Hemolymph bacterial community of Pacific Oyster (*Crassostrea gigas*) in response to long-term hypercapnia.

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Introduction.

It seems inevitable that the increasing emission of carbon dioxide (CO_2) to the atmosphere will result in the phenomenon of ocean acidification (OA) (Joint et al., 2011). As anthropogenic CO_2 increases in the atmosphere, it dissolves in the surface ocean and increases the partial pressure of carbon dioxide (pCO_2) and the concentrations of carbonic acid (H_2CO_3), hydrogen ions (H^+) and bicarbonate ions (HCO_3^-), with a coupled decrease in carbonate ions (CO_3^{2-}). However, pH of the oceans is not constant and there are considerable seasonal, depth and regional variations. Therefore, it should be considered that microorganisms in the present-day ocean experience variable pH, and that most of that variability are consequence of microbial activity itself (Joint et al., 2011). For example, in all the marine regions where respiration exceeds photosynthesis, this aerobic respiration of organic matter implies the release of CO_2 to the surrounding environment having as consequence a local reduction in seawater pH.

Recently, Endres et al. (2014) performed a large-scale mesocosms experiment and considered the effects of OA on a natural plankton community in a Norwegian fjord. On their experiments, the mesocosms were adjusted to a gradient of different pCO2 levels and sampled for 34 days. Their results indicate that this microbial community benefits directly and indirectly from a lower seawater pH. A 5–10% more transparent exopolymer particles were formed in the high pCO_2 mesocosms during phytoplankton blooms, and total and cell-specific aminopeptidase activities were elevated under low pH conditions. These two features most likely influenced the up to 28% higher bacterial abundance in the high pCO_2 treatments (Endres et al., 2014). Thus, the result of this modern experiment suggests a stimulatory effect of OA over the marine bacterial community and an increment of the microbial recycling of organic matter.

These kinds of study are very recent, and most of the researches have been done studying the effects of OA over free-living microorganisms. However considering the close interaction between marine metazoans and their associated microbial communities, studies regarding the effects of OA over this association is also needed. The diverse types of symbioses between microorganisms and metazoans have received increasing attention with regards to many different features of their complex interactions (Zilber-Rosenberg & Rosenberg 2008). Tabal-Fernandez et al., 2014 indicate that research on aquatic organisms has shown that the resident associated

microorganisms (microbiota) are involved in several beneficial roles, including the development of the host gastrointestinal tract, nutrition (providing vitamins, enzymes and essential fatty acids for the host), immune responses and disease resistance (Prieur et al., 1990; Harris, 1993; Moriarty, 1997).

In the case of oysters, transient microbiota can rapidly become residents of their microbiota during the larvae and post-larvae stages (Brown, 1973; Kueh & Chan, 1985; Kesarcodi et al., 2012; cited in Tabal-Fernandez et al., 2014). Bivalves have a persistent hemolymph microbiome that in the case of *C. gigas* consists principally of Proteobacteria (class Alpha-, Delta- and Gamma-Proteobacteria), Bacteroidetes (class Flavobacteria), Spirochaetes (class Brachyspirae) and in a minor proportion Firmicutes (class Bacilli and Clostridia) and many other taxons that represent a small proportion of this diverse community (Lokmer & Wegner, 2014). It was thought that the host genotype influenced the microbiome of *C. gigas,* suggesting that its hemolymph microbiome might assemble in a host dependent manner (Wegner et al., 2013). However, a more recent article by the same authors showed that *C. gigas* hemolymph *Vibrio* populations converged to be similar that of the oysters of their new location 5 months after being moved from their original bed site (Wendling et al., 2014). Thereby, it is most likely that *C. gigas* hemolymph microbiome is a dynamic community, which depends at least to a certain level, on the microorganisms present in its environment (and the factors that modify its composition).

C. gigas is a worldwide distributed oyster that grows in the bottom of coastal estuaries, usually attach to rocks (but can also live buried under the seafloor sediments), at depths between 5m – 40m (www.fao.org/fishery/species/3514/en). In an acidified environment *C. gigas* fertilization success is reduced (Parker et al., 2010), their larvae exhibit developmental delay and shell malformations (Barton et al., 2012; Gazeau et al., 2011; Kurihara et al., 2007; Timmins-Schiffman et al., 2012), the calcification rates of adult oysters decrease linearly with increasing pCO_2 in adults (Gazeau et al., 2007) and affects their energy metabolism (Lannig et al., 2010). Two metabolo-proteomic studies in which *C. gigas* was maintained at a series of high pCO_2 conditions for one month (Timmins-Schiffman et al., 2014; Wei et al., 2014), exposed that although the shells growth was not affected, they diminished the quality of the deposited shell material. In addition, relative amounts of fatty acids and glycogen content, from whole tissue extracts appeared to be unaltered at elevated pCO_2 (Timmins-Schiffman et al., 2014). However a more detailed analysis determined that gills were ATP depleted (and their succinate levels were significantly increased) and hepatopancreas tissue exhibited accumulations of ATP, glucose and glycogen (Wei et al., 2014) at high pCO_2 conditions. These results were in agreement with what had been reported previously (Lannig et al., 2010), and despite whole tissue proteome exhibited higher levels galactose production proteins (Timmins-Schiffman et al., 2014), hepatopancreas proteome showed a decrease in these proteins (which was correlated with the accumulation of ATP and glucose due to a lower production of galactose; Wei et al., 2014). Additionally, whole

tissue proteome showed that high pCO_2 increased the levels of fatty acid metabolism (desaturation and elongation) and transport (Timmins-Schiffman et al., 2014), the desaturation of fatty acids might be justified by their less sensitivity and protective capacities towards oxidative stress. Although these proteomic changes were not observed in *C. gigas* fatty acids profiles after grown for one month at high pCO_2, it has been shown that eleven weeks under these conditions alter both the carbohydrate and lipid reserves of *C. virginica* (Dickinson et al., 2012). Consequently, a more extended exposure to these conditions might affect its energy reserves of *C. gigas* as well. Proteomic studies revealed high levels of mitochondrial ATP production-related, mitochondrial stress-induced and oxidative stress-induced proteins in *C. gigas* grown at high pCO_2 (Timmins-Schiffman et al., 2014; Wei et al., 2014). Wei et al. (2014) showed that elevated pCO_2 exposure might induced osmotic stress in *C. gigas* gills, which were altered in their levels of organic osmolytes (homarinetaurine and dimethylglycine), and low levels of the hydroxyectoine biosynthesis-related protein ectoine hydroxylase (whose product serves as a compatible solute that protects cells against various stresses, and exhibit protein stabilizing properties in vitro; Pastor et al., 2010).

In summary, previous metabolo-proteomic studies shown that, in addition to a general stress response, the high pCO2 levels induce an altered energy production and storage metabolism, vary immune system functioning, and induce a general oxidative stress response in addition to a gill-localized osmotic stress response. These long-term low pH-induced changes in the performance of *C.gigas* imply a reallocation of energetic and metabolic resources. This suggests that standard physiological condition characterized until now will be readjusted and maintained to compensate those changes, being the new standard conditions until evolutionary processes occur. These stress conditions will most likely change the microbiota associated to the hemolymph and its interaction with *C.gigas*, being an additional factor to study in order to complement the predictions about the response of *C.gigas* under OA conditions. In addition to the decrease in pH an increase of seawater temperature in coastal areas, due to the climate change, could increase the frequency of disease events over cultures of *C.gigas.* Wendling et al., 2014, indicate that *Vibrio spp* diversity was positively correlated with mean temperature. *Vibrio spp.* pathogenicity is tightly linked to increased seawater temperature, due to a higher proliferation rate as well as an upregulation of virulence factors (Wendling et al., 2014). Then, the susceptibility of *C.gigas* to *Vibrio* pathogens is higher because of the OA.

With this scenario, antimicrobial compounds produced by the own microbiota of the marine invertebrate represent a good example of beneficial interaction, by improving the response of the host to the emerging pathogens (Defer et al., 2013). Notably, recent studies discovered in *C. gigas* the presence of hemolymph microorganisms with potential anti-pathogenic activity and hemocyte mortality-reducing capacities (Defer et al., 2013; Desriac et al., 2014). The "beneficial" strains isolated from *C. gigas* hemolymph microbiome have been taxonomically affiliated to the *Pseudoalteromonas* and *Vibrio* genus (Gamma-Proteobacteria class) (Desriac et

al., 2014). However, this isolation screening probably excluded several taxons due to it was culture-dependent. All these suggest that associated microbiota may participate in bivalve protection and therefore confer a health benefit to the host.

The Aim of this study

In this paper I will study the changes in the bacterial community associated to *C. gigas* in response to the physiological changes triggered by simulated future pCO2 conditions. Specifically changes of the community associated to the hemolymph will be analyzed in early adults stages of *C.gigas* and its will be correlated with the physiological changes of the oyster under induced long-term hypercapnia.

Materials & Methods.

Sample collection.The *C.gigas* were collected from Carlsbad aquafarm (California, USA) at an early adult stage.

***Low pH condition*.** The oysters were maintained in two controlled pCO_2 conditions: present coastal seawater conditions ~ pH 7.9, pCO2 ~1036 ppm (Control), and Low pH conditions to induce hypercapnia at ~pH 7.6, pCO2 ~2008 ppm (Hypercapnia).
The incubation lasted for 5 weeks, and was performed in an open system circuit of eight tanks with 10L of water capacity, at 12:12 L:D daily light cycle, with a constant influx of seawater pre-filtered (sand and mesh filters from the SIO/UCSD Pier continues system). The flux of seawater was 400 mL min^{-1}, this flux of seawater enables to study the changes of the gut and hemolymph associated microbial community induced just by the physiological changes of the oyster due to the hypercapnia condition, and not due to changes in the composition of microorganisms in the seawater due to OA. The temperature was not controlled, and corresponded to the seawater influx from the environmental seawater from the SIO pier.

The pH was constantly monitored using the IKS Aquastar analyzer system, and the CO_2 concentrations maintained by a computer-controlled CO_2 influx system. The pH was also monitored manually using a portable HACH pH meter in. The oysters were fed twice a day with a co-culture of *Pavlova* and *Tetraselmis*.

Hemolymph collection: After 1 week of acclimation, the oysters were placed in their corresponding treatments tanks and 6 of them were sampled (T0). After 18 days (T1) a second sampling was performed, and finally after 36 days (T2) the last collection was done. An $n=6$ per treatment per time was used.

The valves of each oyster were slightly separated right after their were removed from the water, avoiding breaking the abductor muscle, a knife was move from the tip along the ventral side. A clean metallic stick was used to maintain the valves separated approximately by 8mm, to allow

the sea water removal and then introduce a needle (22 G 1 ½) attached to a 5 mL syringe directly in the abductor muscle to extract approximately 900 µL of hemolymph.
Additionally, 10 L of seawater from the main tanks were collected in 0,22 µm Sterivex (Millipore).

Hemolymph pH measurement. The pH of the hemolymph was measured using microelectrodes pH/mV meter UB-10 Ultra Basic (Denver Instrument) *insitu* right before the extraction of the samples.

Hemolymph HCO_3 content. The bicarbonate (HCO_3) content of the hemolymph was measured in the Corning 965 CO_2 analyzer, according to manufacter's protocol right after the extraction.

Hemolymph glucose content. The free glucose content of the hemolymph was measured by using the MyQubit Amplex® Red Glucose assay (Life Technologies) according to manufacter's protocol right after the extraction.

Hemolymph associated bacteria culturing. A volume of 50 µL of hemolymph was carefully streaked in modified Marine Agar plates.

The modification consisted in replace a half of the standard formula of the Marine Broth 2216 (Difco, Becton Dickinson) for filtered (0.22 µm) sea water from the same source than the water of the tanks, to mimic partially the environmental nutrient content of the seawater. The plates where incubated at 18 ^{0}C per at least 12 hours after the first colonies where visible and re-streaked in a new plate for their isolation. The plates were maintained in the same conditions after 1 week to visualize and isolate bacteria with slower growth rate.

Approximately 120 isolated bacteria where obtained, and the 16S rRNA gene was partially sequenced for 36 of them for phylogenetic assignation.

DNA extraccion and 16S rRNA PCR. Approximately 300 µL of hemolymph was be used to extract DNA by using the Zymo Research Quick-gDNA^{TM} Miniprep kit according to the Whole Blood, Serum and Plasma Samples of the manufactures' protocol with minimum modifications. An additional 1 min bead beating step was performed before the standard protocol.

The DNA extraction from the seawater samples was performed by using the PowerWater Sterivex kit (MoBio).

The PCR reactions were performed in a BioRad DNA Engine thermo cycler, in 35 µl reaction mixture containing 5ng of DNA of the Q5® High-Fidelity 2X Master Mix (New Englad Biolabs), and 0.5 µM (each) primer and PCR grade water.
The bacterial-specific 16S rRNA fragment was amplified using the 358f-GC CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GC CTA CGG GAG GCAG CAG and the 901RM CCG TCA ATT CMT TTG AGT TT. The PCR Touch Down

program was as follows: 98°C for 30 sec, 30 cycles at 98°C for 10 sec, annealing temperature for 30 sec and 72°C for 30 sec. In the first 10 cycles, the annealing temperature decreased by 1°C after every cycle (from 65°C to 55°C). In the last 20 cycles annealing temperature was maintained at 55°C. The final extension time was 2 min at 72°C.

16S rRNA Denaturing Gradient Gel Electrophoresis (DGGE).

DGGE for 16S rRNA fragment PCR products was done as described previously (Diez, 2001; Amer 2009), with minor modifications. Briefly, a linear gradient of denaturing agents from 40–60% was used to resolve the 16S rRNA PCR products. The DGGEs were run at 100 V for 16 h. A matrix of presence/absence of bands was done and a Clusterized Heatmap based in precense/abcense of taxa (bands). Taxa were grouped by divisive hierarchical clustering according to their euclidean distance**,** using R (R Core Team, 2013).

Results.

Hemolymph acid-base

Under hypercapnia the hemolymph had a reduction in its pH compared to the control (from 7.39 [control] to 7.27 [hypercapnia]) Figure 1. A proportional increment of the CO_2 respecting to the seawater in both conditions was measured. Only a minor increases of HCO_3^- was detected, suggesting a passive increase of HCO_3^- similar to the measured in the seawater, indicating that the oyster are not doing anaerobic metabolism under hypercapmia. This imply that *C.gigas* under hypercapnia conditions do not activate a bicarbonate buffering to compensate the drop off in the hemolymph pH. Because the oysters are not actively buffering (no bicarbonate accumulation), their either, can not compensate the drop of the pH or their do not need to compensate it in the hemolymph.

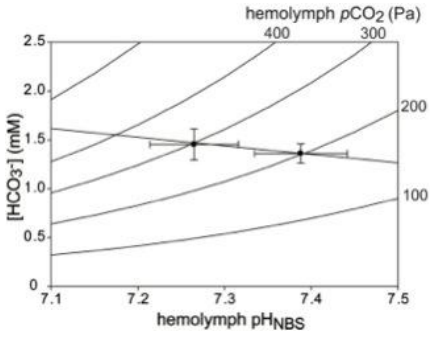


Figure 1: Davenport diagram representing 3 variables (HCO_3^-, pH_{NBS}, and pCO_2) of *C.gigas* hemolymph.The black line indicates the NBB line (non-bicarbonate buffering).

Free Glucose Content

The free glucose content in the of oyster hemolymph tend to be higher in response to hypercapmia (Figure 2). The increment of free glucose might provide more carbon and energy

source for the hemolymph bacterial community.

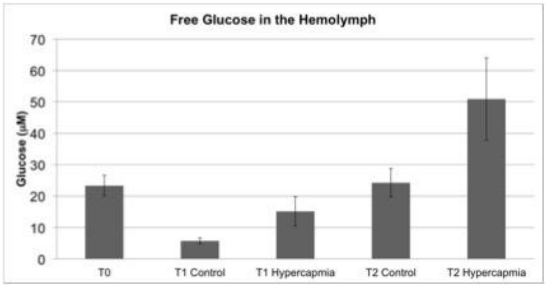


Figure 2: *Free Glucose content in the C.gigas hemolymph*

Bacterial Community

The DGGE patterns shows the loss of of two taxa in the hemolymph of oysters under induced hypercapnia. This two taxa were present in the hemolymph of control oysters. Additionally seven taxa were detected exclusively in the hemolymph of oysters under hypercapnia condition (Figure 3).

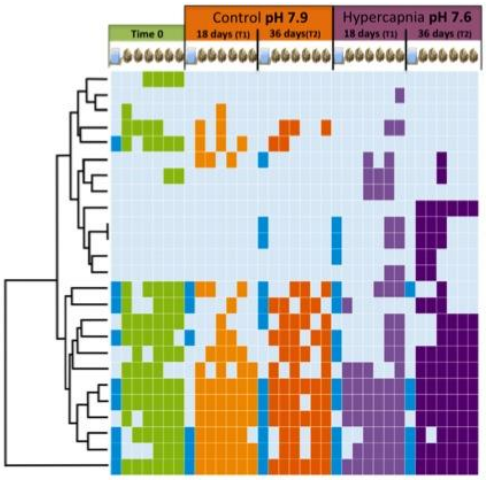
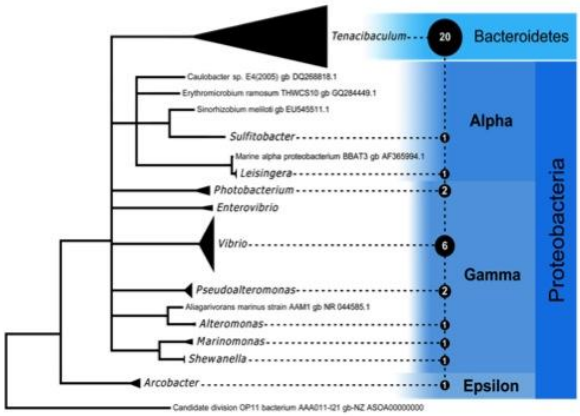


Figure 3: *Clusterized Heatmap based in precense/abcense of taxa (bands). Time 0 in green, control condition in orange, and hypercapnia in purple. Each painted rectangle represent a detected band.*



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