prior to the sampling of zooplankton. Data on water conductivity (salinity), temperature and fluorescence (reflecting the amount of phytoplankton) in the water column were obtained using a SeaBird CTD sonde. A Rosette serial bottle sampler was used for the collection of water at different depths for the analysis of nutrient and oxygen concentrations. Near-bottom water samples (ca. 1 m above the sediment) were obtained separately using a single-sample water bottle. Sediment samples (upper 2 cm layer) for trace metal concentration measurements and whole-sediment toxicity bioassays were collected in 4 replicates using a GEMAX dual corer, and stored at 5°C.

Sampling and analysis of organochlorine compounds in zooplankton

Mesozooplankton were sampled during the annual HELCOM monitoring cruise (COMBINE 3), of the R/V 'Aranda' between 16 and 26 August 2010 for the determination of polychlorinated dibenzodioxins/furans (PCDD/Fs) and PCBs. At each sampling location, the water column was sampled with vertical hauls from close to the bottom to the surface using a 100 µm Hydrobios WP2 closing net. Separate comparable hauls were conducted for the determination of species composition at each sampling location. Samples for chemical analyses were pooled within each basin to ensure that the biomass was sufficient to enable reliable laboratory measurements of PCDD/F and PCB compounds above analytical detection limits. The pooled samples were collected from the BS (4 sampling locations), and the western and eastern GoF (4 and 6 locations, respectively). Concentrations of all 17 toxic congeners of the PCDD/Fs and 37 PCBs were determined in the laboratory of the National Institute for Health and Welfare, Kuopio, Finland (Peltonen et al. 2014). Species composition of the zooplankton samples at each location was determined microscopically in the laboratory of the Marine Research Centre of SYKE following the guidelines of HELCOM (for details, see Annex C in www.helcom.fi/action-areas/monitoring-and-assessment/manuals-and-guidelines/combine-manual).

Analysis of trace metals in sediments

Analysis of sediment trace metal concentration was carried out as described in Kulikova & Seisuma (2005). Briefly, cadmium (Cd), lead (Pb), copper (Cu), zinc (Zn), nickel (Ni), manganese (Mn) and iron (Fe) were extracted from the sediment samples with concentrated HNO_3. The samples were analyzed by atomic absorption spectrophotometry (AAS-1, Carl Zeiss, Jena and Perkin Elmer 403). Mercury (Hg) was extracted by a mixture of concentrated H_2SO_4, HNO_3 and HCI in the presence of $KMnO_4$ and $K_2S_2O_8$, and total Hg was measured by AAS-1 and a Flow Injection Mercury System (FIMS; Perkin Elmer). Standard samples from the National Research Council of Canada were used (BEST-1 for marine sediments, MESS-2 for Hg, HISS-1 and MESS-2 for the other trace metals) with recovery rates varying between 90 and 95%. The measured trace metal concentrations used in the statistical analyses were corrected for organic content (loss-on-ignition) of the sediment.

Statistical treatments

PASW Statistics 22 software (SPSS) was used for investigating the association of variables, with Spearman correlation and principal component analysis (PCA). In the case of a lack of an adequate amount of sample material for analysis, the result was taken to be a missing value. These missing values (n = 2 in GR, n = 3 in GP, n = 1 in SOD) were imputed with the multiple imputation procedure in PASW for the PCA, but not used in other statistical analyses (where unequal sample sizes were allowed). The variation in GR, GST, GP and SOD associated with hydrographical variables, sediment trace metal concentrations and bioassay mortality (*Gmelinoides fasciatus* bioassay data from Berezina et al. 2013) was studied with curve estimation (PASW) and generalized linear mixed models (GLMMs with lognormal error distribution and identity link function) using the SAS statistical software v.9.2 (SAS Institute). Degrees of freedom were calculated with the Kenward-Roger method. Due to high intercorrelation between the hydrographical variables, PCA scores combining all significant hydrographical variables from initial inspections were used as explanatory factors in the GR, GST and GP models. Sampling site was used as a random factor in all these models. The between-site variation in oxidative stress variables GSH/GSSG and LPX was studied with the Kruskal-Wallis multiple comparison in PASW. Throughout the study, results were considered statistically significant at $p < 0.05$.

RESULTS

Physical and chemical water quality characteristics

Hydrographical conditions in the entire study area varied, especially between the different basins (GoF and BS) but also among the different sampling sites (Table 1). The thermocline was observed at a depth of ca. 30 m from the surface at all sampling sites, but other physicochemical conditions below that differed between the basins. In general, the water below 30 m was warmer in the GoF than in BS while oxygen concentrations were markedly higher in the BS than in GoF, especially in the near-bottom layer (ca. 1 m from the bottom). Salinities below 30 m were on average between 6.8 in the GoF and 5.8 in the BS, which in the Baltic Sea is a difference large enough to modify the species composition of the ecosystem. Total nitrogen and phosphorus concentrations and fluorescence

Table 1. Depth, Secchi depth and main hydrographic variables measured at the Gulf of Finland (GoF) and Bothnian Sea (BS) sites; n/a: not available

Site	Secchi (m)	Depth (m)	Temperature (°C)	Salinity (ppm)	Oxygen (ml l^{-1})	CTD-fluorescence (v)	Total nitrogen (µmol l^{-1})	Total phosphorus (µmol l^{-1})
XV1 GoF	4.5	1	17.5	4.2	6.4	0.3	20.1	0.3
		30	6.8	5.3	4.3	0.1	25.2	1.0
		50	4.3	7.0	2.3	0.1	26.3	1.8
		63	4.6		0.8		28.7	3.0
Vindaloo	4.0	1	17.2	4.9	6.2	0.4	22.2	0.4
GoF		30	6.4	5.3	5.5	0.1	22.4	0.8
		60	4.6	8.2	1.8	0.1	25.4	2.6
		73	4.9		0.3		23.8	4.6
F1 GoF	4.0	1	17.1	4.8	6.3	0.5	25.3	0.4
		30	4.2	6.2	5.6	0.1	23.2	1.0
		50	4.2	7.6	3.3	0.1	23.8	1.7
		63	4.5		2.0		25.0	2.5
F56 GoF	4.5	1	17.0	6.0	6.1	0.4	22.1	0.4
		30	16.8	6.0	5.9	0.3	20.1	0.5
		70	5.0	8.9	0.7	0.1	20.4	4.1
		80	5.0		0.5		21.7	4.4
LL5 GoF	n/a	1	16.8	5.1	6.2	0.5	24.7	0.4
		30	6.4	5.6	5.1	0.1	25.1	0.7
		60	3.9	7.0	4.4	0.1	26.5	1.4
		67	4.1		3.1		27.3	2.0
LL7 GoF	4.0	1	16.4	5.6	6.3	0.5	29.2	0.4
		30	4.2	6.3	5.7	0.1	25.6	0.9
		90	4.6	8.4	1.3	0.1	29.2	3.7
		100	4.6		1.4		28.5	3.7
SR7 BS	6.5	1	16.1	5.3	6.0	0.3	18.1	0.2
		30	3.5	5.4	8.0	0.1	13.7	0.2
		60	2.8	5.9	6.9	0.1	16.1	0.5
		77	2.8		6.9		16.9	0.8
MS9 BS	n/a	1	16.1	5.4	6.2	0.3	16.3	0.1
		30	3.2	5.5	8.2	0.1	13.1	0.1
		90	2.7	6.4	6.6	0.1	16.8	0.6
		100	2.7		6.5		18.2	0.9
MS3 BS	7.0	1	13.7	5.1	6.6	0.3	15.0	0.2
		30	2.2	5.4	7.9	0.1	12.8	0.1
		70	3.1	6.0	4.9	0.1	20.5	0.7
		84	3.3		4.6		21.3	0.8

values indicate that the GoF is clearly much more eutrophic than the BS.

Contamination proxies

The BS sites were characterised by higher sediment trace metal concentrations than the GoF sites (Table 2). The concentrations of coplanar PCBs, other PCBs and PCDD/Fs based on the lipid levels in zooplankton were higher in the eastern GoF than in the BS or the western GoF (Table 3). The zooplankton biomass in each marine area mainly consisted of copepods, accounting for 94 and 86% in the western and eastern Gulf of Finland, respectively, and 76% in the BS. *Limnocalanus macrurus* increased towards the less saline areas, constituting ca. 25% of the biomass in the BS and 8% in the eastern GoF.

Oxidative stress biomarkers in *L. macrurus*

Eight different oxidative stress biomarkers were measured from pooled samples of field-collected *L. macrurus* individuals (Table 4). Statistical analysis on the associations among them showed that the bio-

Table 2. Sediment trace metal concentrations (µg g^{-1}) measured at the Gulf of Finland (GoF) and Bothnian Sea (BS) sites. Concentrations are corrected for the organic content (loss-on-ignition) of the sediment

Site	Hg	Cd	Pb	Cu	Ni	Zn
XV1 GoF	0.004	0.04	1.4	1.7	1.3	6.5
Vindaloo GoF	0.01	0.03	1.2	1.4	1.7	6.4
F1 GoF	0.04	0.09	4.8	2.5	3.5	12.4
F56 GoF	0.01	0.03	2.6	1.7	2.3	7.8
LL5 GoF	0.01	0.04	2.4	1.8	1.8	8.3
LL7 GoF	0.004	0.03	1.1	1.2	1.1	5.1
SR7 BS	0.02	0.1	4.7	3.6	11.3	20.4
MS9 BS	0.02	0.1	4.7	3.6	11.3	20.4
MS3 BS	0.01	0.04	2.2	2.9	2.9	12

markers of glutathione metabolism (GST, GR, GP, totGSH and GSH/GSSG) were positively correlated with each other and negatively correlated with LPX (Table 5). Both GSSG and SOD activity were positively correlated with LPX. In the PCA (Fig. 2A), GST, GP, GR, GSH/GSSG and totGSH were all associated with Component 1, while SOD and LPX had inverse relationships with these variables. Component 2 consisted of a positive association of GSSG with totGSH and a negative association with GSH/GSSG. The component matrix is provided in the Appendix. Samples from the GoF and the BS were clearly separated in the PCA score plots (Fig. 2). High activities of glutathione metabolism enzymes and high GSH/GSSG ratios were recorded in *L. macrurus* from the BS, whereas specimens collected from the GoF area had higher levels of SOD activity (Fig. 2A). Samples collected from the GoF also contained more GSSG, had lower GSH/GSSG ratios and higher LPX levels with almost half of these being above the zero line of the *y*-axis.

Effects of hydrographic factors on oxidative stress biomarkers

The higher oxygen content and lower temperature in the BS sites in comparison with the GoF sites were shown to be associated with higher activities of GST, GR, GP, higher levels of totGSH, higher GSH/GSSG ratios and lower levels of SOD activity, LPX and GSSG measured in *L. macrurus* (Fig. 2B). The component matrix is provided in the Appendix.

The effects of temperature, salinity and oxygen metrics on the different oxidative stress biomarkers in *L. macrurus* were inspected, first individually. Significant associations among the hydrographic variables and GST, GR, GP and SOD were found (data not shown). Due to the strong correlations between these variables, a PCA score combining the relevant hydrographic variables for each biomarker was used in the final models. The activity levels of GR, GST and GP were associated with the PCA scores of the selected hydrographic variables (Fig. 3). GR activity was higher in the cooler, more oxygenated and less saline water characteristic of the BS sites (GLMM, $F_{6.56} = 9.8$, p = 0.018) (Fig. 3A). GST and GP activities also were higher under these hydrographic conditions (GLMM, $F_{6.628} = 11.72$, p = 0.0121; GLMM, $F_{6.623} = 16.14$, p = 0.0057, respectively) (Fig. 3B,C). SOD, totGSH, LPX, GSH/GSSG ratios and GSSG did not show significant connections with the hydrographic variables.

Contamination proxies and their relationships with the oxidative stress biomarkers of *L. macrurus*

The levels of Cu and Zn showed significant associations with 5 of the measured oxidative stress bio-

Table 3. Lipid weight concentrations of coplanar polychlorinated biphenyls (PCBs), other PCBs and polychlorinated dibenzodioxins/furans (PCDD/Fs) in zooplankton samples from the Bothnian Sea (BS), western and eastern Gulf of Finland (GoF) (Peltonen et al. 2014)

	Coplanar PCB[a] (pg (g lipid)$^{-1}$)	Other PCB[b] (ng (g lipid)$^{-1}$)	PCDD/F[c] (pg (g lipid)$^{-1}$)
BS	341	91	60
GoF west	410	56	57
GoF east	652	114	121

[a]Sum of CO-PCB-77, CO-PCB-81, CO-PCB-126, CO-PCB-169

[b]Sum of PCB-18, PCB-28/31, PCB-33, PCB-47, PCB-49, PCB-51, PCB-52, PCB-60, PCB-66, PCB-74, PCB-99, PCB-101, PCB-105, PCB-110, PCB-114, PCB-118, PCB-122, PCB-123, PCB-128, PCB-138, PCB-141, PCB-153, PCB-156, PCB-157, PCB-167, PCB-170, PCB-180, PCB-183, PCB-187, PCB-189, PCB-194, PCB-206, PCB-209

[c]Sum of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,7,8,9-HxCDF, 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,7,8,9-HpCDF

Table 4. Oxidative stress biomarkers measured in *Limnocalanus macrurus* from the Gulf of Finland (GoF) and Bothnian Sea (BS) sites. GST: glutathione *S*-transferase; GP: glutathione peroxidase; GR: glutathione reductase; GSH/GSSG: ratio of reduced to oxidized glutathione; totGSH: total glutathione; GSSG: oxidized glutathione; LPX: lipid peroxidation; SOD: superoxide dismutase

Site		GST (µmol min^{-1} mg^{-1})	GP (µmol min^{-1} mg^{-1})	GR (nmol min^{-1} mg^{-1})	GSH/GSSG	totGSH (µM mg^{-1})	GSSG (µM mg^{-1})	LPX (µM cumene-hydroperoxide inhibition equivalents)	SOD inhibition (%)
F1 GoF	Mean	1.29	0.04	17.64	6.73	1.13	7.01	7.44	73.50
	N	10	9	10	10	10	10	10	10
	Min–max	0.65–2.05	0.01–0.09	0.01–37.34	2.01–21.14	0.14–2.42	2.34–16.15	3.84–10.43	49.32–84.28
F56 GoF	Mean	0.81	0.03	13.82	49.85	1.42	3.92	6.68	65.66
	N	11	11	10	11	11	11	11	11
	Min–max	0.65–1.15	0.02–0.05	7.32–18.73	1.78–190.17	0.01–4.41	2.41–5.74	1.77–14.15	43.38–76.46
LL5 GoF	Mean	0.77	0.01	15.39	32.88	1.33	4.73	8.02	67.98
	N	5	5	5	5	5	5	5	5
	Min–max	0.69–0.94	0.01–0.02	6.25–21.29	1.92–156.02	0.02–1.93	2.36–6.20	3.26–11.73	63.15–72.22
LL7 GoF	Mean	0.67	0.14	12.97	2.18	1.93	4.79	16.16	85.66
	N	7	6	6	7	7	7	7	7
	Min–max	0.56–0.74	0.03–0.27	5.04–19.74	1.49–3.03	1.61–2.64	3.59–5.85	6.19–21.32	41.02–96.65
Vindaloo GoF	Mean	1.15	0.03	16.12	5.49	0.60	4.35	7.58	73.78
	N	10	10	10	10	10	10	10	9
	Min–max	0.84–1.38	0.01–0.03	10.14–23.01	3.57–9.05	0.45–1.12	3.25–6.95	5.35–10.61	67.63–78.81
XV1 GoF	Mean	1.73	0.03	25.17	5.73	0.93	6.27	5.92	80.50
	N	10	10	10	10	10	10	10	10
	Min–max	1.13–2.71	0.02–0.05	17.25–36.68	2.50–9.69	0.39–2.22	3.27–10.00	2.01–10.70	70.87–86.34
MS3 BS	Mean	1.98	0.21	36.86	202.83	0.05	5.58	0.64	58.92
	N	11	11	11	11	11	11	11	11
	Min–max	0.95–4.30	0.13–0.29	29.30–47.08	27.06–272.66	0.02–0.17	3.43–8.08	0.10–3.88	44.84–68.55
MS9 BS	Mean	2.14	0.21	42.33	74.36	0.28	6.01	4.38	59.93
	N	11	11	11	11	11	11	11	11
	Min–max	1.27–4.58	0.16–0.27	35.65–55.43	7.14–295.24	0.02–0.63	4.48–7.78	0.10–15.31	52.88–67.16
SR7 BS	Mean	3.21	0.17	36.36	43.83	2.08	8.15	1.12	52.36
	N	8	7	8	8	8	8	8	8
	Min–max	2.47–4.11	0.10–0.21	16.52–60.01	0.37–213.05	0.03–4.42	3.28–14.50	0.23–3.27	19.79–64.88
Total	Mean	1.57	0.10	25.24	51.90	1.00	5.66	6.03	68.20
	N	83	80	81	83	83	83	83	82
	Min–max	0.56–4.58	0.01–0.29	0.01–60.01	0.37–295.24	0.01–4.42	2.34–16.15	0.10–21.32	19.79–96.65

markers. The higher levels of glutathione metabolism variables GR, GST totGSH in samples from the BS were associated with higher sediment Cu and Zn concentrations (Table 6). Furthermore, samples collected from sites with higher concentrations of Cu and Zn had lower activities of SOD (Table 6). LPX levels were lower in samples from sites with higher sediment concentrations of Cu (Table 6), reflecting the negative correlations between glutathione metabolism variables and LPX as well as that between glutathione metabolism variables and SOD (Table 5). GLMM used with both trace metals and hydrographic variables as explanatory factors showed no significant associations. GSH/GSSG ratios and GSSG did not show any significant associations with the sediment trace metal concentrations.

SOD activity levels were significantly associated with the amphipod mortality sediment bioassay results (GLMM, $F_{6.93} = 7.94$, $p = 0.0261$) and were highest in samples from the GoF Sites XV1, Vindaloo, LL5 and LL7 where the amphipod mortality rates were the highest.

Significant between-site differences were found both in GSH/GSSG and LPX (Fig. 4A,B); lower GSH/GSSG ratios or higher LPX both indicate that an organism is experiencing oxidative stress. The ratios of GSH/GSSG were significantly higher in *L. macrurus* samples from BS Site MS3 compared to those from all GoF sites and SR7, and in samples from Site MS9 compared to Site LL7. LPX was higher at all the GoF sites compared to the samples collected from Site MS3 in the BS. Significant differences in LPX levels

Table 5. Spearman rank correlation coefficients and significance (p-values) of oxidative stress biomarkers measured in *Limnocalanus macrurus*. See Table 4 legend for biomarker abbreviations

		GST	GP	GR	GSH/GSSG	totGSH	LPX	SOD
GP (μmol min^{-1} mg^{-1})	Correlation coefficient	0.449						
	p-value	0.000						
GR (nmol min^{-1} mg^{-1})	Correlation coefficient	0.706	0.622					
	p-value	0.000	0.000					
GSH/GSSG	Correlation coefficient	0.342	0.423	0.462				
	p-value	0.002	0.000	0.000				
totGSH (μM mg^{-1})	Correlation coefficient	0.550	0.328	0.567	−0.057			
	p-value	0.000	0.003	0.000	0.607			
LPX (μM cumene-hydroperoxide equivalents	Correlation coefficient	−0.535	−0.461	−0.576	−0.579	−0.169		
	p-value	0.000	0.000	0.000	0.000	0.128		
SOD inhibition%	Correlation coefficient	−0.340	−0.376	−0.463	−0.270	−0.148	0.523	
	p-value	0.002	0.001	0.000	0.014	0.186	0.000	
GSSG (μM mg^{-1})	Correlation coefficient	−0.180	−0.285	−0.283	−0.933	0.309	0.469	0.221
	p-value	0.104	0.011	0.010	0.000	0.004	0.000	0.046

were also found between Site SR7 and GoF sites LL7, LL5 and Vindaloo, and between Sites MS9 and LL7. The GSH/GSSG ratios were low and LPX values high also in the eastern GoF sites where the highest concentrations of coplanar PCBs, other PCBs and PCDD/Fs were measured in zooplankton samples.

DISCUSSION

The Baltic Sea basins GoF and BS are essentially different in regards to their hydrographical characteristics (Voipio 1981, Leppäranta & Myrberg 2009). The GoF is directly connected to the Baltic Proper

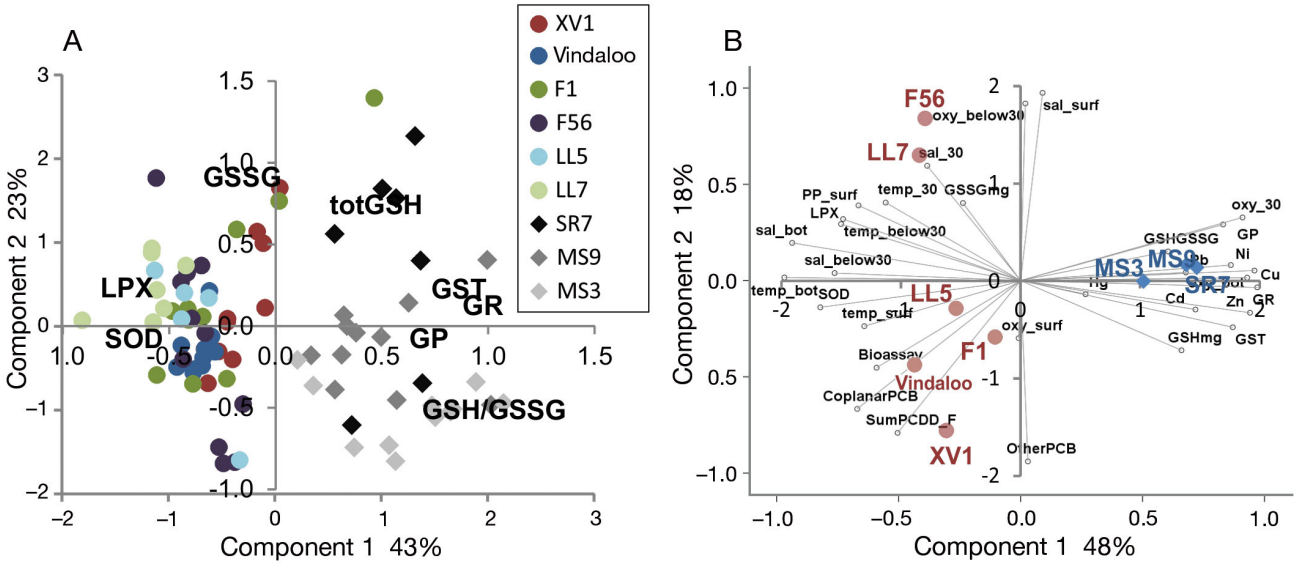

Fig. 2. (A) Component 1 and 2 scores from principal component analysis (PCA) for *Limnocalanus macrurus* samples collected from 6 sites from the Gulf of Finland (GoF) and 3 sites from the Bothnian Sea (BS). The corresponding loading plot (oxidative stress biomarkers) is superimposed on the scores plot. Sea areas are indicated by symbols (BS: diamonds; GoF: ovals), and sites are indicated with by colours. (B) Component 1 and 2 scores from PCA for site-wise hydrographic variables, sediment metal concentrations, sediment bioassay mortality, PCB and PCDD/F concentrations in zooplankton and oxidative stress biomarkers. The corresponding loading plot (sites) is superimposed on the scores plot. Blue diamonds: BS sites; red circles: GoF sites. See Table 4 for biomarker abbreviations; others as follows: oxy: oxygen; sal: salinity; temp: temperature; bot: bottom; surf: surface; 30: depth of 30 m. See Appendix for component matrices

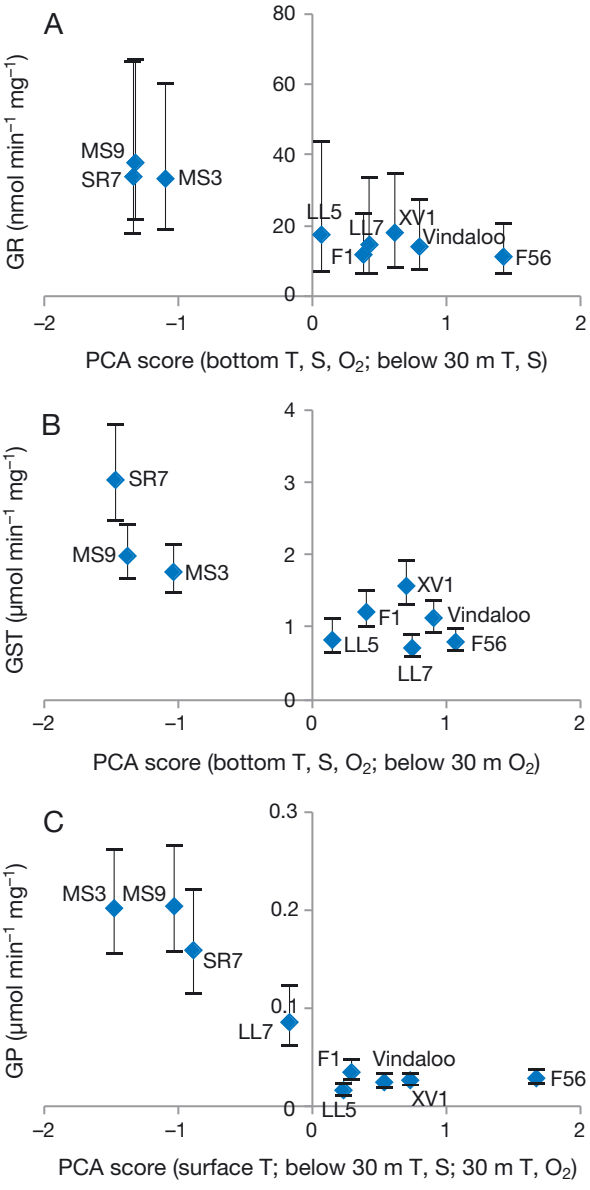

Fig. 3. Estimates of (A) glutathione reductase (GR), (B) glutathione *S*-transferase (GST) and (C) glutathione peroxidase (GP) activity in *Limnocalanus macrurus* from generalized linear mixed model (GLMM) principal component analysis (PCA) score combining the significant hydrographic variables (based on preliminary analysis of single explanatory variables) as the explanatory factor, and site as the fixed factor. Error bars: 95% confidence intervals. See Table 4 legend for biomarker abbreviations; others as follows: O_2 oxygen, S = salinity, T = temperature

from which stagnated saline deep water enters the gulf with subsequent major consequences to the salinity and oxygen conditions in the area. The GoF is stratified due to the salinity difference between the surface layer and the deep water, and the bottom waters are largely deficient of oxygen, which markedly

Table 6. Significant generalized linear mixed model (GLMM) results (F_{df}, p) of oxidative stress biomarkers measured in *Limnocalanus macrurus* samples collected from 6 sites from the Gulf of Finland (GoF) and 3 sites from the Bothnian Sea (BS) with Cu and Zn concentrations (µg g^{-1}) as explanatory factors and site as the fixed factor. See Table 4 legend for biomarker abbreviations

	Cu	Zn
GR	$24.87_{7.265}$, 0.0014	$14.48_{7.22}$, 0.0063
GST	$14.71_{6.918}$, 0.0066	$11.87_{6.956}$, 0.0109
totGSH	$7.15_{7.553}$, 0.0297	$6.18_{7.611}$, 0.0393
SOD	$15.62_{7.345}$, 0.005	$13.19_{7.386}$, 0.0076
LPX	$8.4_{7.178}$, 0.0224	

reduces the occurrence of benthic and mesopelagic fauna. The low oxygen concentration affects the survival of *Limnocalanus macrurus* and limits its geographical distribution (Roff 1973); as a result, the species was absent from some of our intended sampling sites in the GoF, apparently due to the low bottom oxygen concentration observed at those sites. The BS (i.e. the southern part of the Gulf of Bothnia) is separated from the Baltic Proper by shallow sills that limit water exchange and thereby maintain the characteristic hydrographical properties of the basin. Salinity increases slightly with depth in the BS but there is no permanent halocline. The water below the thermocline is often well-mixed, and oxygen deficiency is rarely observed in offshore bottom areas.

In this study, the observed spatial differences in abiotic hydrographic variables among the study sites and basins likely had an effect on the activities of GR, GST and GP in *L. macrurus*. The salinity in the northern Baltic Sea is low, and the biomarker responses measured in this study could differ from copepods living in more marine regimes. Results from previous studies suggest that hydrographic variables modulate the antioxidant defence of zooplankton organisms. Seo et al. (2006) found that salinity modulates transcription of the GR gene in the intertidal copepod *Tigriopus japonicus*, while Cailleaud et al. (2007) reported that salinity and temperature both affect GST activity in the copepod *Eurytemora affinis*. Experimental changes in temperature have been shown to affect the antioxidant capacity and oxidative balance in the calanoid copepod *Acartia bifilosa* from the Baltic Sea (Vehmaa et al. 2013). Abiotic factors, phytoplankton community structure and food availability have also been found to affect the concentrations of the non-enzymatic antioxidants α-tocopherol and astaxanthin in Baltic Sea zooplankton (Holeton et al. 2009, Häubner 2010).

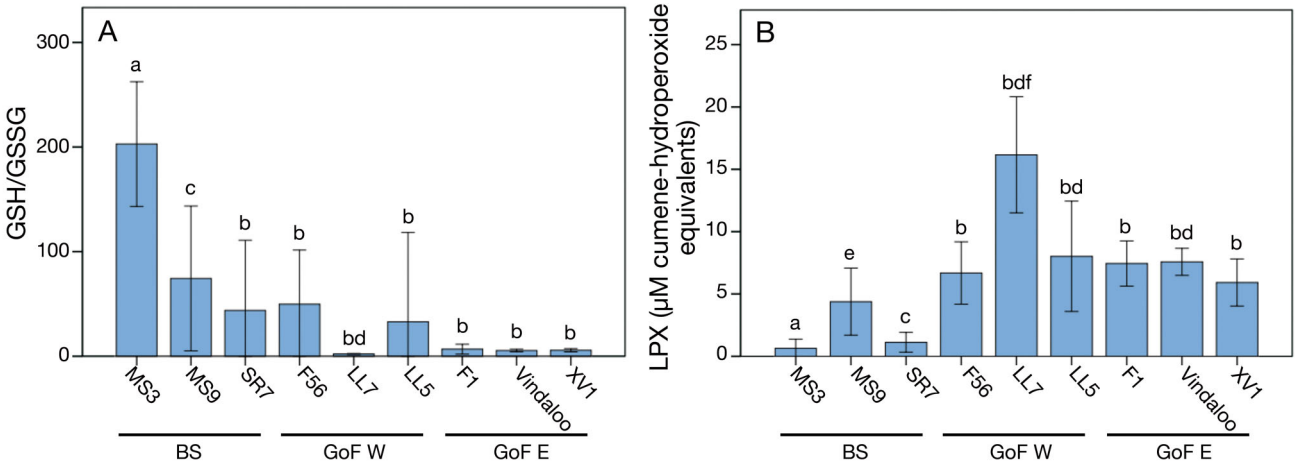

Fig. 4. (A) Ratio of reduced/oxidized glutathione (GSH/GSSG) and (B) lipid peroxidation (LPX) in *Limnocalanus macrurus* collected from 6 sites from the Gulf of Finland (GoF; W = west and E = east) and 3 sites from the Bothnian Sea (BS). Error bars: 95% confidence intervals. Statistically significant differences ($p < 0.05$, Kruskal-Wallis ANOVA) between sites are shown with pairs of letters (a–b, c–d, e–f)

Interestingly, Tremblay et al. (2010) showed that changes in the values of oxidative stress biomarkers were associated with the zoogeographic distribution (and daily vertical migration ranges) of 3 krill species in the Gulf of California. In their study, an efficient enzymatic antioxidant defence was found to have a significant role in the ability of *Euphausia eximia* to inhabit regions with low oxygen concentrations and higher temperatures compared to the 2 other species investigated. Oxidative stress biomarkers of the 3 krill species were also generally elevated in the summer compared to values in the winter, further highlighting the impact of environmental factors on antioxidant defence and oxidative stress (Tremblay et al. 2010).

In the most recent integrated assessment of hazardous substances in the Baltic Sea, the status of most geographical assessment units in the offshore areas of the GoF were classified as 'poor' or 'bad', and the units in the Gulf of Bothnia (including the BS) as 'moderate' (HELCOM 2010). The sediment trace metal concentration data used in this study showed higher levels in the BS compared to the GoF, whereas the highest levels of coplanar PCBs and PCDD/Fs were found from copepod samples collected from the eastern GoF. The mortalities of amphipods in sediment bioassays were high in the GoF sites as well (Berezina et al. 2013). Thus, it is apparent that the profiles of environmental contamination differ between these 2 basins, plausibly leading to differential biological responses in the organisms inhabiting these areas.

The effects of various hazardous substances on copepods have been studied experimentally to some extent, but not in Baltic Sea species. A number of substances such as trace metals, monoethanolamine, water-soluble fractions of oil, naphthalene and diethanolamine have been found to cause increases in the transcription of genes encoding oxidative stress defence-related proteins in copepods (Seo et al. 2006, Hansen et al. 2007, 2008, 2010, Lee et al. 2008), however field studies on the responses of zooplankton to hazardous substances are scarce. Cailleaud et al. (2009b) investigated biomarker responses in *E. affinis* and found significantly increased GST activities at the highest hydrophobic organic contaminant concentrations in the water column. The importance of copepods in the biogeochemical cycles of PAHs, PCBs, brominated diphenyl ethers (BDEs), non-ionic surfactants and synthetic steroids in marine food webs have been reported (Carls et al. 2006, Cailleaud et al. 2009a,b, 2011, Tomy et al. 2009).

In this study, significant correlations between the oxidative stress biomarkers measured in *L. macrurus* and concentrations of selected trace metals in sediments were observed. However, it is not clear how well the concentrations of contaminants in the sediment reflect their concentrations in the pelagic food web, and this obviously needs more research. Factors such as the temperature, oxygen content and pH of a water body can alter the solubility of the metal salts present in it, the occurrence of different species, as well as their bioavailability and toxicity (Green-Ruiz & Paez-Osuna 2004, Guerra-García & García-Gómez 2005, Namiesnik & Rabajczyk 2010). As shown, the 2 basins differ considerably in their hydrographic characteristics, and, for example, a high level of oxygenation of the sediments can minimize the mobility and

bioavailability of pollutants (Namiesnik & Rabajczyk 2010). Thus, although the trace metal concentrations in sediments give a convenient measure of metal pollution, such measures do not necessarily predict the toxicity of these pollutants to organisms detected from the oxidative stress responses. Unfortunately, no comparative data on trace metal concentrations in *L. macrurus* or zooplankton in general in different areas of the Baltic Sea are available. Therefore, the observed statistical correlations between trace metal concentrations and oxidative stress biomarkers presented in this study should be considered with caution since the direct linking mechanisms are yet to be proven. It is, however, noteworthy that several trace elements have been found to be highly concentrated in Baltic Sea zooplankton (Nfon et al. 2009). There are 2 alternative (and partly overlapping) explanations for the higher GR and GST activities and totGSH levels we observed in *L. macrurus* from the BS compared to the GoF: (1) the lower water temperature and higher oxygen concentration may enable more efficient glutathione metabolism- related antioxidant defence, or (2) the higher trace metal concentrations in the area increase the need for an elevated antioxidant defence.

Whereas *L. macrurus* samples from the BS showed high levels of glutathione metabolism-associated variables, those from the GoF had higher levels of SOD activity, LPX, GSSG content and a lower GSH/GSSG ratio. All of these indicate unequivocally higher oxidative stress in the animals of the GoF than in those collected from the BS. This major finding may be associated with the higher concentrations of PCBs and PCDD/Fs measured in zooplankton samples from the GoF compared to those from the BS. The higher organochlorine contaminant concentrations together with higher water temperatures likely contribute to the observed elevated levels of oxidative stress and SOD activity in *L. macrurus* in the GoF, in particular because *L. macrurus* is a cold-water stenoterm species. Increased toxicity of sediments collected from the GoF sites also indicates a positive correlation with SOD activity; the elevated mortality of amphipods in the test sediments may have been caused by increased toxicity of the sediments due to compounds formed under anoxic conditions. How these compounds diffuse and affect organisms living in the water column remains unclear. In regard to the observed correlations between biological endpoints and trace metal concentrations, the actual mechanisms and extent to which toxic sediments affect organisms living in the proximity of the sea bottom requires further study. Finally, the GoF is highly eutrophic (HELCOM 2009), and abundant cyanobacterial toxins may also cause oxidative stress in grazers, especially during the late summer (Karjalainen et al. 2007).

In conclusion, the results obtained here show basin- and site-specific differences in the oxidative stress biomarkers of the zooplankter *L. macrurus*, and their potential linkages to hydrographical factors and chemical contamination. Increased levels of oxidative damage to lipids, lower GSH/GSSG ratios and high concentrations of PCBs and PCDD/Fs indicate the presence of multiple environmental stressors in the GoF. The biomarker response profile observed in the BS appears different with the active functioning of the cellular redox defence machinery (mainly increased efficiency of glutathione turnover) preventing oxidative damage. Previous literature and the data presented here suggest that copepod glutathione metabolism enzyme activities in particular may be affected by hydrographic conditions. In this study, GSH/GSSG, LPX and SOD were, in turn, indicative of possible exposure to PCBs and PCDD/Fs and also correlate with the sediment toxicity of the study locations. Since zooplankton represent an important link between primary producers and fish, it is crucial to address its responses when predicting the effects of environmental stressors on pelagic ecosystems. In the present work, 8 biomarker assays from pooled samples of field-collected individuals of *L. macrurus* were successfully measured and the results were investigated in the light of information on known abiotic and biotic variables. Since *L. macrurus* is a dominant zooplankton species found in the pelagic regions in the BS as well as in the GoF, it could be a suitable model species for studying biological effects of environmental stressors in plankton communities in these sea areas. In addition, the methods presented in this paper can be readily applied to studies on environmental stressors and their effects in other locations, copepod species or even mesozooplankton communities. The possible effects of hydrographic factors on biomarkers and the differences in associations with contaminant proxies should be considered when planning the use of oxidative stress biomarkers for the assessment of environmental stress in zooplankton.

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Appendix. Component matrices for PCAs

Component matrix for Fig. 2A. Biomarker abbreviations are as follows: GST: glutathione *S*-transferase; GP: glutathione peroxidase; GR: glutathione reductase; totGSH: total glutathione; LPX: lipid peroxidation; SOD: superoxide dismutase; GSSG: oxidized glutathione; GSH/GSSG: ratio of reduced to oxidized glutathione

	Component 1	2
GST	0.783	0.247
GP	0.697	−0.049
GR	0.894	0.158
totGSH	0.475	0.746
LPX	−0.700	0.238
SOD	−0.644	−0.077
GSSG	−0.210	0.867
GSH/GSSG	0.589	−0.605

Component matrix for Fig. 2B. Five components were extracted, but only first 2 are shown. oxy: oxygen; sal: salinity; temp: temperature; bot: bottom; surf: surface; 30: depth of 30 m

	Component 1	2		Component 1	2
temp_surf	−0.641	−0.237	GSH/GSSG	0.605	0.151
sal_surf	0.090	0.975	totGSH	0.660	−0.362
oxy_surf	−0.008	−0.299	LPX	−0.736	0.293
temp_30	−0.553	0.405	SOD	−0.821	−0.139
sal_30	−0.383	0.596	GSSG	−0.236	0.402
oxy_30	0.910	0.328	Hg	0.266	−0.071
temp_below30	−0.728	0.319	Cd	0.717	−0.150
sal_below30	−0.763	0.039	Pb	0.677	0.042
oxy_below30	0.020	0.920	Cu	0.970	−0.036
temp_bot	−0.968	0.016	Ni	0.862	0.080
oxy_bot	0.959	0.052	Zn	0.929	0.015
sal_bot	−0.936	0.196	Bioassay	−0.591	−0.452
PP_surf	−0.664	0.390	CoplanarPCB	−0.670	−0.666
GST	0.870	−0.242	OtherPCB	0.030	−0.939
GP	0.830	0.291	SumPCDD_F	−0.504	−0.791
GR	0.941	−0.167			

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