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AVALIAÇÃO DE RESPOSTAS IMUNOLÓGICAS DO MEXILHÃO MARROM *Perna perna* COMO INDICADORES DE POLUIÇÃO FECAL

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Marinha e Ambientes Costeiros da Universidade Federal Fluminense, como requisito parcial para obtenção do Grau de Mestre em Biologia Marinha e Ambientes Costeiros.

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and second (principal component 2) axes produced by the PCA are statistically significant ($p < 0.001$).

RESUMO

O mexilhão *Perna perna* é um bivalve intertidal amplamente distribuído, cultivado e consumido na África do Sul e em países da América do Sul, como Brasil e Venezuela. Dentre os recursos marinhos, os moluscos bivalves são um dos mais impactados pela poluição antropogênica, visto que podem acumular bactérias patogênicas, dentre outros poluentes presentes na água. Os hemócitos são células de defesa dos moluscos que podem ser afetadas em sua abundância e funções em resposta a contaminantes, como carga bacteriana. O objetivo deste estudo foi avaliar parâmetros imunológicos em *P. perna* como indicadores de poluição fecal em sua hemolinfa e na água. Para tal, os mexilhões e água do mar adjacente foram coletados de praias do estado do Rio de Janeiro (Brasil) com diferentes níveis de contaminação fecal: Praia Vermelha (VB); Praia de Icaraí (IB); Praia da Urca (UB); Praia de Jurujuba (JB). Os parâmetros hemocitários (densidade, morfologia, atividade fagocítica e produção de espécies reativas de oxigênio – ROS) foram avaliados por citometria de fluxo. A quantificação de bactérias indicadoras fecais (FIB) em água foi realizada a partir da técnica dos tubos múltiplos, para cada praia, e em hemolinfa por *spread-plate*. Concordando com a avaliação histórica dos níveis de contaminação fecal, UB apresentou o maior número de FIB em água (coliformes termotolerantes, TEC = 1.600 NMP 100 mL^{-1}), e VB o menor (TEC = 17 NMP 100 mL^{-1}). Os mexilhões coletados em UB apresentaram densidade de hemócitos e atividade fagocítica seis e oito vezes maiores, respectivamente, que os mexilhões de VB. Os mexilhões oriundos de VB e IB apresentaram número de coliformes totais em hemolinfa significativamente menor e hemócitos com complexidade interna relativa significativamente maior que os mexilhões coletados em UB e JB ($p \leq 0.01$, PERMANOVA). A produção de ROS pelos hemócitos foi significativamente menor em mexilhões de VB quando comparados aos mexilhões de JB ($p = 0.04$, ANOVA). Os resultados indicam uma relação significativa entre o nível de contaminação fecal em ambientes aquáticos e uma resposta imunológica relacionada aos hemócitos dos mexilhões. Parâmetros imunológicos podem ser utilizados como indicadores da condição de saúde dos bivalves e da qualidade ambiental. A análise das características morfológicas e funções dos hemócitos de *P. perna* por citometria de fluxo e seu papel na resposta imune relacionada a poluição fecal abre uma nova perspectiva para estudos futuros.

PALAVRAS-CHAVE: Carga bacteriana; Bactérias Indicadores Fecais; Hemócito; Sistema imune; Fagocitose; Espécies Reativas de Oxigênio.

ABSTRACT

The mussel *Perna perna* is an intertidal bivalve widely distributed, cultivated and consumed in South Africa and in countries of South America, such as Brazil and Venezuela. Among the marine resources, bivalve mollusks are one of the most impacted by anthropogenic pollution, once they can accumulate pathogenic bacteria, among other pollutants from the water. Hemocytes are molluscan defense cells that can be affected on its abundance and functions as response to contaminants, such as bacterial load. The aim of this study was to evaluate several immune parameters in *P. perna* as indicators of fecal pollution in seawater and in mussels' hemolymph. Thus, mussels and adjacent seawater were collected from beaches in Rio de Janeiro state (Brazil) with different fecal contamination levels: Vermelha Beach (VB); Icaraí Beach (IB); Urca Beach (UB); Jurujuba Beach (JB). Hemocyte parameters (density, morphology, phagocytic activity and production of Reactive Oxygen Species - ROS) were evaluated using flow cytometry. Fecal Indicator Bacteria (FIB) was quantified in seawater by the multiple tubes technique for each beach and hemolymph by spread-plate technique. In agreement to historical evaluation of fecal contamination levels, UB presented the highest number of FIB in seawater (thermotolerant coliforms, TEC = 1,600 NMP 100 mL-1), and VB the lowest (TEC = 17 NMP 100 mL-1). UB mussels showed, respectively, six and eight times higher hemocyte density and phagocytic activity than mussels from VB. Mussels from VB and IB showed significantly lower number of total coliforms in hemolymph and significantly higher relative internal complexity of hemocytes than those from UB and JB ($p \leq 0.01$, PERMANOVA). ROS production by hemocytes was significantly lower in mussels from VB when compared to those from JB ($p = 0.04$, ANOVA). Results indicate a significant relationship between the level of fecal contamination on aquatic environments and response of immune system related to mussels hemocytes. Immune-related parameters may be useful as indicators for bivalve health and environmental quality. Analysis of *P. perna* hemocytes morphological characteristics and functions by flow cytometry and their role in immune fecal pollution response opens a new approach for further studies.

Keywords: Bacterial load; Fecal Indicator Bacteria; Hemocyte; Immune system; Phagocytosis; Reactive Oxygen Species.

INTRODUÇÃO GERAL

As regiões costeiras abrigam cerca de 50% da população mundial e grandes centros urbanos (MMA 2007; Shuval 2003). Estas são áreas favoráveis ao desenvolvimento industrial e socioeconômico, além de possuírem imensurável relevância ecológica (MMA 2007; Shuval 2003). Todavia, os ecossistemas costeiros sofrem demasiada pressão antrópica por conta da urbanização intensa e aumento populacional em descompasso com políticas ambientais adequadas (MMA 2007; Shuval 2003). Em todo o mundo, ao longo da costa, efluentes são lançados diariamente no mar, direta ou indiretamente, com pouco ou nenhum tratamento, podendo transportar milhões de patógenos por metro cúbico (Shuval 2003).

Dentre os recursos marinhos, os moluscos bivalves são os mais impactados pela poluição dos ambientes aquáticos por serem filtradores e sésseis na fase adulta. Como estas características possibilitam o acúmulo de bactérias patogênicas (Antunes *et al.* 2010; Bianchi *et al.* 2014), toxinas marinhas, metais pesados, dentre outros poluentes presentes na água (Pereira 2003), estes organismos são bons bioindicadores para poluição (Abessa *et al.* 2005; Canesi *et al.* 2002; Silva 2000).

Os bivalves acumulam um grande número de bactérias presentes na água, podendo estas ser bactérias nativas do ambiente marinho ou oriundas do escoamento continental (Abessa *et al.* 2005; Antunes *et al.* 2010; Canesi *et al.* 2002). As bactérias podem exercer impactos positivos ou negativos sobre os bivalves quer mediante o estabelecimento de relações simbióticas ou pela promoção de doenças, respectivamente. Graças à eficiência de seu sistema imunológico, esses invertebrados podem sobreviver em um ambiente com grande carga bacteriana (Abessa *et al.* 2005; Antunes *et al.* 2010; Canesi *et al.* 2002).

O sistema imunológico dos bivalves, ao contrário dos vertebrados, é exclusivamente inato ou natural, ou seja, não possui memória imunológica (Gosling 2015). Os principais imunoefetores celulares dos bivalves são os hemócitos. Estas células circulantes compõem sua hemolinfa e são rapidamente acionados durante as reações de defesa e processos inflamatórios. Os hemócitos possuem as seguintes capacidades: produção de metabólitos tóxicos, como espécies reativas de oxigênio (ROS) e óxido nítrico; Liberação de enzimas lisossômicas a partir dos seus grânulos (subtipo celular granular) e atividade fagocítica e de encapsulação

(Donaghy *et al.* 2015; Goedken & De Guise 2004; Gosling 2015; Ladhar-Chaabouni & Hamza-Chaffai 2016).

Um dos principais mecanismos de ação degradativa dos hemócitos é a produção de espécies reativas de oxigênio (“reactive oxygen species” - ROS). Estas atuam principalmente na oxidação de proteínas, carboidratos ou bases de DNA (Donaghy *et al.* 2015; Gosling 2015). Já a defesa contra os agentes infecciosos acontece principalmente por meio da fagocitose ou encapsulamento (Gosling 2015; Sauvé *et al.* 2002). Na fagocitose ocorrem etapas de quimiotaxia (deslocamento celular através de um gradiente de concentração químico), reconhecimento, englobamento e degradação. O encapsulamento, por sua vez, está relacionado a parasitas de maior tamanho, que neste caso, não poderiam ser fagocitados. Nesta resposta os hemócitos se dispõem ao redor do parasita, impedindo sua proliferação (Gosling 2015; Sauvé *et al.* 2002).

Concentrações elevadas de poluentes podem produzir efeitos imunológicos relacionados aos hemócitos, dentre outros, tornando os bivalves mais suscetíveis, o que pode acarretar prejuízos econômicos importantes aos cultivos e ainda atingir a saúde humana (Abessa *et al.* 2005; Canesi *et al.* 2002; Silva 2000). Os parâmetros hemócitários podem apresentar modulações relacionadas a fatores bióticos e abióticos, e, desta forma, podem ser parâmetros utilizados tanto para expressar a condição de saúde do bivalve como a qualidade ambiental. (Abessa *et al.* 2005; Canesi *et al.* 2002).

Diante disso, o presente estudo teve como objetivo avaliar o efeito da poluição fecal no sistema imunológico de mexilhões *Perna perna* (Bivalvia) oriundos de quatro praias do estado do Rio de Janeiro com um gradiente de contaminação fecal.

OBJETIVOS

Avaliar a resposta de parâmetros imunológicos - densidade, morfologia, atividade fagocítica e produção de espécies reativas de oxigênio - de mexilhões *P. perna* como indicadores de poluição fecal.

CAPÍTULO I: Evaluation of immune responses of brown mussel *Perna perna* as indicators of fecal pollution

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Abstract

The mussel *Perna perna* is an intertidal bivalve widely distributed, cultivated and consumed in South Africa and in countries of South America, such as Brazil and Venezuela. Among the marine resources, bivalve mollusks are one of the most impacted by anthropogenic pollution, once they can accumulate pathogenic bacteria, among other pollutants from the water. Hemocytes are molluscan defense cells that can be affected on its abundance and functions as response to contaminants, such as bacterial load. The aim of this study was to evaluate several immune parameters in *P. perna* as indicators of fecal pollution in seawater and in mussels' hemolymph. Thus, mussels and adjacent seawater were collected from beaches in Rio de Janeiro state (Brazil) with different fecal contamination levels: Vermelha Beach (VB); Icaraí Beach (IB); Urca Beach (UB); Jurujuba Beach (JB). Hemocyte parameters (density, morphology, phagocytic activity and production of Reactive Oxygen Species - ROS) were evaluated using flow cytometry. Fecal Indicator Bacteria (FIB) was quantified in seawater by the multiple tubes technique for each beach and hemolymph by spread-plate technique. In agreement to historical evaluation of fecal contamination levels, UB presented the highest number of FIB in seawater (thermotolerant coliforms, TEC = 1,600 NMP 100 mL-1), and VB the lowest (TEC = 17 NMP 100 mL-1). UB mussels showed, respectively, six and eight times higher hemocyte density and phagocytic activity than mussels from VB. Mussels from VB and IB showed significantly lower number of total coliforms in hemolymph and significantly higher relative internal complexity of hemocytes than those from UB and JB ($p \leq 0.01$, PERMANOVA). ROS production by hemocytes was

significantly lower in mussels from VB when compared to those from JB (p= 0.04, ANOVA). Results indicate a significant relationship between the level of fecal contamination on aquatic environments and response of immune system related to mussels hemocytes. Immune-related parameters may be useful as indicators for bivalve health and environmental quality. Analysis of *P. perna* hemocytes morphological characteristics and functions by flow cytometry and their role in immune fecal pollution response opens a new approach for further studies.

Keywords: Bacterial load; Fecal Indicator Bacteria; Hemocyte; Immune system; Phagocytosis; Reactive Oxygen Species.

1. Introduction

The discharge of effluents on marine ecosystems has been a problem in coastal areas worldwide, mainly close to populated cities (Islam & Tanaka 2004; Shuval 2003). Domestic and municipal wastes are generated in large quantities in urban zones reaching daily the coastal seawaters with a wide range of pollutants and nutrients (Islam & Tanaka 2004), such as large amounts of organic matter (Sophonsiri & Morgenroth 2004) and high microbial diversity (Wang *et al.* 2014). Some microorganisms known as Fecal Indicator Bacteria (FIB) that inhabit gastrointestinal tract of warm-blooded animals, including human beings, can be investigated on water monitoring, since their presence and quantity are related to fecal contamination degree and indicate the presence of other pathogenic bacteria (Griffin *et al.* 2001). The most commonly used FIB are total and thermotolerant coliforms, *Escherichia coli* and enterococci (Griffin *et al.* 2001).

Aquaculture activities depends directly on the conditions of the aquatic ecosystems where they are developed. Among the marine resources, bivalve mollusks are one of the most impacted by anthropogenic pollution, once they can accumulate pathogenic bacteria, among other pollutants from the water column, during filter-feeding process (Abessa *et al.* 2005; Canesi *et al.* 2002). The consumption of shellfish harvested from wastewater-polluted areas have direct impacts on economy and public health. It was estimated about 4 million cases of infectious hepatitis, leading to 40 thousand deaths and 40 thousand cases of long-term disability and a spent of about 11.6 billion dollars annually (Shuval 2003).

Bivalves have an efficient immune system activated to deal with bacteria-enriched environments. Hemocytes, circulating cells components of hemolymph, are the major cellular immune effectors. These cells are rapidly activated during defense reactions and inflammatory processes and they have the following capabilities: production of toxic metabolites such as reactive oxygen species (ROS) and nitric oxide; release of lysosomal enzymes from their granules (granular cell subtype) and phagocytic and encapsulation activity (Donaghy *et al.* 2015; Gosling 2015; Ladhar-Chaabouni & Hamza-Chaffai 2016). Bacterial killing is mainly due to phagocytosis by hemocytes, a process that involves the recognition, binding, and internalization of foreign bodies (Canesi *et al.* 2002). Hemocyte modulations may be useful to assess the health condition of the bivalve and the environmental quality, including contamination by sewage and bacterial load (Abessa *et al.* 2005; Canesi *et al.* 2002). Several authors showed modulation on abundance and functions of hemocytes of different bivalve species as response to bacterial challenge (Ciacci *et al.* 2009; Bianchi *et al.* 2014; Bianchi *et al.* 2015; Bianchi *et al.* 2016; Husmann *et al.* 2011; Sabatini *et al.* 2011).

The brown mussel *Perna perna* (Bivalvia, Mytilidae) is an intertidal bivalve widely distributed in warm-temperate regions of the Atlantic, Mediterranean Sea and Indian Ocean (Silva & Barros 2011). This mussel is considered a key species in the aquaculture world, being cultivated and harvested in countries such as Venezuela, South Africa and Brazil (Bravo *et al.* 2003; Calvo-Ugarteburu *et al.* 2017; Narváez *et al.* 2008; Sáenz *et al.* 2010). According to the Fisheries and Aquaculture Department of the Food and Agriculture Organization of the United Nations (FAO 2017), 1,000 tons of mussels *P. perna* were produced in Venezuela in 2011 and 12,000 tons in Brazil in 2007. In this way, the present study aimed to evaluate the response of the immune-related parameters – density, morphology, phagocytic activity and ROS production - of mussels *Perna perna* as indicators of fecal pollution. Mussels were harvested from four urban beaches at the Guanabara Bay (Rio de Janeiro, Brazil) with different historical trends in fecal contamination. *Perna perna* production is an important economic activity in Guanabara Bay, 20 to 65 tons of mussels are harvested per month comprising harvesting of wild mussels and mussels grown in farms, as the Mariculture Association of Jurujuba which represents 50 to 70% of the total mussel production (Lage & Jablonski 2008).

2. Material and methods

2.1 Study areas

Mussel and adjacent seawater sampling was conducted in four urban beaches at Rio de Janeiro state, Brazil connected to Guanabara Bay (Figure 1). Guanabara Bay is an estuarine system in southeast Brazilian coast that has been impacted over decades due to urbanization, demographic growth and industrialization in its surrounding (Fistarol *et al.* 2015; Ribeiro & Kjerfve 2002). The bay receives daily microorganisms, domestic and industrial effluents, organic pollutants, heavy metals and hydrocarbons that cause several environmental impacts, including its eutrophication (Fistarol *et al.* 2015; Ribeiro & Kjerfve 2002). The western and northwestern parts of the Guanabara Bay have poorer water quality, since they receive most of the drainage from metropolitan region of Rio de Janeiro. However, it has quite heterogeneous features, presenting some degraded areas and others with good quality (Fistarol *et al.* 2015; Ribeiro & Kjerfve 2002).

The beaches selected in this study presented different historical degrees of contamination (INEA 2017). They were classified as great (1), regular (2), bad (3) and poor (4) water quality, respectively as follows: (1) Vermelha Beach - VB (22° 57' 18.59" S 43° 9' 52.91" W) is a clean area next to the Natural Monument of the Sugar Loaf and Urca hills; (2) Urca Beach - UB (22° 56' 52.60" S 43° 9' 48.02" W), is located at the foothill of the Urca's hill and has rainwater outlets which sometimes transpose clandestine sewage; (3) Jurujuba Beach - JB (22° 55' 53.45" S 43° 6' 59.61" W), has several clandestine sewage outlets. In this beach there is a Fishermen's Colony with farming mussels *P. perna*; (4) Icaraí Beach - IB (22° 54' 30.20" S 43° 6' 43.26" W), where are two rainwater outlets. When it rains, these rainwater outlets transpose sewage from clandestine canals to the beach. Close to one of these rainwater outlets there is a *P. perna* harvesting area.

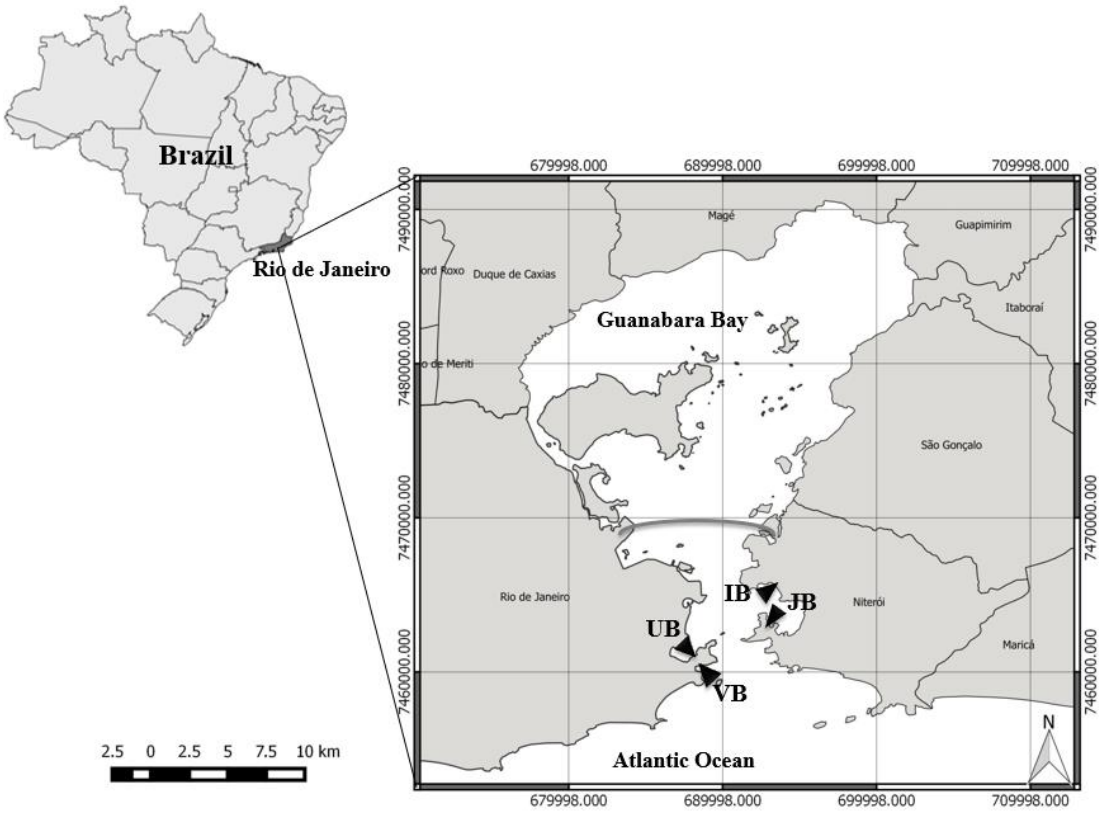

Figure 1. Mussels and seawater sampling locations in Guanabara Bay, Rio de Janeiro, Brazil: Vermelha Beach (VB), Urca Beach (UB), Jurujuba Beach (JB) and Icaraí Beach (IB).

2.2 Mussel sampling

About 20 individuals of *Perna perna* mussels were collected from each beach at rising tide during the cold season. Mussels of approximately 70-100 mm in length were randomly collected at the following points: rocky shore in VB and UB; harvesting area in IB and farming area in JB. The mussels were transported in a 20L container filled with seawater from their original beach. Upon arrival to the laboratory, bivalves were acclimated at 24°C in an aquarium (40L) with forced ventilation for 24-48h before the hemolymph extraction. The aquarium was filled with the seawater in which mussels were transported to keep the conditions of sampling beach.

2.3 Abiotic variables and microbiological analysis in seawater

Salinity, pH and temperature of seawater surrounding the mussels were measured using a multiparameter sensor HANNA Hi 98282.2 at the four beaches during collection. Approximately 150 mL of seawater was collected, using previously

sterilized material, and transported in an ice pack to the laboratory for microbiological analysis (EPA 2007). Fecal Indicator Bacteria (FIB) in seawater was quantified by multiple tubes technique with five replicates of three sets of serial sample dilutions (10^0, 10^{-1}, 10^{-2}) following APHA (2012) methodology. The number of FIB in seawater was reported as Most Probable Number per 100 milliliters (MPN 100 mL^{-1}).

Determination of *E. coli* (EC), total (TC) and thermotolerant coliforms (TEC) was performed according to APHA (2012). Enterococci (ENT) quantification samples were inoculated in Azide dextrose broth (Acumedia, USA) and, after 48h incubation at 35°C, were transferred to m-Enterococcus agar (Acumedia, USA). Light or dark red colonies were selected, inoculated into inclined tubes of Brain Heart Infusion (BHI) agar (Acumedia, USA) and incubated for 24h incubation at 35ºC. Colonies with Gram positive cocci, catalase negative test, growth at BHI broth after 48h at 45ºC (Acumedia, USA), and at BHI broth with 6.5% NaCl (Acumedia, USA) after 72h at 35ºC were considered as enterococci (Koneman *et al.* 1997).

2.4 Hemolymph extraction

About 0.5 to 3.0 mL of hemolymph were withdrawn from the posterior adductor muscle of each mussel using sterile 3 mL syringe and a 21G needle. Collected hemolymph was immediately transferred into sterile micro-tubes and maintained on ice to minimize the cell clumping (Buggé *et al.* 2007; Delaporte *et al.* 2006; Donaghy *et al.* 2009). The quality of hemolymph samples was examined using an inverted optical microscopy to eliminate samples with particle contamination (e.g., gametes, tissue debris). Contamination-free hemolymph samples from 3 to 4 mussels sampled from the same beach were pooled into micro-tubes to reduce inter-individual variation and to provide enough volume for all the assays (final volume of 3 mL) (Buggé *et al.* 2007; Delaporte *et al.* 2006). A total of five replicates of pooled-hemolymph with 3 mL by each sampled beach were evaluated, in which 1 mL of pooled-hemolymph was used for hemocyte analysis and 2 mL was used for the quantification of fecal indicator bacteria.

2.5 Flow cytometry analysis

The characterization of hemocyte morphology (relative size and internal complexity), density and functions (phagocytic activity and production of Reactive

Oxygen Species - ROS) were determined using a FACSCalibur flow cytometer (BD, Bioscience, USA). Samples (n=5 pools by beach) were evaluated at low flow during 1 min on three consecutive counts (analytical replicates) recorded by FL-1 (green) fluorescence detector using 350 nm excitation in flow cytometer. Specific methods for the evaluation of hemocyte parameters are described below. Cytometer flow (volume min^{-1}) was determined before analysis of hemolymph samples by cell counting of a known cultured cell density previously quantified in an inverted optical microscopy.

2.5.1 Morphology and density of hemocytes

Morphology (relative size and internal complexity) and density of hemocytes (number of events per mL) were determined by staining hemocytes double-stranded DNA with SYBR Green I (Sigma-Aldrich, USA). Into 5-ml cytometry tubes, each pooled-hemolymph sample (250 µl) were added to 250 µl of the Alsever anticoagulant solution (Rebelo *et al.* 2013) and 10 µl of SYBR Green I (100 × dilution of the commercial solution, Molecular Probes) and incubated for 30 min in the dark at room temperature (24°C) before flow cytometry analysis (Donaghy *et al.* 2009; Donaghy *et al.* 2010; Labreuche *et al.* 2006). Hemocyte population was discriminated in dot plot according to the relative flow cytometric morphological parameters: side scatter (SSC) that measures internal complexity, and forward scatter (FSC) that measures cell size, using logarithmic scale for both. A dot plot of SYBR Green fluorescence with FSC was used to discriminate debris from hemocytes. Morphological results were expressed in arbitrary units (A.U.) and hemocyte density (cells mL^{-1}) was calculated considering the number of cells counted by min and the cytometer flow (mL min^{-1}) determined before analysis.

2.5.2 Phagocytic activity

Hemocytes phagocytic activity was estimated by the density of hemocytes (cells mL^{-1}) that had engulfed one or more fluorescent latex beads (Fluoresbrite Yellow Green Microspheres, 2 µm, Polysciences, Germany). A volume of 250 µl of each pooled hemolymph sample (n=5 pools by beach) were mixed with 20 µl of the fluorescent beads solution (working solution at a 2.5% dilution) into 5-mL cytometry tubes. After 120 min of incubation at room temperature (24°C) in the dark, 250 µl of filtered sterile seawater (FSSW; Millipore Glass Fiber Filter, Millipore AP-40,

Millipore Brazil) were added to the sample shortly before flow-cytometry analysis (Donaghy *et al.* 2009, Labreuche *et al.* 2006). Hemocytes that phagocyted one or more fluorescent beads were identified on an histogram plot of FSC (relative cell size) and -FL1 (green fluorescence detector) (Donaghy *et al.* 2009, Labreuche *et al.* 2006).

2.5.3 Reactive oxygen species (ROS) production

The ROS production by hemocytes was evaluated using the 2′7′-dichlorofluorescein diacetate marker (DCFH-DA; Sigma-Aldrich, USA) according to a method adapted from Lambert *et al.* (2003). In 5-mL cytometry tubes, 250 µl of each pooled hemolymph sample were mixed to 250 µl of FSSW and 5 µl of the DCFH-DA solution (1 mM stock solution). Samples were incubated in the dark for 120 min at room temperature (24°C). Into the cell, DCFH-DA is oxidized to the DCF fluorescent molecule, which is quantitatively related to total oxidative activity of hemocytes, mainly ROS production (Delaporte *et al.* 2006; Donaghy *et al.* 2010). Relative oxidative activity was expressed as the geometric mean of the green fluorescence intensity (FL-1 fluorescence detector using 710 nm excitation in flow cytometer) and expressed in arbitrary units (A.U.; Donaghy *et al.* 2010).

2.6 Quantification of Fecal Indicator Bacteria (FIB) in hemolymph

For Fecal Indicator Bacteria (FIB) quantification in hemolymph of *Perna perna* mussels, a volume of 150 µl of each hemolymph pool dilutions (n=5 pools by beach) were spread into sterile 6-well flat plates (SPL Life Sciences, Korea) containing 3 mL of ChromoCult® Coliform Agar (Merck, Germany) or m-Enterococcus Agar (Acumedia, USA). Samples were serially diluted (10^0, 10^{-1}, 10^{-2}, 10^{-3}) in phosphate-buffered saline (PBS, pH=6.8, concentration 1x). Grown colonies were counted and the number of FIB in hemolymph of *P. perna* was reported as Colony Forming Units per milliliter (CFU mL^{-1}).

For quantification of total coliforms (TC) and *E. coli* (EC), hemolymph samples were spread into ChromoCult® Coliform Agar (Merck, Germany). After incubation at 37°C during 24h, colony growth indicated the presence of total coliforms. Colonies colored with dark blue to violet were considered as *E. coli* and transferred to Tryptic Soy Broth (TSB). Thus, they were incubated at 37ºC for 24h and identified by Voges

Proskauer, methyl red test and production of gas from lactose fermentation in EC broth (44.5°C - 48h) followed by the indole test (APHA 2012; Koneman *et al.* 1997).

For enterococci (ENT) quantification in hemolymph, samples were spread on the m-Enterococcus Agar (Acumedia, USA). After incubation at 35°C for 48h, the growth of light or dark red colonies indicated the presence of enterococci. Typical colonies of enterococci were isolated into inclined tubes of BHI agar (Acumedia, USA; 24h - 35°C) and then were submitted to confirmation tests: Gram staining (Gram-positive cocci) and Catalase test (catalase negative); bacterial growth in BHI broth (Acumedia, USA; 48h - 45°C) and in BHI broth with 6.5% NaCl (Acumedia, USA; 72h - 35°C; Koneman *et al.* 1997).

2.7 Statistical analyses

One-way analysis of variance (ANOVA) and Tukey test *a posteriori* was carried out to evaluate the influence of the sampling beach on the following hemocyte parameters: relative size (FSC), density, phagocytic activity and ROS production. Homogeneity of variance and normality were assessed by the Levene and Kolmogorov-Smirnov tests, respectively, to confirm the parametric test assumptions. When required, the data were previously transformed as square root or Log_{10}. Since the assumptions of parametric test were not reached, a one-way permutational multivariate analysis of variance (PERMANOVA) was applied to evaluate the influence of sampling beach on relative internal complexity of hemocytes (SSC) and density of total coliforms in hemolymph, the tests were performed based on the Euclidean distance and 9999 permutations. Significant differences between the treatments were tested by a pair-wise PERMANOVA comparison with 9999 permutations (Anderson 2005). ANOVA and PERMANOVA analyses were performed using the software Statistica 8.0 (StatSoft) and software PERMANOVA 1.6, respectively. All results were expressed as mean ±standard deviation (SD). The graphs were plotted using the software GraphPad Prism 5.0 (GraphPad).

Principal component analysis (PCA) was applied as a multivariate analysis to integrate immunological parameters of *P. perna* hemocytes (density, relative intracellular complexity - SSC, relative size - FSC, phagocytosis and ROS production) and fecal pollution (FIB values in hemolymph and seawater surrounding the mussels). Significance of the correlation between variables and factorial axes

was evaluated using equilibrium distance according to Legendre & Legendre (1998). The equilibrium radius is calculated by $(2/m)^{0.5}$, in which m = number of variables. Coordinates above the equilibrium radius were considered significant contributors to the axis. Multivariate analysis was performed using Statistica 8.0 software (StatSoft).

3. Results

The number of FIB in water surrounding the mussels *P. perna* were low in all beaches, except in Urca Beach (TC = 900 and TEC = 1,600 MPN 100 mL^{-1}) (Figure 2). and within the value of sanitary standards recommended by Brazilian Environment Council (TEC = 1,000, EC = 800, ENT = 100 MPN 100 mL^{-1} in 80% or more of the samples; Brasil, 2000). Regarding to abiotic variables measured in the water surrounding the mussels, the pH values ranged around 8.0 in all beaches (VB= 8.2; UB= 7.8; JB= 8.0), except in Icaraí Beach (IB = 9.2). Vermelha Beach (VB) presented the highest values of temperature (27.9°C) and salinity (36.6), while Urca Beach (UB) showed the lowest values, 23.5°C and 32.9, respectively. In Jurujuba Beach (JB) the values of temperature and salinity were 26.9°C and 34.8, and in Icaraí Beach were 26.4°C and 35.0.

Mussels from Vermelha and Icaraí beaches presented significantly lower number of total coliforms in their hemolymph than those from Urca and Jurujuba beaches (one-way PERMANOVA, $F_{3,16}$= 4.83, p=0.01; Figure 2). The fecal indicator bacteria *E. coli* and enterococci weren't found in the hemolymph of mussels from the four studied beaches.

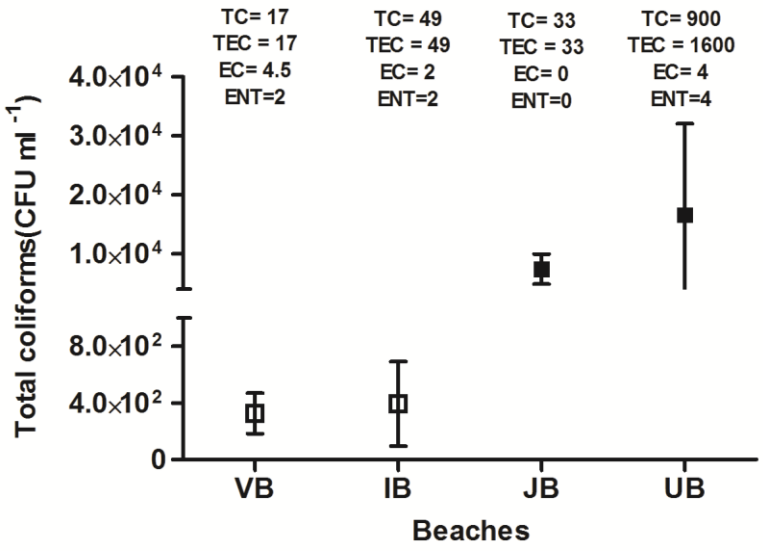

Figure 2. Values of Fecal Indicator Bacteria (FIB) in the seawater surrounding the mussels and plot with the number of total coliforms (CFU mL^{-1}) measured in the hemolymph of *Perna perna* collected from the four urban beaches: VB - Vermelha Beach, IB - Icaraí Beach, JB - Jurujuba Beach and UB - Urca Beach. TC - Total coliforms, TEC - Thermotolerant coliforms, EC - *Escherichia coli* and ENT - Enterococci - in seawater are expressed in MPN 100 mL^{-1}. Graphical results are expressed as means (± standard deviation), n = 5 polls by beach. Different square color shows significant differences ($p < 0.05$) among the beaches.

Hemocyte parameters of mussels *P. perna* (means ± standard deviation) by studied beach are presented in Table 1. Density of hemocytes was significantly higher in the mussels collected from Urca beach than in the mussels from the other beaches (one-way ANOVA, $F_{3,16}= 19.53$, $p< 0.001$; Figure 3a). Regarding to the hemocyte morphological parameters, no significant difference was observed in the relative hemocyte size (FSC) from mussels harvested in the four beaches (one-way ANOVA, $F_{3,16}= 1.71$, $p= 0.20$; Figure 3.d), unlike relative intracellular complexity (SSC). Hemocytes of mussels harvested from Vermelha and Icaraí beaches showed significantly higher internal complexity (SSC) than the hemocytes of mussels from Urca and Jurujuba beaches (one-way PERMANOVA, $F_{3,16}= 8.51$, $p< 0.01$; Figure 3.b). Regarding to hemocyte functions, phagocytosis was significantly different among the hemocytes of mussels from the four beaches and followed the order: Urca> Icaraí> Jurujuba> Vermelha (one-way ANOVA, $F_{3,16}= 93.77$, $p< 0.01$; Figure 3.c). ROS production by hemocytes was significantly lower in mussel from Vermelha Beach when compared to those from Jurujuba Beach (one-way ANOVA, $F_{3,16}= 3.45$, $p= 0.04$; Figure 3.e).

Table 1. Immune-related parameters and total coliforms (means ± standard deviation) measured in *Perna perna* hemolymph by studied beach.

	Hemocyte parameter					TChemo
	Density cells mL^{-1}	FSC A.U.	SSC A.U.	ROS A.U.	Phagocytosis cells mL^{-1}	CFU mL^{-1}
Vermelha	$1.6x10^4±3.8x10^3$	243.4±18.7	5.7±0.5	32.3±5.9	$6.4x10^4±1.8x10^3$	$3.3x10^2±1.4x10^2$
Urca	$9.5x10^4±4.0x10^4$	265.3±13.0	4.8±0.1	52.3±6.7	$5.3x10^4±1.1x10^4$	$1.6x10^4±1.5x10^4$
Jurujuba	$1.9x10^4±7.2x\ 10^3$	241.6±12.2	4.9±0.1	77.4±44.8	$1.4x10^4±2.1x10^3$	$7.4x10^3±2.5x10^3$
Icaraí	$2.9x10^4±1.4x\ 10^4$	241.9±29.8	5.3±0.4	42.5±20.1	$2.0x10^4±3.1x10^3$	$3.9x10^2±3.0x10^2$

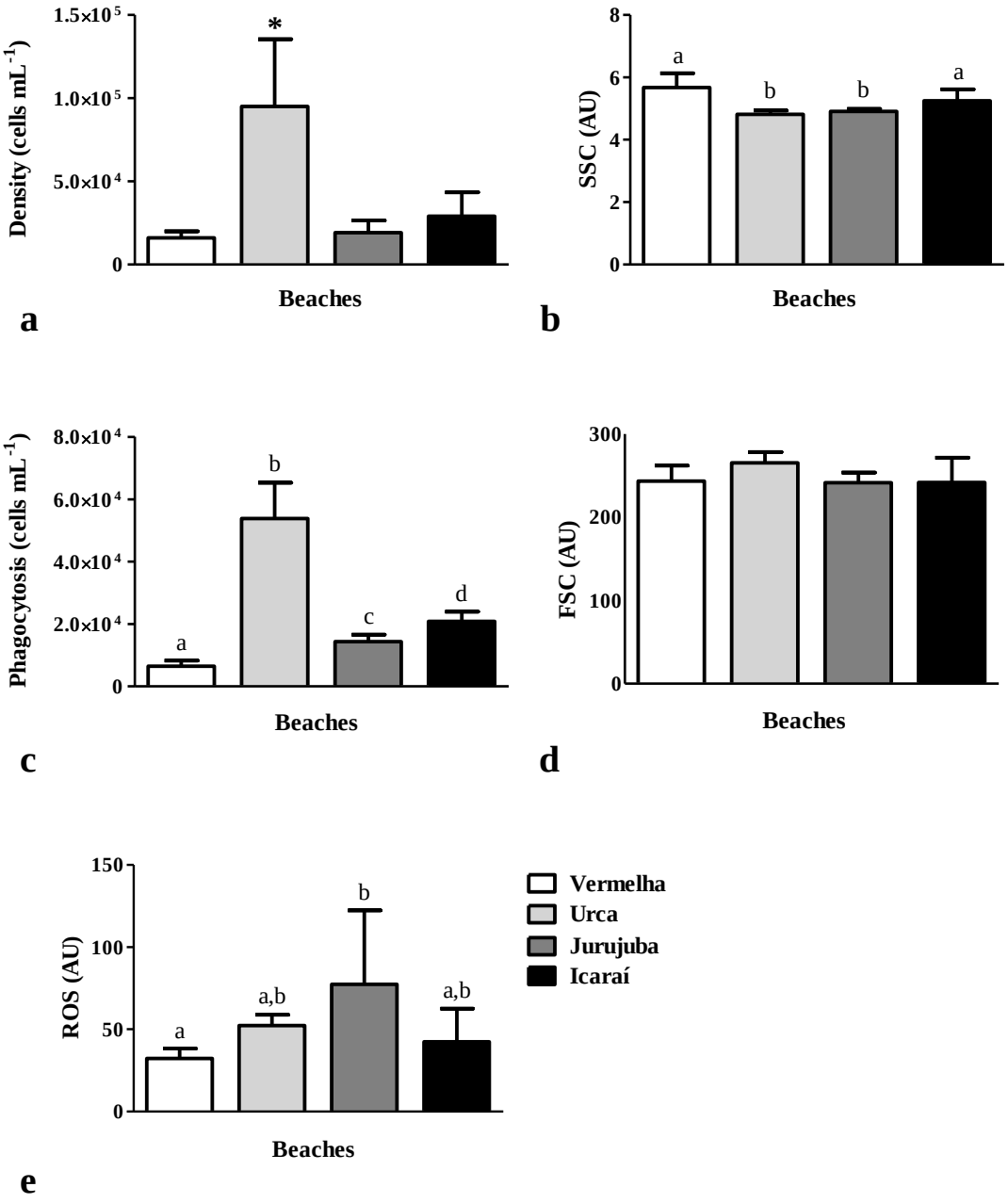

Figure 3. Hemocyte parameters measured in *Perna perna* mussels collected from the four urban beaches with different historical levels of fecal pollution in Rio de Janeiro state, Brazil. a) Density; b) Relative intracellular complexity (SSC); c) Phagocytic activity; d) Relative cell size (FSC); e) Production of Reactive Oxygen Species (ROS). Results are expressed as mean (± standard deviation), n = 5 replicates. Different letters or * show significant differences ($p < 0.05$) of a given parameter among the beaches.

The immunological responses of *P. perna* mussels to fecal pollution was summarized by the multivariate analysis of principal components (Figure 4). The first (PC1) and second (PC2) axes were statistically significant ($p < 0.001$) and represented 47.5% and 25.1% of the variance, respectively. Principal Component 1

(PC1) was strongly negatively correlated to the hemocyte and bacterial parameters: density (Dens, correlation coefficient, cc= -0.88) and phagocytic activity of hemocytes (Phago, cc= -0.96), number of total coliforms in hemolymph (TChemo, cc= -0.76), and total and thermotolerant coliforms in water surrounding the mussels (TC, cc= -0.91 and TEC, cc= -0.91, respectively). Moreover, PC1 was positively correlated to internal complexity of hemocytes (SSC, cc= 0.66). All these variables were significantly related to the axis 1, thus these variables are able to be used for axis interpretation as their projections on PC1 (descriptors projections of 0.93, 0.97, 0.76, 0.94, 0.94 and 0.89 respectively) were higher than the radius of equilibrium (d= 0.45).

Principal Component 2 (PC2) was positively correlated with the morphological features of hemocytes – relative internal complexity (SSC, cc= 0.59) and relative size (FSC, cc= 0.35) - and with the number of *E. coli* (EC, cc= 0.68) and enterococci (ENT, cc= 0.57) in water surrounding the mussels. In addition, production of Reactive Oxygen Species was highly negatively correlated to PC2 (ROS, cc= -0.80). All these variables were significantly related to this axis, thus these variables are able to be used for axis interpretation as their projections on PC2 (descriptors projections of 0.89, 0.55, 0.69, 0.85 and 0.84, respectively) were higher than the equilibrium radius (d= 0.45).

Samples ordination on PC1 and PC2 were determined by the sampling beach. Samples from beaches with less fecal pollution (Vermelha, Icaraí and Jurujuba) showed more similarity among them (aggregated) and they were mainly distributed in the positive side of the first axis (PC1). Most of the samples from VB were highly related to the positive projection of the variable SSC. While most of the samples from JB and IB, less influenced by SSC, were distributed in the negative projection of variables related to bacteria indicator, density, phagocytosis and relative size of hemocytes. In this way, mussels from Vermelha and Icaraí beaches (better quality) showed a tendency to present hemocytes with higher internal complexity (SSC), since there was an inverse relationship between hemocyte internal complexity (SSC) and fecal bacterial load, both in water surrounding the mussels and in hemolymph.

Samples collected at Urca Beach were grouped in the negative side of PC1 (fourth quadrant of the graph) with high values of hemocytes density, phagocytosis, FSC and number of FIB, primarily TC and TEC in water surrounding the mussels

and TC in their hemolymph. These immune-related parameters, mainly density and phagocytosis, seems to have positive relation with fecal bacterial load.

On the second axis (PC2), samples from Jurujuba Beach were distributed in the negative side with high values of ROS. The variable of ROS production by the hemocytes was oriented toward the third quadrant of the graph, in which the points related to the beaches with poor water quality were the most representative, Jurujuba and Urca. Samples of the other beaches (Vermelha, Icaraí and Urca) were gradually placed in direction to the positive side of PC2 with high values of density of hemocytes, phagocytosis, FSC, SSC and FIB in hemolymph and in water surrounding the mussels.

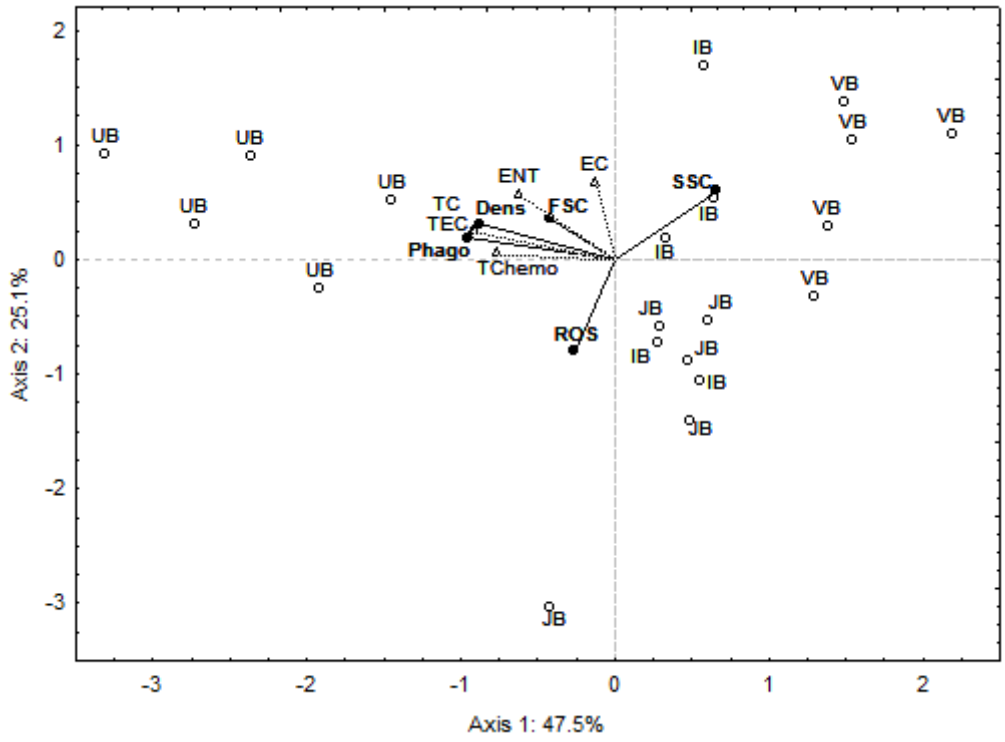

Figure 4. Biplot of the immune responses of *P. perna* mussels from the four urban beaches with different levels of fecal pollution in Rio de Janeiro state (unfilled circles): Vermelha Beach (VB), Urca Beach (UB), Jurujuba Beach (JB) and Icaraí Beach (IB). The numbers of Fecal Indicator Bacteria (FIB) are pointed by unfilled triangles: total (TC) and thermotolerant (TEC) coliforms, *Escherichia coli* (EC) and enterococci (ENT) in water surrounding the mussels and total coliforms in hemolymph (TChemo). Hemocyte parameters are indicated by filled circles: density (Dens), internal complexity (SSC), relative size (FSC), phagocytosis (Phago) and production of Reactive Oxygen Species (ROS). First (principal component 1) and second (principal component 2) axes produced by the PCA are statistically significant ($p < 0.001$).

4. Discussion

Mussels from Vermelha and Icaraí beaches presented lower number of TC in their hemolymph when compared with those from Urca and Jurujuba beaches. Bacteria numbers in mussel fluids seems to be a consequence of fecal contamination (FIB number in the seawater). Historically, domestic sewage has been discharged onto the beaches of Urca, Jurujuba and Icaraí. Sediment samples from Botafogo Cove, which has communication with Urca beach, confirmed accumulation of domestic sewage by coprostanol concentration and bacteria counts in sediments (Costa & Carreira 2005). In addition, Jurujuba and Icaraí beaches have been characterized as eutrophic beaches with the presence of total and thermotolerant coliforms (Marques Jr *et al.* 2006; Silva *et al.* 2010). However, their hydrodynamic conditions suggest to reflect directly in their bacteriological conditions, since greater circulation of water bodies tends to improve water quality (Cunha *et al.* 2006; He & He 2008; Zhu *et al.* 2011). Jurujuba-Urca and Icaraí-Vermelha beaches share similar environmental conditions, sheltered and more exposed to wave action, respectively (Baptista Neto *et al.* 2000; Franco *et al.* 2016). In this way, as expected, beaches with greater circulation showed less bacteria load in the seawater.

Considering the report of water quality monitoring (INEA 2017) that is based on CONAMA resolution (Brasil 2000), the four urban beaches covered in this study were considered safe for bathing during our sampling since 80% or more of the samples results from the previous five weeks showed TEC <1,000 MPN 100 mL^{-1}. Vermelha beach, considered the reference area in the present study, showed excellent water quality throughout the 2017 year. Urca beach, due to sanitation program "Sena Limpa" (CEDAE 2017), presented an improvement in water quality during the year of 2017. However, there was an event of contamination four days before our sampling in this beach, when it was considered unfit for bathing with TEC >1,000 MPN 100 mL^{-1} in two or more of the last five collections, or TEC numbers >2,500 MPN 100 mL^{-1} in last sampling. The water quality of the JB and IB varied widely throughout the year of 2017, being considered unfit for bathing during most of the year. On both beaches, there were events of fecal contamination six days before our samplings.

Phagocytosis is one of the most important primary cellular immune responses of bivalves (Gosling 2015), being important for pathogen clearance and immune

surveillance (Song *et al.* 2010). This immune-related parameter has been used in aquatic organisms as an indicative of the health status and indicator of environmental toxic conditions (Fournier *et al.* 2000; Donaghy *et al.* 2016). In the present study, fecal pollution in urban beaches significantly increased the density of circulating hemocytes and, mainly, the phagocytic activity of hemocytes in the marine mussel *Perna perna*. Mussels from Urca beach, which presented the highest fecal bacteria load, showed values of density of hemocytes and phagocytosis six and eight times higher, respectively, than mussels from the beach with the lowest FIB number - Vermelha Beach (reference area). Similarly, the freshwater mussels *Elliptio complanata* harvested at the downstream of sewage outfall showed an increase in two times in the hemocyte numbers and in 10% of phagocytosis efficiency compared to mussels from the reference area (Farcy *et al.* 2011). Bianchi *et al.* (2014) observed that the freshwater mussel *Diplodon chilensis* inhabiting an area affected by sewage showed phagocytic response and hemocyte number almost twice higher than mussels from an unpolluted area. According to Bianchi *et al.* (2014), this effect may be a long-term response, enabling mussels' immune system to cope with mild bacterial load (TEC = 950 MPN.100 mL^{-1}). In addition, it was observed that mussels from both, unpolluted and polluted areas, were able to clear bacteria from the water and reduce enteric bacteria loads. Thus, increases in total number and phagocytic activity of hemocytes in mussels *Perna perna* exposed to increasing fecal pollution in urban beaches may be a strategy to deal with increasing rates of bacteria clearing and a defense against the bacterial infection.

In this study, *E. coli* and enterococci were not found in any of *P. perna* mussels hemolymph although these bacteria were detected in seawater of Vermelha, Urca and Icaraí beaches. This result can be related to a capacity of differential clearance of bacteria by *P. perna* hemolymph as demonstrated by Antunes *et al.* (2010) in biological fluids of the freshwater mussel *Anodonta cygnea*. *Escherichia coli* and enterococci were periodically detected in water, but not in *A. cygnea* fluids, suggesting that this mussel has the ability to filter and eliminate *E. coli* present in the surrounding environment through an active phagocytic process conducted by the hemocytes. In this way, it is possible that despite the filtering process of *E. coli* and enterococci from seawater *P. perna* mussels have capability to eliminate these pathogenic bacteria from their hemolymph.

Mussels *P. perna* present two blood cell types in the circulation: hyalinocytes and granulocytes, in which hyalinocytes are more abundant comprising approximately 60% of hemocytes in mussel hemolymph (Barracco *et al.* 1999). Granulocytes present many cytoplasmic granules, whereas few or no granules are observed in hyalinocytes (Donaghy *et al.* 2009; Gosling 2015; Ladhar-Chaabouni & Hamza-Chaffai 2016). Granulocytes and hyalinocytes may present differences in proportion and functions such as phagocytic activity and H_2O_2 production (Donaghy *et al.* 2009; Goedken & De Guise 2004). Both hemocyte types, but especially the granulocytes, were actively phagocytic and contained acid phosphatase in mussels *P. perna* (Barracco *et al.* 1999). In the present study, hemocytes in mussels from Vermelha and Icaraí beaches presented greater internal complexity (SSC) compared with hemocytes in mussels from Urca and Jurujuba beaches. This trend may be related to differential recruitment or deregulation of hemocyte subtypes. Proportions of the hemocyte cell types may vary according to bacterial challenge such as Gram(−) *Vibrio anguillarum* and *V. splendidus* and Gram(+) *Micrococcus lysodeikticus*, as showed in mussels *Mytilus galloprovincialis*, suggesting a differential involvement of mussel hemocyte sub-populations in the clearance of bacteria (Parisi *et al.* 2008; Ciacci *et al.* 2009). In the present study, differences on internal complexity of circulating hemocytes according to fecal bacteria load and TC number in mussel hemolymph, lower in mussels from Vermelha and Icaraí beachs compared to Urca and Jurujuba beaches, suggest a change in proportions and involvement of the hemocyte sub-types in mussels *P. perna* in relation to fecal pollution exposure.

ROS production by mussel's hemocytes followed a rising pattern according to increase of fecal pollution. However, the production of reactive oxygen species (ROS) by hemocytes was higher in mussels harvested from Jurujuba, which showed the second highest TC number in hemolymph. The rate of cellular reactive oxygen and nitrogen species (ROS/RNS) generation in mollusks is closely related to environmental stress, such those related to inflammatory response (Almeida *et al.* 2007). Thus, higher ROS production by mussel hemocytes are closely related to a defense against bacteria in their fluids. Although Jurujuba Beach presented the highest ROS production, Urca shows the highest FIB values in seawater and hemolymph. Considering that this was a study performed in natural environment,

other components present in Jurujuba Beach should have affected ROS production of these *P.perna* hemocytes.

The present study was the first to analyze morphological characteristics and functions of *P. perna* hemocytes by flow cytometry and evaluate the response of immune-related parameters as indicators of fecal pollution. Finally, our results showed a significant relation of immune responses of the brown mussel to fecal pollution and suggest a tendency to immune stimulation in *P. perna* mussels with the increase in fecal bacteria load, highlighted by the increase in density and phagocytic activity of hemocytes. Future studies are needed for the evaluation of differential capacity of the mussel *P. perna* to deal with bacteria in their hemolymph and the potential use of mussel filtration as a strategy for bioremediation in aquatic ecosystems.

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CONCLUSÃO GERAL

Os mexilhões oriundos das praias Vermelha e Icaraí, com maior hidrodinâmica, apresentaram menor número de bactérias indicadoras fecais na hemolinfa quando comparados aos coletados nas praias da Urca e Jurujuba, com baixa hidrodinâmica. Mexilhões coletados em praias mais contaminadas, isto é, com maior número de bactérias indicadores fecais em água e hemolinfa apresentaram um aumento na densidade e atividade fagocítica dos hemócitos. Estes mexilhões também apresentaram hemócitos com menor complexidade interna, maior produção de ROS e ausência de *E. coli* e enterococos na hemolinfa analisada. Estas respostas podem estar relacionadas à capacidade de defesa contra infecção bacteriana e depuração diferencial. Portanto, os resultados indicam um efeito da circulação local na qualidade da água e sugerem um efeito significativo da contaminação fecal sobre os hemócitos de *P. perna*. Estas modulações hemocitárias podem ser utilizadas como parâmetro para avaliação da saúde do bivalve e da qualidade ambiental. Futuras investigações sobre a capacidade de depuração diferencial de bactérias em hemolinfa de *P. perna* podem trazer novas perspectivas para o uso deste bivalve em biorremediação de ambientes contaminados com carga fecal.

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APÊNDICE

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Manuscript Draft

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Title: Evaluation of immune responses of brown mussel "Perna perna" as indicators of fecal pollution

Article Type: Full Length Article

Keywords: Bacterial load; Fecal Indicator Bacteria; Hemocyte; Immune system; Phagocytosis; Reactive Oxygen Species.

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Abstract: The mussel "Perna perna" is an intertidal bivalve widely distributed, cultivated and consumed in South Africa and in countries of South America, such as Brazil and Venezuela. Among the marine resources, bivalve mollusks are one of the most impacted by anthropogenic pollution, once they can accumulate pathogenic bacteria, among other pollutants from the water. Hemocytes are molluscan defense cells that can be affected on its abundance and functions as response to contaminants, such as bacterial load. The aim of this study was to evaluate several immune parameters in "P. perna" as indicators of fecal pollution in seawater and in mussels' hemolymph. Thus, mussels and adjacent seawater were collected from beaches in Rio de Janeiro state (Brazil) with different fecal contamination levels: Vermelha Beach (VB); Icarai Beach (IB); Urca Beach (UB); Jurujuba Beach (JB). Hemocyte parameters (density, morphology, phagocytic activity and production of Reactive Oxygen Species - ROS) were evaluated using flow cytometry. Fecal Indicator Bacteria (FIB) was quantified in seawater by the multiple tubes technique for each beach and hemolymph by spread-plate technique. In agreement to historical evaluation of fecal contamination levels, UB presented the highest number of FIB in seawater (thermotolerant coliforms, TEC = 1,600 NMP 100 mL-1), and VB the lowest (TEC = 17 NMP 100 mL-1). UB mussels showed, respectively, six and eight times higher hemocyte density and phagocytic activity than mussels from VB. Mussels from VB and IB showed significantly lower number of total coliforms in hemolymph and significantly higher relative internal complexity of hemocytes than those from UB and JB ($p \leq 0.01$, PERMANOVA). ROS production by hemocytes was significantly lower in mussels from VB when compared to those from JB ($p = 0.04$, ANOVA). Results indicate a significant relationship between the level of fecal contamination on aquatic environments and response of immune system related to mussels hemocytes. Immune-related parameters may be useful as indicators for bivalve health and environmental quality. Analysis of "P. perna" hemocytes morphological characteristics and functions by flow cytometry and their role in immune fecal pollution response opens a new approach for further studies.