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► **To cite this version:**

Rafael Trevisan, Fabrício Flores-Nunes, Euler Dolores, Jacó Mattos, Clei Piazza, et al.. Thiol oxidation of hemolymph proteins in oysters *Crassostrea brasiliana* as markers of oxidative damage induced by urban sewage exposure. *Environmental Toxicology and Chemistry*, 2017, 36 (7), pp.1833-1845. 10.1002/etc.3543 . hal-03908224

HAL Id: hal-03908224

<https://hal.science/hal-03908224>

Submitted on 20 Dec 2022

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1 Running head

2 **Protein thiol oxidation in oysters exposed to urban sewage**

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6 **Thiol oxidation of hemolymph proteins in oysters *Crassostrea brasiliana* as markers of**
7 **oxidative damage induced by urban sewage exposure**

8

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23 **Abstract**

24 Urban sewage is a concerning issue worldwide, threatening both wildlife and human health. The
25 present study investigated protein oxidation in Mangrove Oysters (*Crassostrea brasiliiana*)
26 exposed to seawater from Balneário Camboriú, an important tourist destination in Brazil that is
27 affected by urban sewage. Oysters were exposed for 24 h to seawater collected close to
28 Camboriú river (CAM1) or 1 kilometer away (CAM2). Seawater from an aquaculture laboratory
29 was used as a reference. Local sewage input was marked by higher levels of coliforms, nitrogen,
30 and phosphorus in seawater, as well as polycyclic aromatic hydrocarbons (PAHs),
31 polychlorinated biphenyls (PCBs), linear alkylbenzenes (LABs), and fecal steroid in sediments at
32 CAM1. Exposure of oysters to CAM1 caused marked bioaccumulation of LABs and decreased
33 PAHs and PCBs concentrations after exposure to both CAM1 and CAM2. Protein thiol oxidation
34 in gills, digestive gland, and hemolymph was evaluated. Lower levels of reduced protein thiols
35 were detected in hemolymph from CAM1, and Actin, Segon, and Dominin were identified as
36 targets of protein thiol oxidation. Dominin susceptibility to oxidation was confirmed in vitro by
37 exposure to peroxides and hypochlorous acid, and 2 cysteines residues were identified as
38 potential sites of oxidation. Overall these data indicate that urban sewage contamination in local
39 waters has a toxic potential and that protein thiol oxidation in hemolymph could be a useful
40 biomarker of oxidative stress in bivalves exposed to contaminants.

41

42 **Keywords:** Sewage; Biomarkers; Protein oxidative damage; Oxidative stress; Bivalve.

43

1. INTRODUCTION

44 According to recent statistics, only 64% of the world population had access to improved
45 sanitation (hygienically separation of human excreta from human contact) in 2012 [1].
46 Additionally, rapid urbanization, fast population growth, migration into urban centers, and
47 insufficient urban coverage of sanitary sewage collection system, contribute to the sanitary issues
48 [2]. Consequently, urban sewage contamination is an increasing concern not only due to its
49 hazards to human health but also due its potential adverse effects on aquatic ecosystems. Raw or
50 untreated urban sewage is a complex mixture, typically composed of pathogens, inorganic
51 nitrogen and phosphorous, metals, chlorides, organic compounds (including proteins,
52 carbohydrates, and urea), surfactants, phenols, polycyclic aromatic hydrocarbons (PAHs),
53 polychlorinated biphenyls (PCBs), pesticides, and emerging contaminants such as phthalates,
54 pharmaceuticals, and personal care products [3,4]. Moreover, as only 64% of the world
55 population had access to improved sanitation in 2012, lower rates of access to sewage treatment
56 (primary, secondary and tertiary) are expected. But even if sewage treatment options are
57 available, none of the sewage treatment plants are 100% efficient [5]. Furthermore, sewage
58 treatment process generates a sludge that contains toxic by-products, which can contaminate
59 groundwater when applied as a fertilizer [3]. Therefore, both raw and treated urban sewage may
60 threaten aquatic ecosystems, thus raising concerns within the ecotoxicology and environmental
61 health scientific community.

62 Sewage effluent contains various classes of contaminants, and many of these
63 contaminants possess pro-oxidant properties [6], which may lead to oxidative damage and
64 subsequent reversible and/or irreversible alterations in the structure and function of various

65 cellular components, thereby negatively impacting cellular metabolism [7]. Cysteine (Cys)
66 residues are among the main targets of reactive oxygen species (ROS), as their thiol groups are
67 very sensitive to alterations in the cellular redox state. Reversible post-translational
68 modifications of proteins include intra or intermolecular disulfide bridges (-SS-),
69 glutathionylation (-SSG) or S-nitrosylation (-SNO), and oxidation to sulfenic acid [7].
70 Irreversible oxidation forms can also be produced, such as sulfinic (-SO₂⁻) and sulfonic acid (-
71 SO₃⁻) [7]. Critical Cys residues on proteins are well evolutionary conserved, and due to this
72 chemical flexibility of the thiol group and its oxidation states, Cys residues are frequently
73 associated with important protein characteristics, such as enzyme catalysis, transcriptional
74 regulation, protein folding, and metal coordination [7].

75 Given the sensitivity of thiol groups to ROS, assessment of protein oxidation in bivalves
76 could be a useful biomarker for aquatic contamination, and several recent studies support this
77 idea. For example, Cys oxidation after exposure to contaminants has been identified in proteins
78 involved with cellular and metabolic functions, including glycolysis, citric acid cycle, stress
79 response, lysosomal processes, and muscle contraction [8,9]. However, the direct link between
80 thiol oxidation, loss of protein function/activity, and metabolic impairment in bivalves is yet to
81 be fully understood. This is not the case for mammals, as Cys oxidation has been linked to
82 conformational changes in proteins, leading to impaired function [e.g. microtubule elongation
83 (tubulin) and DNA-binding (human estrogen receptor)] [10]. Since oxidative stress is a common
84 toxicity mechanism to several classes of contaminants, identifying proteins targets of oxidative
85 damage in bivalves could help to identify cellular and metabolic functions that may be impaired
86 due to contaminants (pro-oxidants) exposure.

87 As for detecting protein oxidation in various organs, studies in mussels suggest that
88 hemolymph can be an important target of protein oxidation in bivalves [11]. This tissue also calls
89 the attention to the presence of abundant thiol-containing proteins in different bivalve species.
90 For example, in oysters *Crassostrea gigas* and *Crassostrea virginica*, the major abundant
91 hemolymph protein is the Extracellular Superoxide Dismutase or Cavortin (*C. gigas*) and
92 Dominin (*C. virginica*) [12–14]. Both proteins have similar molecular weight (~20 kDa), contain
93 4 Cys residues, one lipopolysaccharide (LPS), and one integrin-binding domain, and are able to
94 sequester iron. Interestingly, both Cavortin and Dominin have minimal (if at all) superoxide
95 dismutase activity, despite having domains related to copper-zinc superoxide dismutase.
96 Although these proteins comprise ~50% of the plasma protein abundance, their functions remain
97 unclear, but it is thought that they could be involved in bacterial recognition and phagocytosis
98 events, antioxidant protection via superoxide dismutase activity, as well as metal transport. As
99 abundant thiol-containing proteins, these and other hemolymph proteins may be susceptible to
100 oxidation events due to contaminants and/or pathogens, leading to loss of protein function and
101 potentially adverse outcomes.

102 Balneário Camboriú is an important tourist destination in southern Brazil, especially
103 during summer. Although not a very populous city (~124,000 residents), Balneário Camboriú
104 receives several millions of tourists over the course of a summer. Despite having urban sewage
105 collection and treatment facilities that serve most of the regular population, this significant
106 increase in population due to tourism may be a challenge to sewage treatment and to the water
107 quality along the bay. In fact, microbiological seawater analyses by the local environmental
108 agency indicate that Balneário Camboriú bay has poor water quality in several regions along the
109 shoreline (available at <http://www.fatma.sc.gov.br/>). Notably, the water quality in these regions

110 is persistently poor (i.e. not only during peak tourist season), suggesting that the sewage
111 treatment facility is likely overwhelmed. Continuous worsening of water quality could pose a
112 threat to many economic activities in the region.

113 Thus, taking into account that the urban sewage is a concerning issue and that Cys
114 oxidative damage is a mechanism of toxicity shared by different class of contaminants, the
115 present study investigated for the first time the effects of urban sewage on protein thiol oxidation
116 in bivalves, and aimed to identify proteins sensitive to Cys oxidation in the hemolymph as
117 potential biomarkers of oxidative stress. Consequently, we examined the extent of urban sewage
118 contamination at Balneário Camboriú coast by assessing standard water quality parameters and
119 organic contaminants. In addition, oysters *Crassostrea brasiliana* were exposed to seawater
120 samples collected at Balneário Camboriú, followed by analysis of total protein thiol status in the
121 gills, digestive gland, and hemolymph. The thiol oxidation profile of specific abundant
122 hemolymph proteins was further explored as a simple and efficient method to assess pro-oxidant
123 effects of environmental contaminants in bivalves.

124

125 2. MATERIAL AND METHODS

126 2.1. Study area and seawater sample collection

127 Two sites located at Balneário Camboriú, Santa Catarina State, southern Brazil (27° S
128 and 48°38' W) were investigated (Fig 1): (a) an area near the mouth of Camboriú River (CAM1),
129 which receives urban effluents along its course and is a known source of pollutants to the
130 Balneário Camboriú bay; and (b) an area approximately 1 kilometer away from the mouth of

131 Camboriú River (CAM2). According to the local governmental environment agency (FATMA –
132 SC), water quality at CAM1 or close regions was considered inadequate in approximately 20 -
133 25% of the analyzed periods (available at <http://www.fatma.sc.gov.br>), demonstrating the
134 negative impact of urban sewage in this region.

135 On December (1st) 2014, seawater samples (70 L) were collected at CAM1 and CAM2
136 and stored in 100 L high-density polyethylene gallons, and an aliquot was collected for water
137 quality analysis. In addition, approximately 250 g of sediment were collected at each site for
138 chemical analysis using a Van Veen Grab Sampler.

139 2.2. *Animals and exposure conditions*

140 Oysters *C. brasiliiana* (males and females, 6 – 8 cm length) were obtained from an
141 aquaculture laboratory at Federal University of Santa Catarina (Marine Mollusks Laboratory,
142 LMM). Animals were maintained at LMM in a flow through system for 11 d prior to the
143 experiments, using filtered (0.45 µm) and UV treated seawater with constant temperature (25 °C)
144 and salinity (33 ‰). During this period, animals were fed with a mixture of microalgae (50%
145 *Isochrysis galbana* and 50% *Chaetoceros muelleri*, 20×10^4 cells/mL).

146 Following their transport to the laboratory, the animals were acclimated for 24 h in high-
147 density polyethylene tanks containing filtered seawater (0.45 µm, 25 °C and 33 ‰ salinity, 1
148 animal/L) with no further feeding. At the same time, seawater samples collected at Balneário
149 Camboriú were maintained in the same high-density polyethylene plastic gallons used for water
150 sampling and were allowed to stabilize to 25 °C for 24 h in the same laboratory.

151 Following the acclimation period, a group of oyster (1 pool of 10 animals) was collected
152 for chemical analyses (see section 2.3) and was considered the Pre-exposure group. In addition,
153 oysters were further exposed to seawater samples from Balneário Camboriú in 60 L glass aquaria
154 (15 animals and 20 L of seawater per aquarium, 2 aquaria per group, 25 °C). An additional group
155 was used as a reference, composed of animals exposed to seawater from the aquaculture
156 laboratory (REF group). All groups had salinity adjusted to 33 ‰ (initial salinity of 33, 30 and
157 25 ‰ for REF, CAM1 and CAM2 groups, respectively) with commercial sea salt (Instant
158 Ocean® Sea Salt, Spectrum Brands, Blacksburg - USA). After 24 h of exposure, 3 animals per
159 aquarium were randomly selected (total of 6 animals per group, n = 6), and gills, digestive gland,
160 and hemolymph were collected. Hemolymph was obtained from the adductor muscle using a
161 cooled-down 1 mL syringe with a 23-gauge needle. Tissues were immediately frozen in liquid
162 nitrogen and stored at -80 °C for further analyses.

163 2.3. *Microbiological and chemical analyses*

164 Water samples (REF, CAM1, and CAM2) were analyzed by a commercial laboratory
165 (QMC Laboratório de Análises, Florianópolis - Brazil) registered by the local governmental
166 environmental agency (FATMA – SC). The following assays were performed according to the
167 American Public Health Association (APHA), the American Water Works
168 Association (AWWA), and the Water Environment Federation (WEF) Standard Methods for the
169 Examination of Water and Wastewater [15]: total coliforms (TC, method 9221 E),
170 thermotolerant coliforms (TtC, method 9221 E), biochemical oxygen demand (BOD, method
171 5210 B), chemical oxygen demand (COD, method 5220 D), detergents (method 5540 C), total

172 phenols (method 5530 C), total phosphorous (method 4500-P E), Kjeldhal nitrogen (method
173 4500-N_{org} B) and total suspended solids (TSS, method 2540 D).

174 Levels of organic contaminants (PAHs, PCBs, organochlorine pesticides, and linear
175 alkylbenzenes - LABs) were also measured in oysters prior to exposure (Pre-exposure group),
176 and after the 24 h exposure period (REF, CAM1, and CAM2). The presence of organic
177 contaminants (PAHs, PCBs, organochlorine pesticides, LABs, and steroids) was also analyzed in
178 the sediments collected at Balneário Camboriú (CAM1 and CAM2). Samples (pool of 10
179 oysters per group or 250 g sediment) were freeze-dried (Thermo Savant lyophilizer – Modulyo
180 D) for 72 h and then crushed and homogenized with a pestle. The analyses of total PCBs, PAHs,
181 LABs, steroids and organochloride pesticides were carried out according to the procedures
182 detailed elsewhere [16,17]. Briefly, samples (1 g for oysters and 20 g for sediments) were
183 Soxhlet-extracted with a 50% mixture of residue grade n-hexane and dichloromethane. The
184 organochlorine extract was purified in an alumina chromatographic column. The hydrocarbon
185 extracts were fractionated into F1 (LABs), F2 (PAHs), and F3 (steroids, only for sediment) by
186 silica gel-alumina column chromatography. F3 was evaporated to dryness, and steroids were
187 derivatized to form trimethylsilyl ethers using bis(trimethylsilyl) trifluoroacetamide with 1 %
188 trimethylchlorosilane. Organochlorine pesticides were analyzed in an Agilent Technologies gas
189 chromatograph in splitless mode using an electron capture detector (GC-ECD). Steroids were
190 determined on a gas chromatography with flame ionization detector (GC-FID). PAHs, PCBs, and
191 LABs were analyzed by an Agilent 6890 gas chromatograph coupled to a 5973N mass
192 spectrometer (GC/MS) in a selected ion mode (SIM). Certified standards at five different
193 concentrations were used for calculations. PAH, PCB, and LAB identification were based on

194 mass/charge ratio of the individual quantitation ion (m/z) and retention times of certified
195 standards.

196 2.4. *Sample preparation*

197 Gills and digestive gland (approximately 100 mg wet weight each) were homogenized
198 (1:4 w:v) in 10 mM TRIS buffer pH 7.2, containing 1 mM EDTA, 0.5 M sucrose, 0.15 M KCl,
199 and 1 mM PMSF, followed by centrifugation (12,000 g for 60 min at 4 °C). The supernatant was
200 collected and used for further procedures. Hemolymph was used without any additional
201 treatments. Total protein content was determined using Coomassie blue [18] and serum bovine
202 albumin standards.

203 Reduced protein thiol groups (PSH) were assayed using the fluorophore 5-
204 iodoacetamide-fluorescein (5-IAF) based on a protocol with marine bivalves [19], with minor
205 modifications. For this purpose, 100 µg protein for gills and digestive gland or 50 µg protein for
206 hemolymph were incubated at room temperature for 30 min with 0.1 M TRIS pH 7.5 containing
207 0.2 mM 5-IAF. This fluorophore specifically recognizes reduced PSH. Excess 5-IAF was
208 removed by acidification with 10% trichloroacetic acid (TCA) for 5 min at 4 °C, centrifuged
209 (11,000 g for 3 min at 4 °C) and washed twice with cold acetone. Samples were further
210 resuspended in SDS-PAGE sample buffer containing 5% β-mercaptethanol and stored at -20°C
211 for further analyses.

212 Reversibly oxidized protein thiol groups (R-PSX) were also assayed using the
213 fluorophore 5-IAF, but with the addition of steps for PSH alkylation and reduction of R-PSX
214 [20]. Briefly, 100 µg protein for gills and digestive gland or 50 µg protein for hemolymph were

215 incubated with 100 mM N-ethylmaleimide (NEM) and 1 % sodium dodecyl sulfate (SDS) for 15
216 min at room temperature for PSH blocking, followed by NEM removal by protein precipitation
217 with 10 % TCA and cold acetone washing. Proteins were resuspended in 6 M urea containing 1
218 % SDS and 10 mM dithiothreitol (DTT) for 30 min for the reduction of R-PSX. DTT was
219 removed by 10 % TCA precipitation and the newly reduced thiols groups (originated from the
220 reduction of R-PSX by DTT) were labeled by incubation for 30 min at room temperature in 6 M
221 urea containing SDS 1 % and 0.2 mM IAF. Excess 5-IAF was removed by 10 % TCA
222 precipitation and cold acetone washing. Samples were resuspended in SDS-PAGE sample buffer
223 containing 5% β -mercaptoethanol and stored at -20°C for further analyses.

224 2.5. In vitro exposure of hemolymph to oxidants

225 Hemolymph from reference animals (REF) was used for further exposure to hydrogen
226 peroxide, cumene hydroperoxide (CHP) or hypochlorous acid (HOCl). A total of 4 samples (n =
227 4) were used for this experiment. Proteins extracts (60 μ g) were incubated with 0.01, 0.1, 1, and
228 10 mM (in phosphate saline buffer pH 7.4 adjusted to 3 % NaCl) of each oxidant for 1.5 h in the
229 dark and at room temperature. By the end of the incubation period, samples were immediately
230 separated in two aliquots (30 μ g each): the first was used for PSH determination, with the
231 addition of 10 % TCA, while the second was used for R-PSX determination, with the addition of
232 100 mM NEM. Samples were further prepared and analyzed as stated in sections 2.4 and 2.5. An
233 additional group was maintained in 3 % NaCl - phosphate saline buffer pH 7.4 under the same
234 conditions described above for 1.5 h, and used as control (Ctl) for each sample.

235 2.6. *Quantification of PSH and R-PSX*

236 After 5-IAF labeling, protein extracts (10 µg, except for the in vitro experiments with
237 hemolymph which used 5 µg) were assayed by SDS-PAGE in 12 % polyacrylamide gels and
238 immediately scanned for 5-IAF signal in a FUJIFILM FLA-9000 scanner (473/510
239 excitation/emission wavelengths). Gels were further stained with colloidal Coomassie and
240 protein abundance was quantified in a Bio-Rad ChemiDoc MP. The 5-IAF signal was
241 normalized to the Coomassie staining signal, and this ratio was used to quantify PSH and R-PSX
242 levels. Total PSH and R-PSX were based on the signal intensity of the whole electrophoresis
243 lane, while PSH and R-PSX of individual proteins were based on the signal intensity of the
244 individual bands (n = 4 for in vitro experiments and n = 6 for in vivo experiments).

245 2.7. *Protein digestion and MALDI-TOF and MALDI-TOF/TOF analyses of hemolymph*
246 *proteins*

247 After SDS-PAGE, proteins of interest from hemolymph samples were excised and
248 digested in-gel with trypsin [21]. Briefly, samples were reduced with 10 mM DTT in 100 mM
249 ammonium bicarbonate pH 8.5 for 30 min at 56 °C, washed with acetonitrile, and alkylated with
250 55 mM iodoacetamide in 100 mM ammonium bicarbonate pH 8.5 for 20 min at room
251 temperature. Samples were further destained with 50 % acetonitrile and 45 mM ammonium
252 bicarbonate pH 8.5 at 37 °C, dehydrated with 100 % acetonitrile and air dried. Gel pieces were
253 saturated with trypsin (20 µg/mL in 40 mM ammonium bicarbonate pH 8.5 and 9 % acetonitrile)
254 and digested overnight at 37 °C. Remaining peptides were extracted from the gel with 0.1 %
255 trifluoroacetic acid and 50 % acetonitrile at 37 °C for 30 min and samples were dried out in a
256 vacuum centrifuge. The α -cyano-4-hydroxy-cinnamic acid matrix (5 mg/mL in 0.1 %

257 trifluoroacetic acid and 50 % acetonitrile) was used for MALDI-TOF and MALDI-TOF/TOF
258 analyses, with an internal peptide calibration standard for MS (Bruker Daltonics 206195). Mass
259 spectra of peptides were collected with positive reflectron MS and MS/MS on a MALDI auto
260 flex III smart beam (Bruker Daltonics). The resulting mass spectra were searched by MASCOT
261 v2.3 software installed on a local server. A *C. brasiliiana* protein database was constructed based
262 on an in-house *de novo* assembled transcriptome (gills and digestive gland) of *C. Brasiliiana*
263 (kindly provided by Dr. Karim H. Lüchmann, Santa Catarina State University, Brazil). The
264 following parameters were adopted: tryptic peptides with ≤ 1 missed cleavage site, complete
265 carbamidomethylation of cysteines and partial oxidation of methionines, peptide mass tolerance
266 ranging from ± 75 to 250 ppm (MS) or ± 1 Da (MS/MS).

267 2.8. Computational analysis of *C. brasiliiana* Dominin

268 After protein identification by mass spectrometry, the most abundant protein in the
269 hemolymph of *C. brasiliiana* (Dominin, see section 3.4) was investigated for amino acid
270 sequence similarity with homologous extracellular superoxide dismutase in other oyster species
271 by molecular phylogeny and multiple alignments analysis. The presence of reactive and surface-
272 exposed Cys residues was also investigated by 3D structural modeling. These analyses aimed to
273 detect Cys residue prone to oxidation events in *C. brasiliiana* Dominin, and based on the
274 similarity of their amino acid sequences and domains, to predict such events in homologous
275 oyster proteins.

276 The molecular phylogenetic analysis was carried out using the Maximum Likelihood
277 Method [22]. Protein sequences were initially analyzed by multiple alignments at MUSCLE 3.8
278 [23], and suitable alignment blocks were filtered on GBlocks Server [24] using default

279 parameters. Resulting blocks were of filtered sequences were re-aligned at MUSCLE 3.8 and
280 analyzed by MEGA6 [25], testing for the substitution model and using the lowest BIC scores
281 (Bayesian Information Criterion) to define the best substitution pattern. Based on this model, a
282 bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary
283 history of the analyzed taxa.

284 Multiple alignments of *C. brasiliiana* Dominin protein sequence against homologous
285 proteins from *Ostrea edulis* (extracellular SOD, ADF80415.1), *Saccostrea glomerata*
286 (extracellular SOD, ACQ73551.1), *C. virginica* (dominin, BAF30874.1), *Crassostrea gigas*
287 (extracellular Cu-Zn SOD-like, 011414395.1; cavortin, AAT44352.1) were carried out using
288 MUSCLE 3.8. The protein sequence was also analyzed for the presence of peptide signal [26]
289 and putative conserved domains [27]. Motifs related to lipopolysaccharide (LPS) and integrin
290 binding were identified based on *C. gigas* extracellular superoxide dismutase [12], a homologous
291 protein to *C. brasiliiana* Dominin.

292 The protein 3D model was built using I-Tasser suite [28–30] for *ab initio* modeling
293 following default parameters including multiple SOD sequence alignments. The quality of PDB
294 structural model was checked by Procheck [31] and LigPlot software was used to map copper
295 and zinc coordinated amino acids [32].

296 2.9. Statistics

297 All data were checked for normality by Kolmogorov-Smirnov test prior to analyses. PSH
298 and R-PSX levels from the in vivo exposure to water samples from Balneário Camboriú were
299 analyzed by one-way ANOVA followed by Duncan *post hoc* or Kruskal-Wallis ANOVA &

300 Median test when appropriated. Data from the *in vitro* exposure to oxidants were analyzed by
301 repeated one-way ANOVA followed by Dunnet *post hoc* test. Differences were considered
302 statistically significant at $p < 0.05$.

303

304

3. RESULTS

305 *3.1. Water quality and chemical analysis*

306 Analysis of seawater samples (Table 1) indicates an input of urban sewage at CAM1:
307 higher coliforms levels (both TC and TtC), as well as higher organic nitrogen and total
308 phosphorous. Suspended solids, detergents, and total phenol concentrations were similar among
309 all groups while REF had the lowest BOD and highest COD.

310 Levels of organic contaminants were also measured in sediments collected at the two
311 sites of Balneário Camboriú (CAM1 and CAM2), as well as in oysters prior to exposure (Pre-
312 exposure) and after the laboratory exposure (REF, CAM1, and CAM2) (Table 2). Detailed tables
313 presenting all analyzed organic compounds in sediments and in oysters are available as
314 supplementary material (Supplementary Data, Tables S1-S5). Total PAHs concentrations were 4-
315 fold higher in the sediment sampled at CAM1 compared to CAM2. PCBs were detected in
316 sediments from the CAM1 group, but not at other sites. Low concentrations of organochlorine
317 pesticides were also detected, as traceable amounts of DDT in sediments from CAM2 (0.04 ng/g
318 dry weight). LABs, an important group of markers of urban sewage, were also found in
319 sediments from CAM 1 (52 ng/g), but not CAM2 (Table 2). Steroids concentrations were also
320 detected in the sediments, presenting higher concentrations at CAM1 (4.92 $\mu\text{g/g}$ dry weight),

321 compared to CAM2 (1.45 $\mu\text{g/g}$ dry weight). Steroids related to animal feces (cholesterol,
322 coprostanol, epicoprostanol, and cholestenol) were detected at higher concentrations in sediment
323 from CAM1 (3.39 $\mu\text{g/g}$) compared to CAM2 (0.08 $\mu\text{g/g}$).

324 Pre-exposure organisms had the highest total PAHs concentrations (540 ng/g dry weight).
325 This value decreased 67% at REF and was almost completely eliminated (>95% decrease) after
326 24 h of exposure to water sampled at both CAM1 and CAM2 sites. Pre-exposure organisms
327 presented the highest concentrations (35 ng/g dry weight) of total PCBs, which decreased to 60
328 % at REF and to 22 – 25 % at CAM1 and CAM2, respectively. Organochloride pesticides were
329 relatively stable in oysters of all exposed groups (0.99 – 1.85 ng/g dry weight), except at CAM2,
330 where they were not detected. LABs were not detectable in pre-exposure oysters, or in the
331 CAM2 group, but reached 180 ng/g dry weight in CAM1 group. Steroids were not analyzed in
332 oysters.

333 3.2. Total PSH and R-PSX in tissues of oysters exposed to seawater samples from Balneário 334 Camboriú

335 Levels of total protein thiol status in gills, digestive gland, and hemolymph (Figure 2) of
336 *C. brasiliiana* exposed in the laboratory (24 h) to water samples collected at Balneário Camboriú
337 (CAM1 and CAM2) or from a university aquaculture facility (REF) were investigated. After
338 24 h of exposure, tissues were collected and analyzed by fluorescent labeling and 1D SDS-
339 PAGE. The fluorophore 5-IAF was used to label protein thiols by two different approaches: (i)
340 direct labeling, where 5-IAF reacts specifically with reduced protein thiols (PSH) [19], and (II)
341 indirect labeling, where PSH are first blocked by NEM, and reversibly oxidized protein thiols
342 (R-PSX) are reduced by DTT and tagged by 5-IAF [20]. Total PSH levels remained unaltered in

343 gills and digestive gland after 24 h of exposure to Balneário Camboriú water samples (Figure 2A
344 and C), while in hemolymph there was a 42 % decrease in CAM1 group in comparison to REF
345 (Figure 2E). Total R-PSX levels did not change significantly in any analyzed tissues (Figure 2B,
346 D and F).

347 3.3. PSH and R-PSX levels of hemolymph proteins after oyster exposure

348 As hemolymph contained proteins sensitive to PSH oxidation, the profile of protein thiol
349 was assessed in the most abundant proteins detected in 1D SDS-PAGE gels (Figure 3).
350 Coomassie staining indicated 3 abundant proteins, namely P1 (~35 KDa), P2 (~55 KDa) and P3
351 (~71 KDa) (Figure 3A). While P1 and P3 had significant and measurable 5-IAF signal for both
352 PSH and R-PSX, P2 had detectable signal only for PSH (Figure 3A).

353 Quantification of PSH levels indicated a decrease in 5-IAF signal for P1 (25%, Figure
354 3B), P2 (50%, Figure 3D) and P3 (47%, Figure 3E) in the CAM1 group when compared to REF,
355 similarly to total PSH levels in hemolymph (Figure 2E). R-PSX levels remained unaltered in P1
356 (Figure 3C) while it decreased in P3 at CAM1 group when compared to REF (Figure 3F).

357 The bands labeled as P1, P2, and P3 (Figure 3A) were excised, and in-gel trypsinized for
358 later protein identification by MALDI-TOF and MALDI-TOF/TOF. Based on MASCOT
359 analysis using a *C. brasiliensis* protein database (see section 2.7), P1 was further identified as
360 Dominin, P2 as Actin, and P3 as Segon (Table 3).

361 3.4. Sensitivity of Dominin thiols to oxidants

362 *C. brasiliensis* Dominin accounted for approximately 60 % of total protein in coomassie-
363 stained SDS-PAGE gels, with an easily detectable 5-IAF signal for both PSH and R-PSX. Data
364 showed decreased PSH levels for Dominin after exposure to CAM1 water samples. Therefore, it
365 was considered a good candidate for protein thiol oxidation studies in oysters and the reactivity
366 of its Cys residues was further assessed in vitro using H₂O₂, CHP, and HOCl.

367 Hemolymph sampled from REF organisms was further exposed in vitro for 90 min to
368 oxidants, and Dominin PSH and R-PSX levels were analyzed by 5-IAF labeling, followed by 1D
369 SDS-PAGE (Figure 4). Data indicate that free/reduced Cys at Dominin are promptly oxidized by
370 all tested oxidants, as detected by the PSH levels (Figure 4A): 33 – 40% decrease for H₂O₂, 40-
371 56% for CHP and 70-72% for HOCl. On the other hand, no changes in R-PSX levels were
372 observed for all oxidants and concentrations tested (Figure 4B). It was not possible to investigate
373 the effects of 1 and 10 mM HOCl due to the high protein degradation rate at these concentrations
374 (data not shown).

375 3.5. Computational analysis of Dominin

376 While reducing SDS-PAGE suggests a molecular weight of approximately 35 kDa for the
377 Dominin, non-reducing electrophoresis revealed a 25 kDa band (Supplemental Data, Figure S1).
378 This shift in the apparent molecular weight indicates the presence of internal disulfide bridges, as
379 already demonstrated for homologous proteins [12,14]. Intramolecular disulfide bridges can
380 maintain a compact structure under non-reducing conditions, allowing for faster migration in the

381 SDS-PAGE. Disulfide bridges can be readily disrupted by the addition of reducing agents, such
382 as DTT and β -mercaptethanol.

383 The localization of the 4 Cys residues in the mature Dominin (without signal peptide)
384 was investigated by 3D structural modeling through multiple alignment sequences (Figure 5). By
385 assuming the presence of Cys residues as disulfide bridges, which are not further oxidized by
386 ROS, it was expected the presence of free Cys residues close to the protein surface, which would
387 be available and prone to oxidation events. The 3D structural model predicts a high conserved β -
388 sheet core with two pairs of cysteines (30-165 and 94-164) connecting a short helix, similar to
389 the human extracellular Superoxide Dismutase (Hsapiens EcSOD, PDB ID: 2JLP) [33]. Only 1
390 of these pairs was close enough to form a disulfide bridge (94-164), as the distance between the
391 Cys 30-165 lateral chains is 2.4 Å and presents uncertainties about the feasibility of a disulfide
392 bridge (Figure 5A; upper box). Assuming that the model was built using multiple alignments we
393 are also considering the possibility of two disulfide bridges on *C. brasiliensis* Dominin, but our in
394 vivo and in vitro experimental data support the idea of the existence of at least 1 free and
395 accessible cysteine residue. This hypothesis is based on our experimental data showing a direct
396 5-IAF labeling of Dominin, indicating the presence of reduced thiol groups.

397 Additionally, Dominin presents several loops, some of them involved in LPS and integrin
398 binding sites (Figure 5A). The loop and short helix involved in Cu coordination are shorter,
399 lacking the key residues Asn180, Asn182 and Arg 186 (Figure 5B; upper box). Other residues
400 related to Cu bound are also substituted in *C. brasiliensis* (His113 and His163 to Leu, His96 to
401 Gln), as well as to Zn binding (His121 and His124 to Asp) (Figure 5; lower panel). Dominin also

402 has a shorter C-terminus with no α -helix in the heparin binding site, as compared to Hsapiens
403 EcSOD (Figure 5B). Dominin 3D.

404 Phylogenetic analysis of complete Dominin protein sequence (including peptide signal)
405 revealed that it is homologous to extracellular SOD-like proteins from oysters, fishes, birds,
406 amphibians and mammals (Supplemental Data, Figure S2). This same analysis grouped Dominin
407 with extracellular SOD-like proteins from other oysters while sequences from vertebrates were
408 grouped as another branch. Multiple alignments of Dominin against homologous proteins from
409 other oyster species (Supplemental Data, Figure S3) indicate that the 4 Cys residues are
410 conserved among the studied species, with *O. edulis* containing an additional Cys residue at the
411 N-terminal end, and that both LPS and integrin-binding motifs are conserved among the genus
412 *Crassostrea* while they are not shared with *O. edulis* and *S. glomerata*. In addition, while protein
413 sequences related to the Cu/Zn SOD domain are similar among the oyster species, many amino
414 acids are not conserved within the family domain (Figure 5).

415

416 4. DISCUSSION

417 Water analyses indicated that CAM1 is influenced by urban sewage more than CAM2.
418 CAM1 TC values were 15 and 175 times higher than those of REF and CAM2, respectively.
419 These data are in accordance with the higher concentrations of total phosphorous and nitrogen at
420 CAM1, compounds commonly associated with urban sewage discharge [34]. CAM1 also
421 presented the highest TtC levels, an indication of fecal contamination in this area. These levels
422 were an order of magnitude lower than the TtC detected at the mouth of Bücheler river, an area

423 heavily contaminated by urban sewage at Santa Catarina State [16,17]. TtC levels detected in the
424 present study in REF and CAM2 groups are in the same range as the ones found in aquaculture
425 sites at Florianópolis (Santa Catarina State), the major oyster producer in Brazil [16].
426 Interestingly, data pertaining to BOD and COD were contrasting. According to BOD
427 measurements CAM1 and CAM2 had higher concentrations of organic compounds relative to
428 REF; however, according to COD measurements, REF had higher concentrations of organic
429 compounds. It is noteworthy that although BOD and COD are similar in function, BOD is more
430 specific as it measures the levels of only the biodegradable organic matter. Thus, in an effort to
431 address the contrasting BOD and COD data, more specific chemical analyses were performed in
432 sediments and oysters.

433 Sediments from CAM1 had the highest concentrations of total PAHs, PCBs, LABs, and
434 steroids, while CAM2 had the highest concentrations of DDT. Notably, total PAHs were
435 considerably lower (1 – 3 orders of magnitude) when compared to contaminated areas with
436 known petrogenic and pyrolytic inputs [35,36], and similar to values found at major oyster
437 farming areas in Brazil [16]. Total PCBs and DDTs were also detected at relatively low
438 concentrations when compared to other contaminated areas [35,37–39]. Moreover, PAHs, PCBs
439 and organochlorines pesticide concentrations in sediments at both sites were below the maximum
440 concentration recommended by MMA, the Brazilian environmental agency
441 (<http://www.mma.gov.br/port/conama/legiabre.cfm?codlegi=693>). LABs and steroid
442 concentrations in sediments are not included in the Brazilian environmental agency regulations,
443 but total LAB concentrations in the sediment of CAM1 were close to the values detected in
444 industrialized areas of China [40] and areas with pronounced input of urban sewage in Brazil
445 [16], suggesting a significant sewage input in the region. Total steroid concentrations in CAM1

446 were also high. In fact, it is possible to estimate the steroids specific to fecal contamination by
447 applying the $5\beta/(5\alpha+5\beta)$ stanol index and the coprostanol/epicoprostanol (c/e) ratio. The
448 $5\beta/(5\alpha+5\beta)$ index aids to discriminate between sewage and biogenic sources of steroids, where
449 values greater than 0.7 indicate urban sewage pollution [41]. For the c/e ratio, values above 1.55
450 indicate human waste as the fecal source of coprostanol [41]. CAM1 presented values of 0.79 for
451 the $5\beta/(5\alpha+5\beta)$ and 10.4 for c/e ratio. It was not possible to calculate these ratios at CAM2 as
452 epicoprostanol concentrations were below the limit of detection. Together, these data indicate a
453 low input of effluents related to industry or agriculture at Balneário Camboriú, but the moderate
454 contribution of urban sewage effluents as indicated by the concentrations of LAB and steroids
455 (and the $5\beta/(5\alpha+5\beta)$ and c/e ratios) at CAM1.

456 The presence of contaminants at CAM1 and CAM2 was also investigated in oysters
457 exposed for 24 h under laboratory conditions to water samples collected at both sites. Oysters
458 had relatively higher concentrations of total PAHs (mostly containing 2 or 3 rings) and PCBs
459 before the exposure, which decreased in all groups after the 24 h exposure. It is not clear what
460 would be the cause of such bioaccumulation in pre-exposure animals. Oysters were obtained
461 from an aquaculture laboratory located in a bay with weaker circulation than Camboriú Bay, and
462 motorized boats are used in the area for the aquaculture activities and by the local community.
463 This could be one of the causes of the higher PAHs and PCBs concentrations in pre-exposure
464 oysters. In fact, di and trimethylated forms of naphthalene accounted for 67% of total PAHs in
465 oysters prior to exposure, indicating a possible source of petrogenic activity (such as diesel fuel
466 for boats), while other PAHs remained at considerably lower concentrations at pre-exposure
467 organisms. Either way, according to the environmental assessment criteria used by the OSPAR
468 Commission (<http://www.ospar.org/documents?v=7167>), PAHs and PCBs concentrations in pre-

469 exposure animals can be considered low and acceptable, and suggests that they may not cause
470 great effects on cellular function and animal physiology. Following exposure to REF, CAM1,
471 and CAM2 water samples, oysters had a fast depuration of PAHs and PCBs, indicating that these
472 classes of contaminants were not major components of the exposure protocol. It is not clear if the
473 storage of water samples in high-density polyethylene plastic containers may have released
474 chemicals to the water samples, what could contribute to the chemical bioaccumulation and
475 toxicity observed in this study. On the other hand, the urban sewage input is clear at Balneário
476 Camboriú bay, as the most significant bioaccumulation was related to total LABs concentrations
477 at CAM1 (from <0.88 ng/g to 180 ng/g dry weight). Together with the higher LABs
478 concentrations in the sediment from CAM1, these data reinforce the urban sewage input in the
479 region. Even if pre-exposure animals were subjected to a pre-existent PAHs and PCBs
480 contamination, the urban sewage-related exposure in animals from the CAM1 group was evident.
481 The potential toxicity of such effluents was further addressed by analyses of protein oxidative
482 modifications in oysters *C. brasiliiana*.

483 Considered as a complex mixture of chemicals and microorganisms, exposure of bivalves
484 to urban sewage could result in adverse effects due to oxidative stress and damage as reported
485 previously [17,42–44]. As such, protein thiol groups are likely the primary targets of chemicals
486 present in the urban sewage; however, this has not been addressed extensively in aquatic species.
487 In the present study, PSH levels decreased in the hemolymph of oysters exposed for 24 h to
488 seawater samples collected at CAM1. This decrease suggests the occurrence of thiol oxidation
489 events in this tissue. Unchanged levels of R-PSX together with decreased PSH levels indicate
490 that these oxidation events in hemolymph may lead to irreversible protein thiol modification. In
491 fact, there are few studies regarding protein oxidative damage in hemolymph or hemocytes of

492 invertebrates and most of them analyzed protein carbonylation. For example, increased protein
493 carbonyl concentrations were reported in crabs and mussels in response to seasonal changes [45]
494 and exposure to organic contaminants, metals [11] and nanoparticles [46]. Protein oxidation in
495 other tissues, including gills and digestive gland, is also studied. In this context, it was suggested
496 that thiol levels, such as PSH and glutathione (the major cellular non-protein thiol) in gills or
497 digestive gland are important markers of toxicity in bivalves in oxidative stress scenarios [47–
498 52]. We did not find alterations in PSH or R-PSX levels in these tissues, but our data include
499 hemolymph as another potential tissue for investigating the toxicity of environmental
500 contaminants and protein thiol oxidation.

501 SDS-PAGE analysis indicated that the hemolymph of *C. brasiliensis* contains 3 highly
502 abundant proteins that are sensitive to thiol oxidation. They were identified by MALDI-TOF and
503 MALDI-TOF/TOF as Dominin (ANB49597.1), Actin (ANB49595.1) and Segon
504 (ANB49596.1). Actin may be related to the presence of a cellular fraction (hemocytes, as total
505 hemolymph was used for the present study), and it is also a protein known to be a target for thiol
506 oxidation [8]. Dominin and Segon (as well as homologous proteins) are the two most abundant
507 plasma proteins that are presumably associated with the immune system, antioxidant defenses,
508 wound repair, metal transport, and shell mineralization [12,14,53,54]. PSH levels of these
509 proteins decreased at CAM1 group while Dominin R-PSX levels remained unaltered. R-PSX
510 levels of Segon also decreased at CAM1 group. These data also reinforce the existence of
511 oxidation events in the hemolymph of animals exposed to CAM1 water samples, probably
512 leading to irreversible modifications. As PAHs, PCBs and organochlorine pesticides levels at
513 CAM1 appear to be low, it is possible that the oxidation could be associated with other
514 compounds, such as LABs or bacterial pathogens (due to fecal contamination at this site), as well

515 as metals, personal care products, pharmaceuticals, and nanomaterials (compounds not analyzed
516 in the present study but commonly detected at urban sewage [4]). The toxic effects of LABs to
517 aquatic species are still unknown, but it may cause upregulation of antioxidant defenses (e. g.
518 glutathione reductase, glucose 6-phosphate dehydrogenase, superoxide dismutase) in oysters [44]
519 as a possible response to a pro-oxidative scenario. Sewage-related bacterial pollution can cause
520 accumulation of pathogens in bivalves [55], causing an immune response to bacterial infection
521 that may induce the production of reactive oxygen and nitrogen species by hemocytes [56] with
522 the potential to cause unspecific damage to plasma proteins [57]. The other cited substances can
523 have different mechanisms of toxicity, but commonly include induction of oxidative stress and
524 cellular damage [11,58–60]. It is not clear which contaminants could be acting as pro-oxidants at
525 CAM1 group, but our data point to hemolymph as a favorable tissue for the analysis of protein
526 oxidative damage in bivalves exposed to urban sewage, and possibly to other contaminants. It is
527 possible that higher PAHs and PCBs concentrations in pre-exposure oysters could have resulted
528 in a pre-existing pro-oxidant scenario that decreased the sensitivity of thiol-containing proteins to
529 further oxidative challenge. However, according to the OSPAR environmental assessment
530 criteria, pre-existing concentrations of PAHs and PCBs would have minimal effects on basal
531 protein oxidation levels. Even though, the susceptibility of Actin, Dominin, and Segon to Cys
532 oxidation remains clear according to the current exposure protocol.

533 *C. brasiliiana* Dominin was further studied in vitro to understand how the thiol groups
534 might be oxidized by pro-oxidants, such as peroxides (H_2O_2 and CHP) and HOCl. The sensitivity
535 to thiol oxidation was higher for HOCl and CHP, which are reported as potent thiol oxidants
536 [61,62]. The unchanged R-PSX levels indicate the possible irreversible thiol oxidation in
537 Dominin after H_2O_2 , CHP or HOCl. In both in vivo and in vitro experiments, this could be easier

538 achieved in the plasma due to the absence/low abundance of thiol reducing systems such as the
539 glutathione and thioredoxin systems [63]. Furthermore, the extracellular environment is typically
540 oxidative, which would favor maintaining thiols in oxidized forms [64]. The presence of
541 abundant proteins with thiols as sites of oxidation in hemolymph resembles the serum albumin,
542 the most abundant plasma protein in mammals. It also has a partially accessible cysteine (Cys34)
543 on the surface, whose oxidation state is associated with oxidative stress and commonly used as a
544 biomarker of thiol oxidation in many pathological conditions [65].

545 Detailed computational analysis of *C. brasiliensis* Dominin suggest that 2 (Cys30 and
546 Cys165) of the 4 available Cys residues would not be pairing as an intramolecular disulfide
547 bridge, and the direct 5-IAF labeling points to the presence of reduced thiols in Dominin. It is
548 possible that one or both Cys30 and Cys165 would be in the reduced state and could be the target
549 of thiol oxidation detected at CAM1 group and by the in vitro experiments. As this protein
550 appears to be a target of reactive oxygen and nitrogen species in the hemolymph, additional
551 studies are necessary to identify which Cys residues are reactive to oxidants in Dominin.
552 Moreover, phylogenetic analysis indicates that these four Cys residues are also present in
553 homologous proteins of *O. edulis*, *S. glomerata*, *C. gigas* and *C. virginica*. Therefore, it is
554 possible that such events of protein thiol oxidation may also occur in other oyster species, not
555 only after exposure to urban sewage but also to other environmental contaminants, biotic and
556 abiotic stressors. Taking into account the suggested multiple functions of this protein in oysters,
557 it could be a promising molecule for toxicity studies in marine bivalves.

558

5. CONCLUSIONS

559 The present study investigated the water quality of two sites at Balneário Camboriú, an
560 important tourist destination at Santa Catarina State (southern Brazil) and an area with emerging
561 fishing and aquaculture activities. The investigation was based on standard water quality
562 measurements, organic contaminants, and laboratory exposure of oysters to water samples
563 collected in the area. Data suggest the influence of urban sewage, mostly near the mouth of
564 Camboriú River (CAM1), which is probably related to an urban input. Such effluents were able
565 to induce protein oxidation in oysters *C. brasiliiana*. In fact, our data showed for the first time the
566 use of 1DE SDS-PAGE to identify thiol oxidation in 3 hemolymph proteins. Additional studies
567 with one of them (Dominin) indicated the presence of Cys residues (Cys30 and Cys165) as sites
568 of thiol oxidation by classical pro-oxidant agents. These data were indicative of a potential
569 toxicity of such urban sewage input to the local aquatic species and call for protective and
570 preventive actions in the region. Moreover, Dominin computational analyses indicated that Cys
571 oxidation events could also occur in homologous proteins of other oysters species, suggesting the
572 use thiol oxidation of hemolymph proteins as a viable tool and a new biomarker of toxicity in
573 bivalves exposed not exclusively to pro-oxidant contaminants or cellular stressors.

574

575 **Acknowledgment**

576 This work was supported by INCT-Toxicologia Aquática, CNPq grant #573949/2008-5.
577 R. Trevisan, G. Toledo-Silva, and F. Flores-Nunes are postdoc fellows of CNPq, CAPES, and
578 Petrobras, respectively. E.S. Dolores, C.E. Piazza, I.M.M. Reis, F.L. Zacchi, B.N.M. Othero,
579 D.F. Mello and A.P.M. Fraga are recipients of scholarships awarded by CAPES. A.L. Dafre,
580 M.R.F. Marques, R.C. Montone, M.C. Bícigo, and A.C.D. Bainy are research fellows of CNPq.

581 We are thankful to CEBIME – UFSC for the technical assistance. We are grateful to Dr. A.
582 Massarsky (Duke University) for kindly revising the manuscript.

583

584 **DISCLOSURE**

585 All authors declare to have no actual or potential conflict of interest including financial,
586 personal or other relationships with other people or organizations. The work described has not
587 been published previously (except in the form of an abstract); it is not under consideration for
588 publication elsewhere, and its publication is approved by all authors and tacitly or explicitly by
589 the responsible authorities where the work was carried out, and if accepted, it will not be
590 published elsewhere in the same form, in English or any other language, without the consent of
591 the copyright holder. The authors also declare that all experiments were carried out according to
592 the local Research Ethics Committee at the Federal University of Santa Catarina.

593 All authors declare to have participated in this study, as following: project coordinators
594 AB, MRFM and ACDB; experimental design, field work and sampling FFN, ESD, JJM, CEP
595 and CMRM; chemical analyses STS, ST, RCM and MCB; animal exposure and tissue sampling
596 FFN, ESD, JJM, CEP, IMMR, FLZ, BNMO and CLVB; sample processing, protein
597 electrophoresis and in vitro studies RT, DFM and ALD; protein identification by mass
598 spectrometry RT, APMF and GTS; bioinformatics and computational analyses RT, NW, GTS
599 and GR; manuscript preparation all authors.

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771 **Figures subtitles**

772

773 Figure 1: Area of study at Balneário Camboriú (Santa Catarina, southern Brazil). Two sites were
774 analyzed: one close to the mouth of Camboriú River (CAM1), which contributes to the input of
775 urban sewage to the bay, and another site approximately 1 kilometer away from the mouth of the
776 river (CAM2) that could potentially be influenced by urban discharge.

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778 Figure 2: Protein thiol status in tissues of oysters *Crassostrea brasiliiana* exposed for 24 h in the
779 laboratory to seawater collected at Balneário Camboriú (Santa Catarina, southern Brazil). Water
780 sampling sites consisted of CAM1 and CAM2 (see legend to Figure 1 for details). The reference
781 group (REF) consisted of water collected at the local university aquaculture laboratory from
782 where the oysters were obtained. 1D SDS-PAGE analysis of reduced protein thiol (PSH, left) by
783 direct 5-IAF labeling and reversibly oxidized protein thiol (R-PSX, right) by NEM/DTT
784 incubation followed by 5-IAF labeling. PSH (left) and R-PSX (right) were assayed in gills (A

785 and B), digestive gland (C and D) and hemolymph (E and F). Data were analyzed by one-way
786 ANOVA followed by Duncan *posthoc* or Kruskal-Wallis ANOVA & Median test. Groups not
787 sharing letters are significantly different at $p < 0.05$ ($n = 6$).

788
789 Figure 3: Thiol status of abundant proteins in the hemolymph of oysters *Crassostrea brasiliiana*
790 exposed for 24 h in the laboratory to seawater collected at Balneário Camboriú (Santa Catarina,
791 southern Brazil) at CAM1 and CAM2, as well as REF group (see legend to Figure 1 for details).
792 (A) A representative SDS-PAGE of hemolymph from 3 *C. brasiliiana* individuals (1 – 3) after
793 colloidal Coomassie staining, and visualization of reduced protein thiols (PSH) or reversibly
794 oxidized protein thiols (R-PSX). The 3 most abundant proteins from hemolymph are shown as
795 P1, P2, and P3 in the Coomassie-stained gel, with their respective signals for PSH and R-PSX,
796 indicated by arrows. Molecular weight markers are shown as (M). Levels of PSH (left) and R-
797 PSX (right) were assayed in P1 (B and C), P2 (D) and P3 (E and F) proteins. It was not possible
798 to quantify R-PSX for P2 due to the faint fluorescent signal. Data were analyzed by one-way
799 ANOVA followed by Duncan *post hoc* test. Groups not sharing letters are significantly different
800 at $p < 0.05$ ($n = 6$).

801
802 Figure 4: Thiol status of Dominin from the hemolymph of oysters *Crassostrea brasiliiana*
803 exposed in vitro to oxidants. Hemolymph from reference animals was incubated with hydrogen
804 peroxide, cumene hydroperoxide (CHP) or hypochlorous acid (HOCl) at the indicated
805 concentrations for 1.5 h. Samples were further assayed for reduced protein thiols (PSH) and
806 reversibly oxidized protein thiols (R-PSX) by 1D SDS-PAGE. Data ($n = 4$) were analyzed by
807 paired one-way ANOVA followed by Dunnet *posthoc*. * ($p < 0.05$), ** ($p < 0.01$) and *** ($p <$
808 0.001) indicate significant differences to the respective control group ($n = 4$).

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810 Figure 5: *Crassostrea brasiliiana* Dominin 3D model. (A) Dominin overall folding showing the
811 β -sheet core in green and α -helices in red. The LPS binding site (DDED) and the integrin binding
812 site (RGB) motifs are represented by sticks. The upper box shows the Cys pairs between
813 positions 30-165 and 94-164. Only Cys 94-164 pair forms a disulfide bridge. (B) Overlay of
814 Dominin and human Extracellular Superoxide Dismutase 3 (Hsapiens EcSOD, PDB ID:2JLP, in

815 purple). The Hsapiens EcSOD structure has no information about the N-terminal part. C-terminal
816 helix related to heparin binding is present only in human. The upper box shows the absence of
817 the electrostatic loop and the twisted β -sheet with CYS 164 and 165 in Dominin, and the metal
818 binding sites (copper and zinc) from Hsapiens EcSOD. The conserved amino acids involved in
819 copper coordination are not present in Dominin, suggesting different dynamic properties on SOD
820 domain. At the bottom panel, alignment of the Dominin and Hsapiens EcSOD mature protein
821 sequences. Cysteines (C) are highlighted at both sequences by gray boxes and residues related to
822 Cu or Zn binding at SOD3 are highlighted by black and white boxes, respectively. LPS (DDED)
823 and integrin (RGD)-binding sites at Dominin are underlined. Conserved amino acid residues are
824 indicated by the * symbol.

825

826 Figure 1.



838

839 Table 1.

840

841	Parameter	REF	CAM1	CAM2
842	TC ^b	240	3500	20
	TtC ^b	210	2400	Not detected
843	BOD ^c	<2.25	3.02	3.09
	COD ^c	1460.0	285.0	395.0
	Detergents ^c	0.76	0.6	0.84
844	Total phenols ^c	0.22	0.36	0.41
	Total phosphorous ^c	<0.01	0.51	<0.01
	Nitrogen ^c	<1.0	3.7	<1.0
845	Oils ^d	Virtually absent	Virtually absent	Virtually absent
	Suspended solids ^c	52.4	48.4	52.0

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^aValues shown with the symbol < (less than) indicate the detection limit for that specific analysis.

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^bMost probable number/100 mL.

^cIn mg/L.

^dMeasured by visual inspection.

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REF = reference group, water from the Federal University of Santa Catarina aquaculture laboratory, where oysters were obtained for the study; CAM1 = water collected near the mouth of the Camboriú River, at Balneário Camboriú; CAM2 = water collected 1 km away from the river mouth; TC = total coliforms; TtC = thermotolerant coliforms; BOD = biochemical oxygen demand; COD = chemical oxygen demand.

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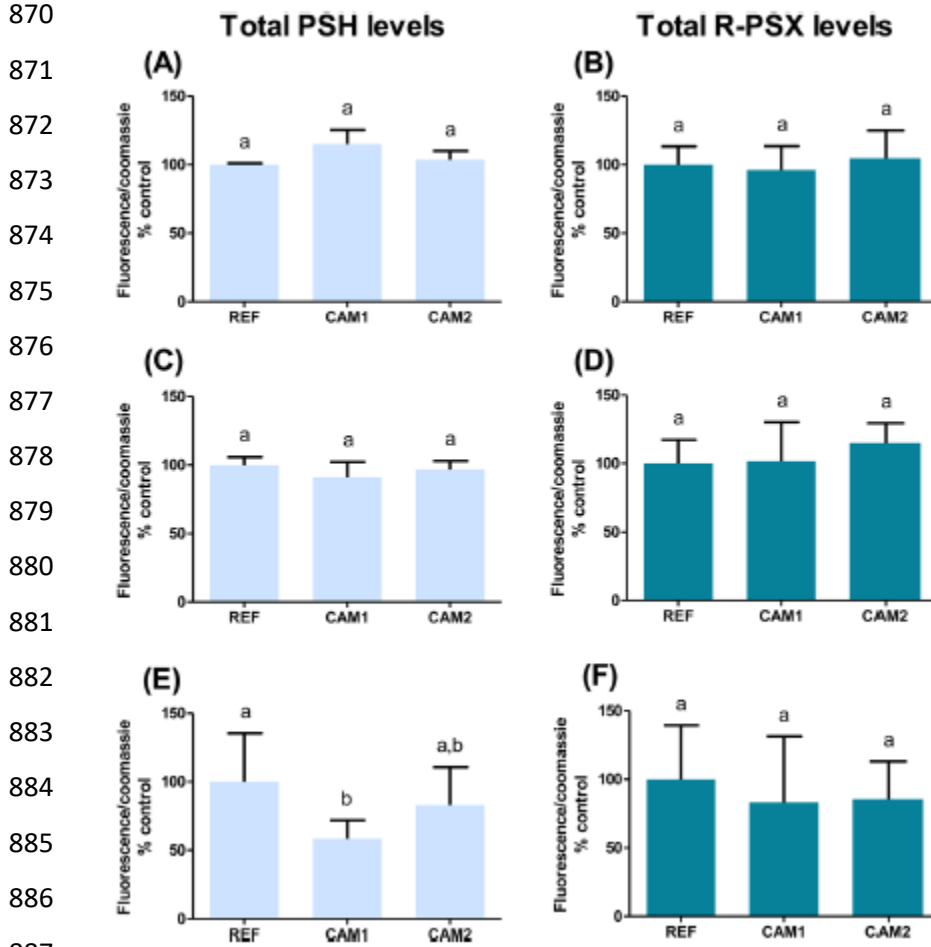
857 Table 2.

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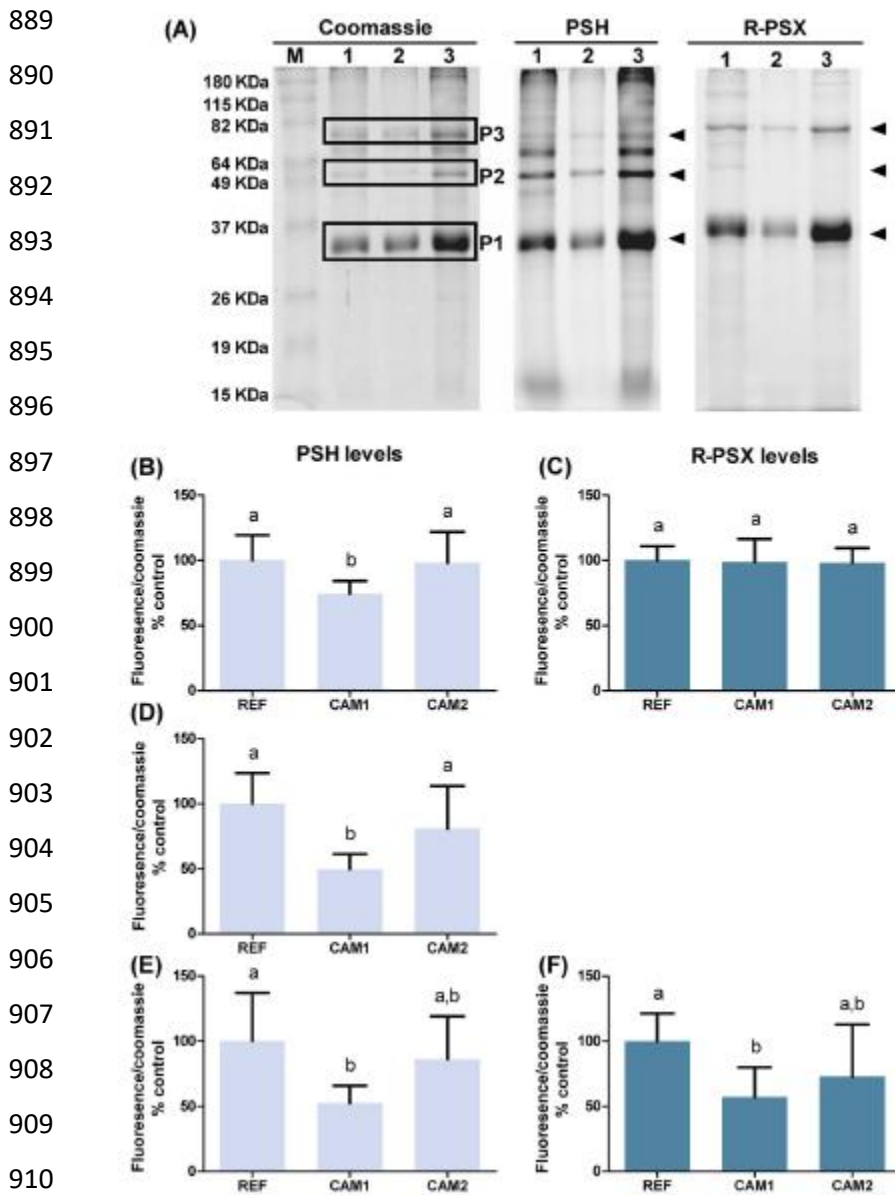
859 Parameter	Sediment		Oysters			
	CAM1	CAM2	Pre-exposure	REF	CAM1	CAM2
860 Total PAH (ng/g)	65.8	15.9	540.1	233.3	22.0	15.0
Total PCB (ng/g)	0.72	<0.02	35.0	21.2	7.61	8.87
861 Total hexachlorocyclohexane (ng/g)	<0.11	<0.11	<0.31	<0.31	<0.31	<0.31
Total chlordanes (ng/g)	<0.03	<0.03	<0.19	<0.19	<0.19	<0.19
Total DDT (ng/g)	<0.01	0.04	0.99	1.85	1.95	<0.20
862 Total endosulfan (ng/g)	<1.14	<1.14	<0.42	<0.42	<0.42	<0.42
Total drins (ng/g)	<0.03	<0.03	<0.22	<0.22	<0.22	<0.22
863 Hexachlorobenzene (ng/g)	<0.03	<0.03	<0.30	<0.30	<0.30	<0.30
Methoxychlor (ng/g)	<0.13	<0.13	<0.32	<0.32	<0.32	<0.32
Mirex (ng/g)	<0.13	<0.13	<0.41	<0.41	<0.41	<0.41
864 Total LAB (ng/g)	52.2	<0.08	<0.88	<0.88	180	<0.88
Total steroids (μg/g)	4.92	1.45	N/A	N/A	N/A	N/A

865 ^aValues with the symbol < (less than) indicate the detection limit for that specific analysis.
 866 Pre-exposure = levels of organic contaminants in oysters prior exposure to water samples; REF = reference group, water from the Federal University of Santa
 867 Catarina aquaculture laboratory, where oysters were obtained for the study; CAM1 = water collected near the mouth of the Camboriú River, at Balneário
 868 Camboriú; CAM2 = water collected 1 km away from the river mouth; PAH = polycyclic aromatic hydrocarbon; PCB = polychlorinated biphenyl;
 869 DDT = dichlorodiphenyltrichloroethane; LAB = linear alkylbenzenes; N/A = not analyzed.

869 Figure 2.



888 Figure 3.



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912 Table 3.

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Protein ^b	MALDI-TOF (MS)			MALDI-TOF/TOF (MS/MS)			Identification	
	Score ^c (peptides ^d)	E-value ^e	Coverage ^f	Score ^g	E-value ^e	Peptide ^h	Protein ⁱ	Accession no. ^j
P1	N/A	N/A	N/A	137	9.4e-14	TFDWLTIGHESGILGR	Dominin	ANB49597.1
P2	65 (8)	1.8e-3	31	N/A	N/A	N/A	Actin	ANB49595.1
P3	N/A	N/A	N/A	72	3.4e-7	EFDWLQINLADGILGR	Segon	ANB49596.1

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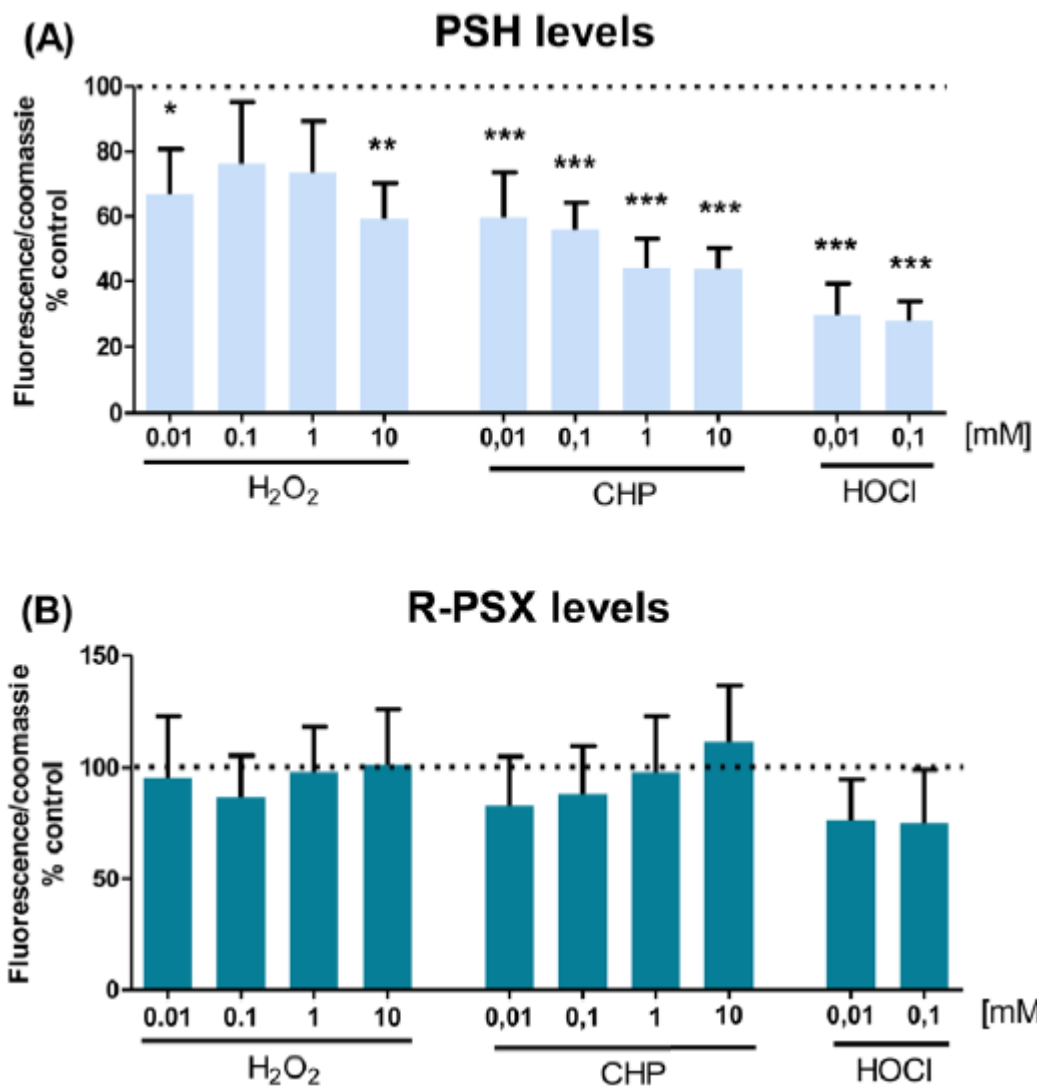
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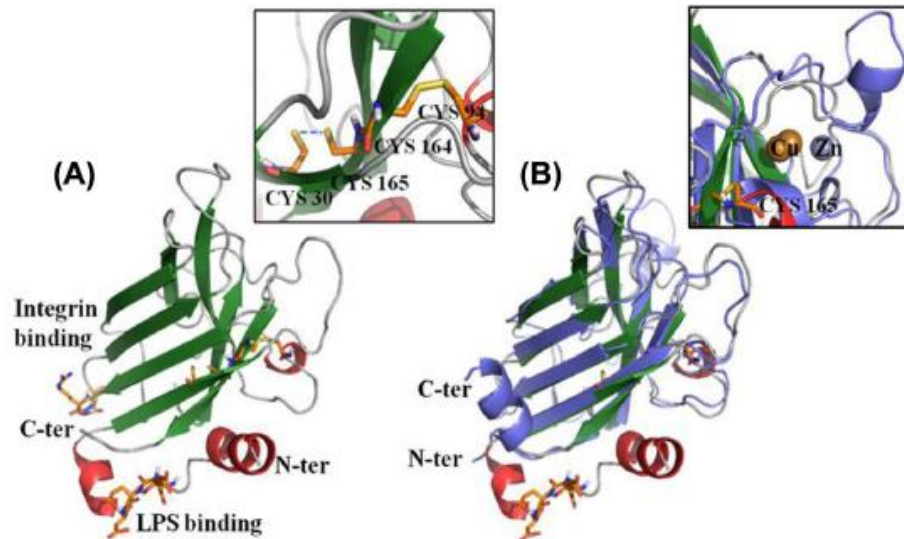
919 Figure 4.

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950 Figure 5.

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(C)

Cbrasiliana Dominin	1	SRNEANV-----NIYLHLS-----DEDPNYANTMHYAQCEMEPNPMPT	40
Hsapiens EcSOD	1	WTGEDSAEPNSDSAEWIRDMYAKVTEIWQEVMQRRDDD----GTLH-AACQVQPSATLDA	55
		. * : * : : : : : * : * . : * * * * : : : : : *	
Cbrasiliana Dominin	41	TLHHKVHGS IEMHQ RGD -GPLSMSFHLTG FNTSEDFAAHNLGLQIHEYGDMEHGCDTIGE	99
Hsapiens EcSOD	56	A-QPRVTGVVLF RQLAPRAKLDAFFALEGFPTEPN--SSSRAIIVIQFGDLSQGCSTGP	112
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Cbrasiliana Dominin	100	LYHNEHAPDHADPGDLGDLHDDEHGEVNSTRTFDWLTIGHESGILGRSLAILQGDHT---	156
Hsapiens EcSOD	113	IYNPLAVFIPCPGIFG NFAVRD-GSLWRYRAGLAASLAGPHSIVGRAVVVIAGEDDLGR	171
		* : . * . . . * : * : * : : * : . . . : * : * : * : * : * : * : *	
Cbrasiliana Dominin	157	--SHTAI-----IACCVIG-----RSHAH-----	173
Hsapiens EcSOD	172	GGNQASVENGNAGRRLACC VVGCGPGLWERQAREHSERKKRRRESECKAA	222
		. : : : : : : * * * * : * : * : * : * : * : * : *	