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## Differential gene expression in oyster exposed to sewage

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### Abstract

In order to investigate the influence of domestic sewage on the gene expression of Pacific oysters *Crassostrea gigas*, Suppression Subtractive Hybridization (SSH) method was employed. Oysters were sampled at a farming area and, after 10 days of acclimation in the laboratory, were exposed to untreated domestic sewage diluted 33% for 48h. Gills of male oysters were excised for total RNA extraction. mRNA was purified and the differential gene expression was analyzed by SSH. We obtained 61 cDNA sequences but only 15 were identified, which includes Fatty Acid Binding Protein, Multidrug Resistance Protein, Omega Glutathione S-Transferase,

cytochrome P450 isoform CYP356A1, among others. The identified genes are associated with different metabolic functions like biotransformation, membrane transport, aerobic metabolism and translational machinery, evidencing the potential toxic effects elicited by these effluents.

*Keywords:* Crassostrea gigas; SSH; Gene expression; Sewage; Biomarker; Monitoring.

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Sewage discharges in marine and estuarine ecosystems are a common practice around the world. Besides the high input of organic matter, sewage may contain several kinds of persistent or non-persistent contaminants, such as pharmaceuticals, personal and home care products, surfactants, plasticizers, polycyclic aromatic hydrocarbons and trace metals, which are continuously delivered and its negative effects on the organisms must be considered (Petrovic et al., 2003). Bivalves are frequently employed in biomonitoring programs because they are filter-feeders, easy to collect and largely distributed. The areas of bivalve genomics and transcriptomics are emerging fields considering their application to aquaculture, fisheries and environmental sciences (Saavedra and Bachère, 2006). The aim of this study was to investigate differential gene expression in the Pacific oyster *Crassostrea gigas* after sewage exposure, under controlled laboratory conditions, in order to identify new potential biomarkers of exposure. For this purpose, we employed suppressive subtractive hybridization (SSH).

Pacific oysters (two years old) were supplied by the oyster culture facility from the Universidade Federal de Santa Catarina and transferred to the laboratory. Individuals were kept in two 45L tanks (n=11) with aerated filtered marine water (salinity 24ppt; temperature  $24 \pm 2^\circ\text{C}$ )

for a 10 days acclimation period. After that, one group of animals was exposed for 48h to untreated domestic sewage, diluted 33%. The sewage sample was collected at the influent duct of the downtown wastewater treatment plant (Florianópolis, SC, Brazil) after solid material grid removal. The light gray color as well as the physical and chemical parameters were characteristic of fresh untreated domestic sewage (BOD 360mg/L; COD 393mg/L; SS 148 mg/L) (Katsoyiannis and Samara, 2007; Al-Shammiri, 2004). A control group was kept in a tank containing filtered marine water diluted similarly in charcoal-filtered freshwater. The water was changed daily and the organisms were fed with microalgae. Likewise, the oysters were monitored daily by visual inspection to determine that they were alive and opening their valves. After the exposure period, the oysters were sacrificed, their sex was identified and the gills were excised and kept in RNAlater (Ambion) at -20°C until RNA extraction. Oyster gills from male organisms from exposed and control groups were pooled (n=5, each group) and total RNA was extracted in TRIZOL (Invitrogen Life Technologies, Carlsbad, CA). PolyA mRNA was isolated using Oligotex mRNA Mini kit (Qiagen, Valencia, CA). Sample purity, mRNA concentration and integrity were checked at 260 and 280 nm and in 2% agarose gel electrophoresis. SSH was performed according to PCR Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) and only the forward subtraction was performed, to identify up-regulated genes. Subtracted libraries were obtained from 2 µg mRNA of pooled gills, respectively, from exposed and control oyster groups (control group cDNA was referred to as the *driver* and the exposed group cDNA as the *tester*). Secondary (nested) PCR-amplified subtracted cDNA populations were cloned into the pGEM-T Easy Vector System and transformed into JM-109 *E. coli* cells (Promega, Madison, WI). Plasmids were extracted and purified using Perfectprep Plasmid Mini kit (Eppendorf, Hamburg). Sixty-one clones were sequenced using ABI Prism Big Dye Terminator Cycle Sequencing kit

(PE Applied Biosystems). Deduced protein sequences were subjected to homology search through the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

From the sixty-one cDNA sequences, 15 were identified (Table 1). The data showed that different pathways of the oyster metabolism were changed after sewage exposure. Biotransformation genes, such as the new cytochrome P450 subfamily CYP356A1 (Toledo-Silva, this volume), omega glutathione S-transferase (GST-O) and multidrug resistance protein (MDR) were up-regulated in the gills of sewage exposed oysters. Besides these biomarkers of exposure, other genes belonging to the transporters family, translational machinery, aerobic metabolism, and nuclear proteins were also induced in the exposed group. One of the most redundant clones contained the cDNA of fatty acid binding protein (*FABP*). *FABP* are small cytosolic proteins (12 – 15 kDa) that bind non-covalently to hydrophobic ligands, mainly fatty acids. It has been suggested that the sub-type L-*FABP*, originally identified in vertebrate liver, is associated with xenobiotic uptake and metabolism (Velkov et al., 2005). *FABP* is also associated with increased fatty acid oxidation, which in turn, could provide energy required for protein synthesis and detoxification.

A subsequent study using semi-quantitative analysis showed that the expression of *CYP356A1*, *GSTO*, *MDR*, *FABP* and aminolevulinic acid synthase (*ALAS*) were induced by 1.9, 3.3, 3.3, 43.6 and 2.9-fold, respectively in the gills of oysters exposed to sewage (Medeiros et al., 2007).

The use of SSH in environmental studies allows the identification of multiple genes that are differentially expressed that can help to elucidate biochemical strategies of adaptation and survival used by mollusks exposed to toxic compounds in domestic sewage. The applicability of these genes as potential biomarkers is being investigated through field experiments before they can be adopted in environmental programs.

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Table 1.

List of up-regulated genes in gills of oyster *Crassostrea gigas* exposed for 48 h to untreated domestic sewage

Putative match	GenBank Accession number*	Species match	Length	e-value	GenBank Accession number**
Cytochrome P450 3561A	EF645271	<i>Squalus acanthias</i>	509 aa	3e-24	AAB34256
Glutathione S-transferase omega class	-	<i>Crassostrea gigas</i>	243 aa	4e-22	CAD89618
Multi drug resistance protein	EU073425	<i>Gallus gallus</i>	1307 aa	1e-52	XP_418636
Acyl CoA oxidase similar protein	EU108716	<i>Danio rerio</i>	660 aa	1e-38	AAH83524
Alternative oxidase isoform A	EU108720	<i>Acanthamoeba castellanii</i>	370 aa	1e-91	ABB04277
5-Aminolevulinate synthase	EU073062	<i>Sepia officinalis</i>	603 aa	1e-51	AAD20808
40S ribosomal protein S2	EU108718	<i>Ictalurus punctatus</i>	277 aa	2e-23	AAK95183
Ribosomal protein L18a similar protein	EU108719	<i>Branchiostoma belcheri</i>	176 aa	1e-20	AAN52374
Asparaginyl tRNA-synthetase similar protein	EU108715	<i>Drosophila melanogaster</i>	558 aa	2e-94	NP_609948
Fatty acid binding protein	EU069496	<i>Caenorhabditis elegans</i>	136 aa	1e-09	NP_491928
Solute carrier family 27 similar protein	EU108714	<i>Canis familiaris</i>	913 aa	1e-08	XP_531894
Sterile alpha motif with 8 domain	EU108712	<i>Mus musculus</i>	478 aa	3e-36	NP_080559
PHD finger similar protein	EU108713	<i>Canis familiaris</i>	1955 aa	5e-33	XP_867023
C8orf1 (hT41) similar protein	EU108711	<i>Pan troglodytes</i>	549 aa	1e-42	XP_528185
Räs homolog gene family	EU108717	<i>Rattus norvegicus</i>	205 aa	1e-12	AAH61760

\*Genbank access number deposited after this work. \*\*Genbank access number of the closest match (e-value) obtained after blasting to National Center of Biotechnology Information