

Genomic methodologies for bivalve pathobiome characterization and detection

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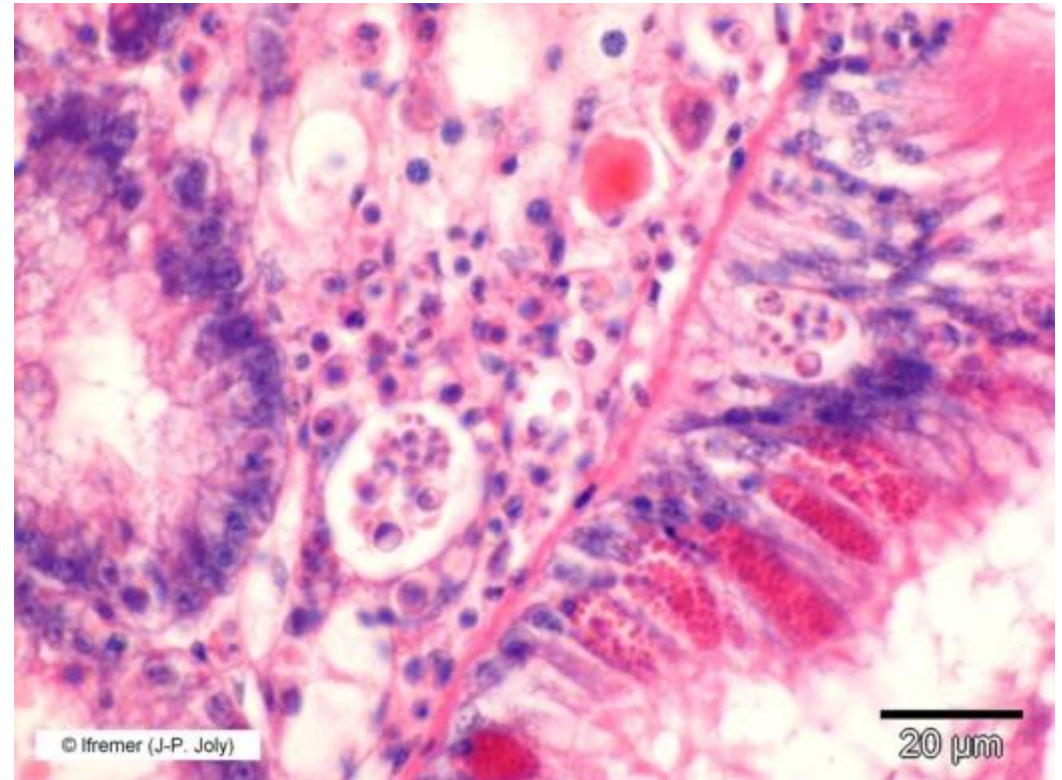


FINAL CONFERENCE
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From histopathology to molecular tools

Histology provides informations about the detection of a wide range of pathogens specially protozoan parasites associated to mortalities or lesions associated to the interaction of the pathogen with the molluscs immune system.

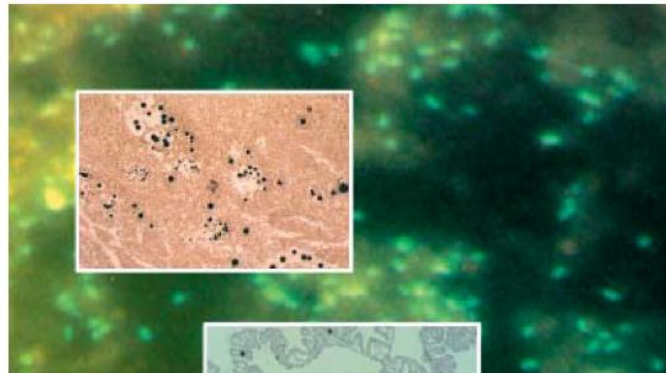


From histopathology to molecular tools

A process started
more than 20
years ago

DNA-based Molecular Diagnostic Techniques

Research Needs for Standardization and
Validation of the Detection of Aquatic
Animal Pathogens and Diseases



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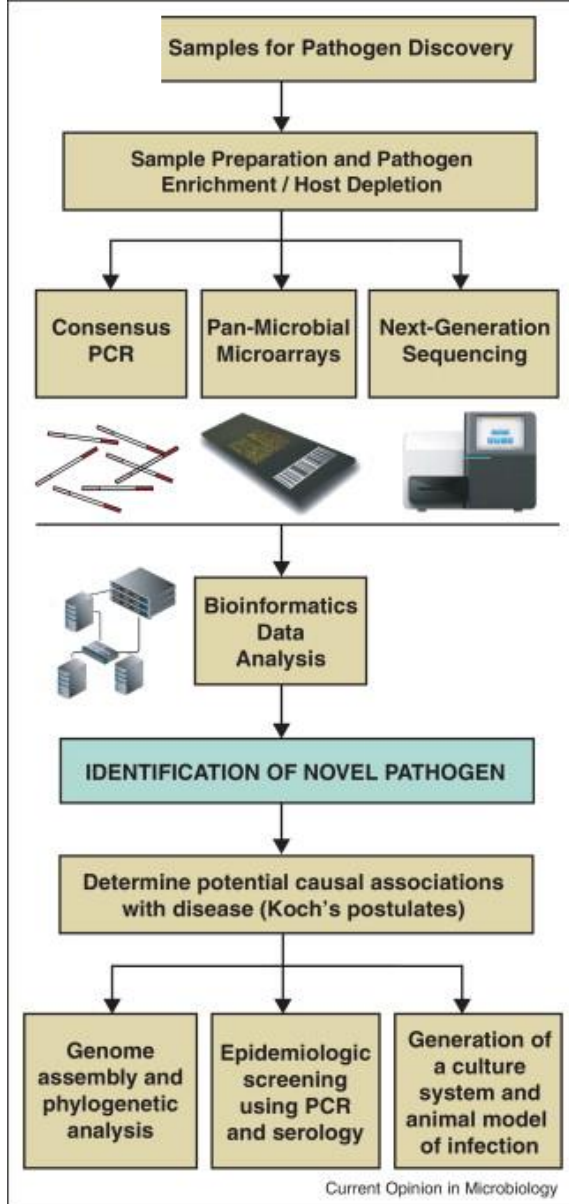
From histopathology to molecular tools

TABLE 2 | Available diagnostic techniques recommended by O.I.E. (2016) for molluscs pathogens listed by EU.

	Targeted surveillance		Presumptive diagnosis		Confirmatory diagnosis	
	Recommended method	Standard method	Recommended method	Standard method	Recommended method	Standard method
<i>B. ostreae</i>	PCR (L, P, J, A) qPCR (L, P, J, A) Histopathology (J, A) Tissue imprints (J,A)		Tissue imprints PCR qPCR	Histopathology	TEM sequencing	PCR-RFLP ISH
<i>B. exitiosa</i>	PCR (L, P, J, A) qPCR (L, P, J, A) Histopathology (J, A) Tissue imprints (J,A)		Tissue imprints PCR qPCR	Histopathology		
<i>M. refringens</i>	PCR (L, P, J, A) Histopathology (J, A)	Tissue imprints (J, A)	PCR tissue imprints	Histopathology	PCR sequencing	ISH TEM
<i>M. mackini</i>	Histopathology (A)	PCR (J, A)		PCR Histopathology	ISH sequencing	Histopathology TEM PCR
<i>Perkinsus marinus</i>	PCR (L) RFTM (J, A)	Histopathology (P, J, A) PCR (J, A) ISH (J, A)	PCR	ISH Histopathology RFTM	ISH	PCR Sequencing
Herpes virus μ var	PCR (L, J, A) qPCR (L, J, A)		PCR (L, J, A) qPCR (L, J, A)		Sequencing	PCR qPCR ISH TEM

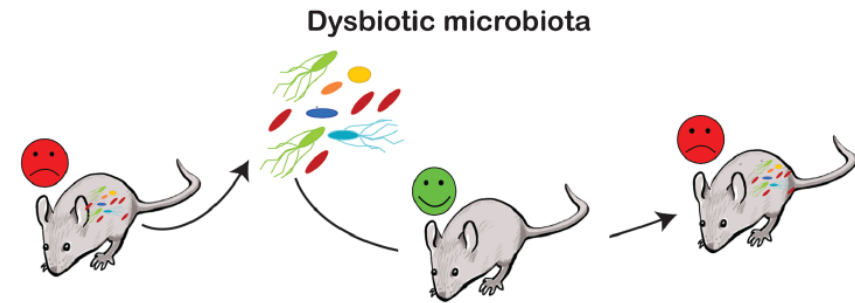
A, Adults; P, post-larvae; L, Larvae; J, Juveniles.

Genomics tools



Advances in the development of molecular tools with the use of new-generation sequencing technologies (NGS) and their ability to produce large volumes of data allow to increase the knowledge of novel pathogens.

Ecological Koch's postulate



«A dysbiosis, a disease»

Not a single pathogen

- Rapid improvements in the technology used to assess microbial communities have led to an expansion of the breadth and scope of microbial pathogen research over the past 20 years.
- The rapid increase in microbiome research, spurred by next generation amplicon sequencing, has allowed researchers to characterize the microbial communities of organisms and environments which were previously poorly understood.

DNA sequencing for microbial community analysis

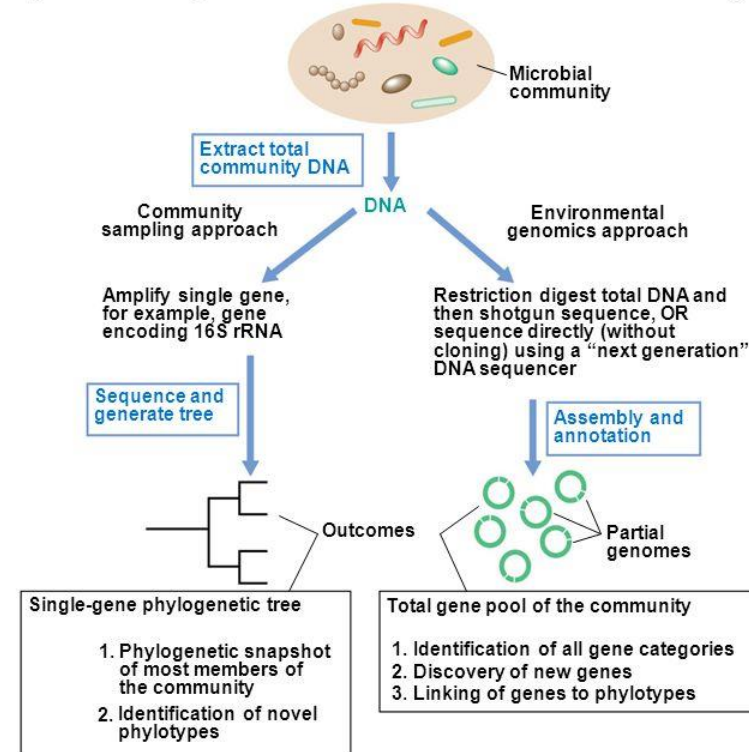


Figure 22.16

Bivalve species	Location	Water temperature °C	Time of year	Tissue type	Operational taxonomic units (OTUs) reported	Shannon index	Bacterial phyla	Reference
<i>Crassostrea virginica</i>	Long Island Sound, CT	4–21	September, November, March, and July	Gut	781	4	11	Pierce and Ward (in review)
<i>Crassostrea virginica</i>	Atlantic Beach, NC	n/a	July	Gut	477–552	1.06–1.33	2	Arfken et al. (2017)
<i>Crassostrea virginica</i>	Gulf of Mexico, LA	n/a	August and September	Gut	243–304	3.96–4.05	12	King et al. (2012)
<i>Crassostrea virginica</i>	Gulf of Mexico, LA	n/a	August and September	Stomach	138–172	1.27–3.63	12	King et al. (2012)
<i>Crassostrea coteziensis</i>	Gulf of California, Mexico	26–29	July–September	Gut	117–368	3.2–4.5	13	Trabal et al. (2014)†
<i>Crassostrea sikamea</i>	Gulf of California, Mexico	26–29	July–September	Gut	79–367	2.57–4.5	13	Trabal et al. (2014)†
<i>Crassostrea gigas</i>	Gulf of California, Mexico	26–29	July–September	Gut	234–305	1.87–4	13	Trabal et al. (2014)†
<i>Crassostrea gigas</i>	Wadden Sea, Germany	2	January	Gills	4,464	2.5–4	10	Wegner et al. (2013)
<i>Crassostrea gigas</i>	Wadden Sea, Germany	8 and 21	August and November	Hemolymph	2,622	4.2–4.8	18	Lokmer and Wegner (2015)
<i>Crassostrea gigas</i>	Wadden Sea, the Netherlands and Germany	13–22	June–August	Hemolymph	100	Avg 4.4 and 3.8 at 2 sites	n/a	Lokmer et al. (2016a)
<i>Crassostrea gigas</i>	Wadden Sea, the Netherlands and Germany	14	n/a	Hemolymph	500–600	n/a	n/a	Lokmer et al. (2016b)
<i>Crassostrea gigas</i>	Wadden Sea, the Netherlands and Germany	14	n/a	Gills	200–400	n/a	n/a	Lokmer et al. (2016b)
<i>Crassostrea gigas</i>	Wadden Sea, the Netherlands and Germany	14	n/a	Gut	100–300	n/a	n/a	Lokmer et al. (2016b)
<i>Crassostrea gigas</i>	Wadden Sea, the Netherlands and Germany	14	n/a	Mantle	200–400	n/a	n/a	Lokmer et al. (2016b)
<i>Crassostrea gigas</i>	Gulf of La Spezia, Italy	26.7	August	Gut	600	700*	n/a	Vezzulli et al. (2018)
<i>Crassostrea gigas</i>	Gulf of La Spezia, Italy	26.7	August	Hemolymph	1,200	1,300*	n/a	Vezzulli et al. (2018)
<i>Crassostrea hongkongensis</i>	Hailing Bay, China	n/a	March–December, monthly	Gut	n/a	2.2–2.8	n/a	Wang et al. (2016)
<i>Mytilus edulis</i>	Long Island Sound, CT	4–21	September, November, March, and July	Gut	989	4–6.5	22	Pierce and Ward (in review)
<i>Mytilus edulis</i>	Barneгат Bay, NJ	n/a	n/a	Gut	178	1.4–4.0	n/a	Schill et al. (2017)
<i>Mytilus edulis</i>	Barneгат Bay, NJ	n/a	n/a	Gills	68	0.3–1.9	n/a	Schill et al. (2017)
<i>Mytilus galloprovincialis</i>	Gulf of La Spezia, Italy	26.7	August	Gut	600–700	800*	n/a	Vezzulli et al. (2018)
<i>Mytilus galloprovincialis</i>	Gulf of La Spezia, Italy	26.7	August	Hemolymph	1,000	1,100*	n/a	Vezzulli et al. (2018)
<i>Brachidontes</i> sp.	Kakaban and Maratua islands, Indonesia	28–32	August	Whole organism	3,553	n/a	44	Cleary et al. (2015)

Not a single pathogen: microbial community analysis

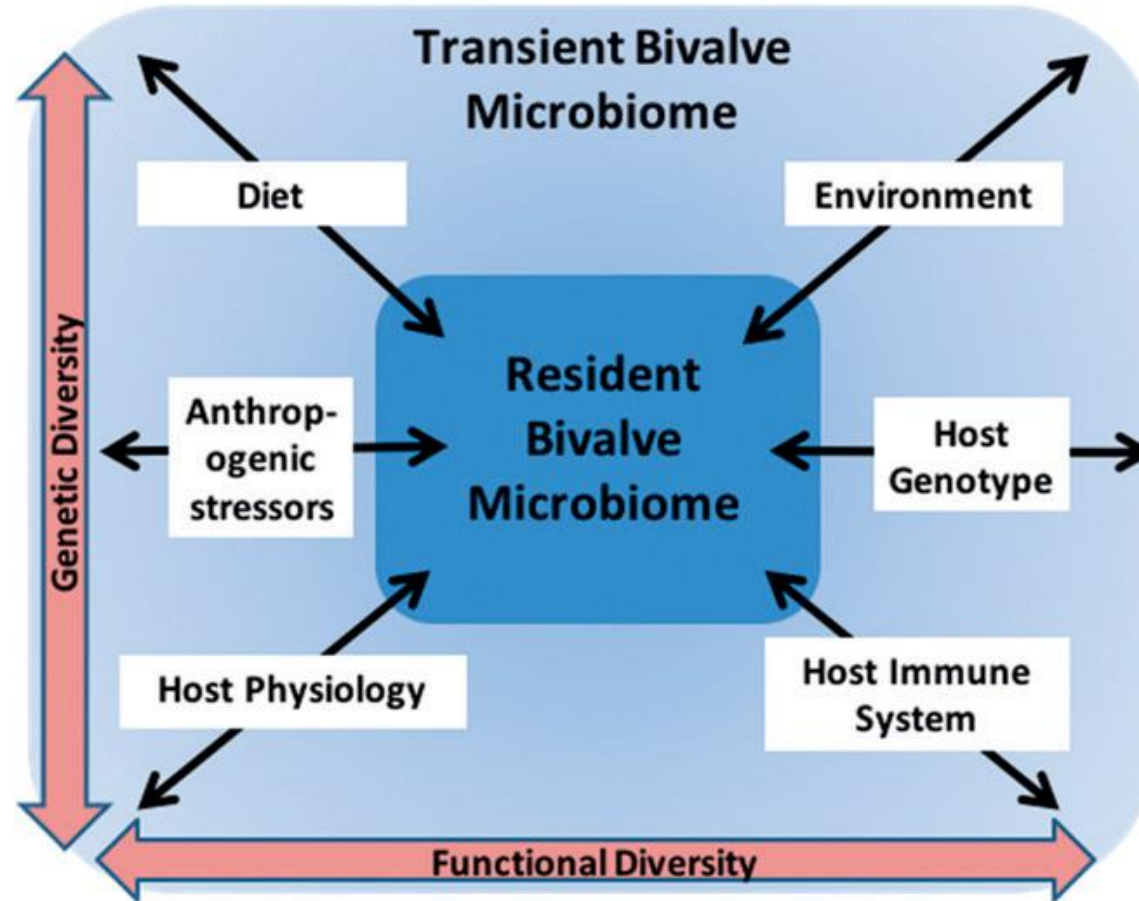
VIVALDI: WP1 Subtask 1.2.1

Bivalve patho-biome will be investigated in selected contrasting samples of oysters and mussels from the key sites indicated above and in clams, cockles and scallops from additional relevant sites. Pathobiome analysis will be extended, when possible, to larvae and/or juveniles. Bacterial and microeukaryote parasite diversity and load in bivalves will be estimated using metagenetics and lineage-specific PCR. Complementary, bivalve “vibriome” will be evaluated in oysters by high throughput sequencing analyses (e.g. whole metagenome/targeted DNA sequencing).

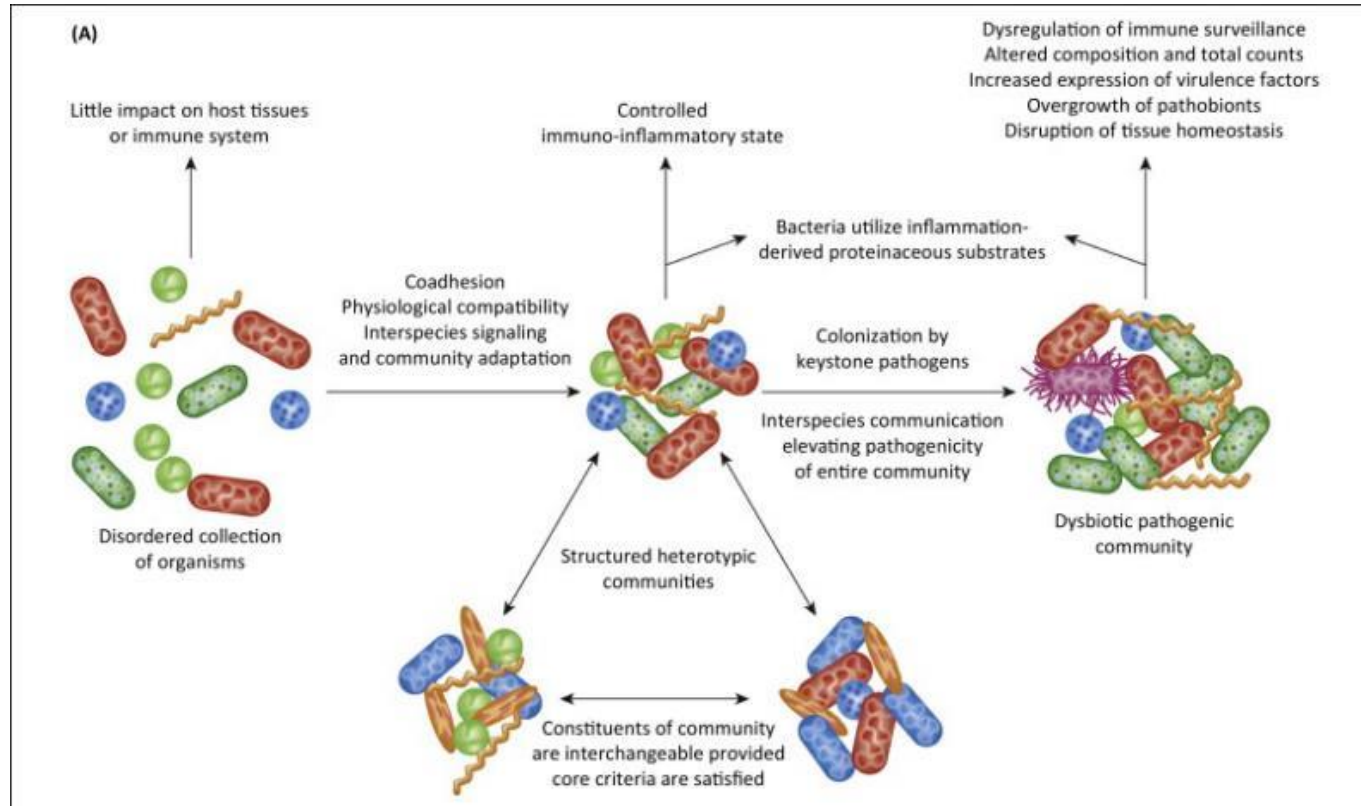
Diseased and non-diseased samples will be stored until the most interesting/relevant samples can be analysed for patho-biome investigation.

Not a single pathogen: microbial community analysis

The microbial communities of suspension-feeding bivalves include both resident and transient microbiota.



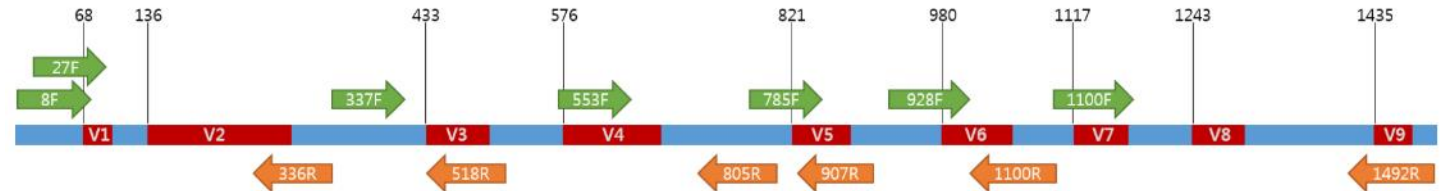
Polymicrobial diseases defined as pathologic manifestations induced by the presence of multiple microorganisms affecting the host are held responsible for infections of marine animals suffering mass mortality episodes such as those repeatedly affecting the Pacific oyster (*Crassostrea gigas*) in European shellfish farms.



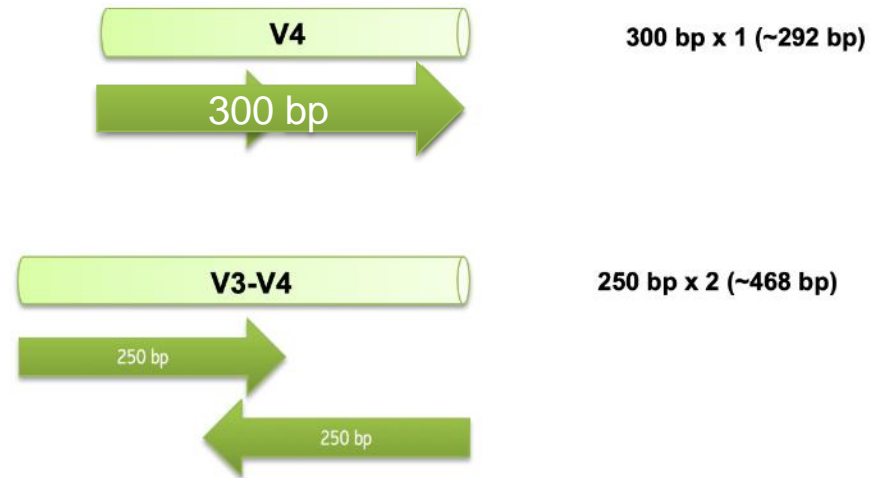
WS: CO-INFECTION
AND MULTIPLE
STRESSORS
D. Bass: pathobiome

Not a single pathogen: microbial community analysis

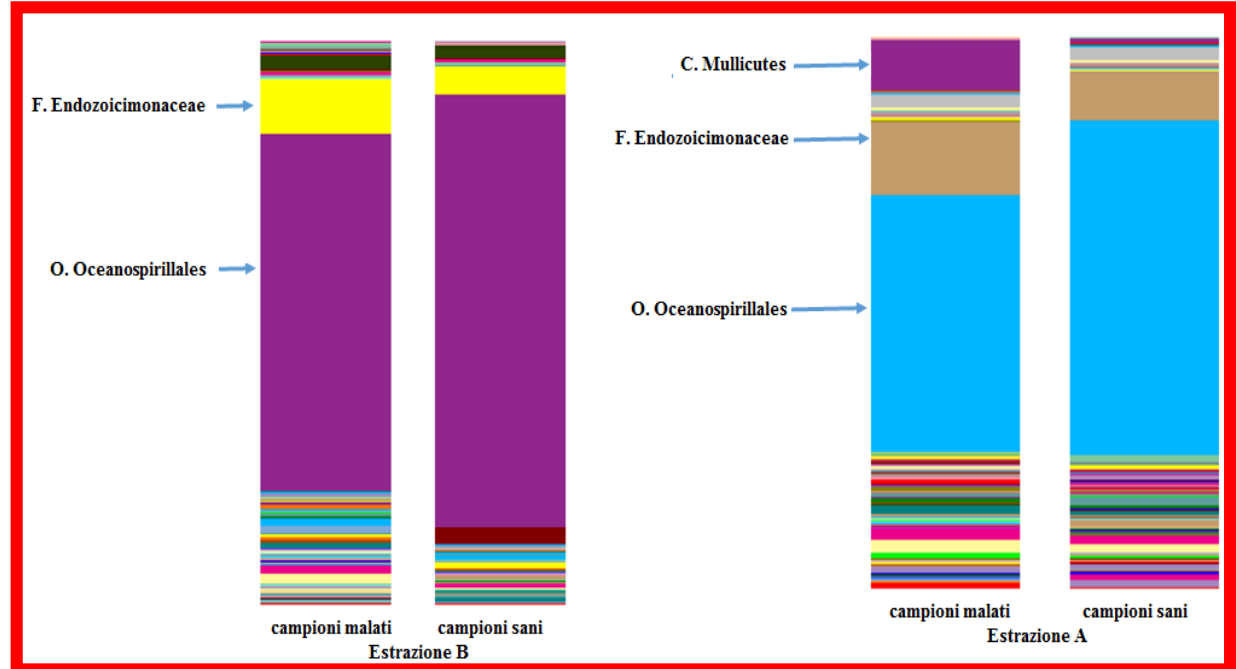
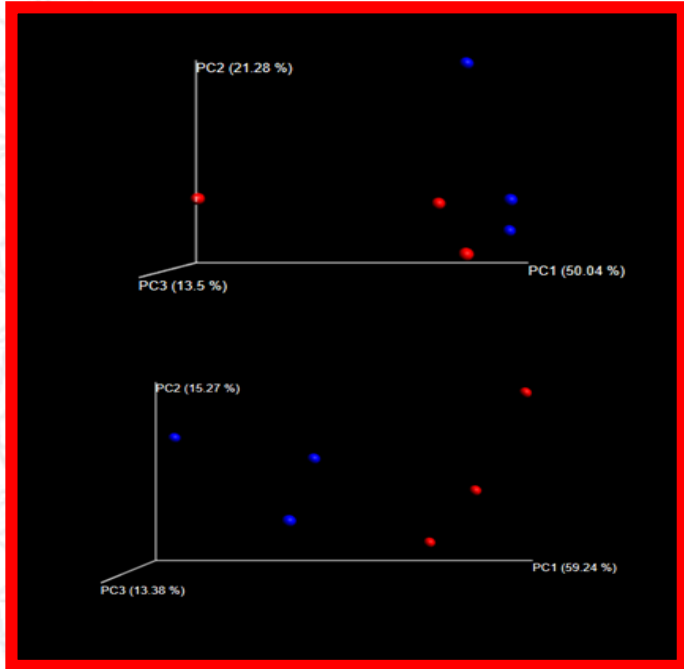
We started 4 years ago setting up and comparing protocols:



- DNA extraction
- 16S region to amplify
- Database to adopt

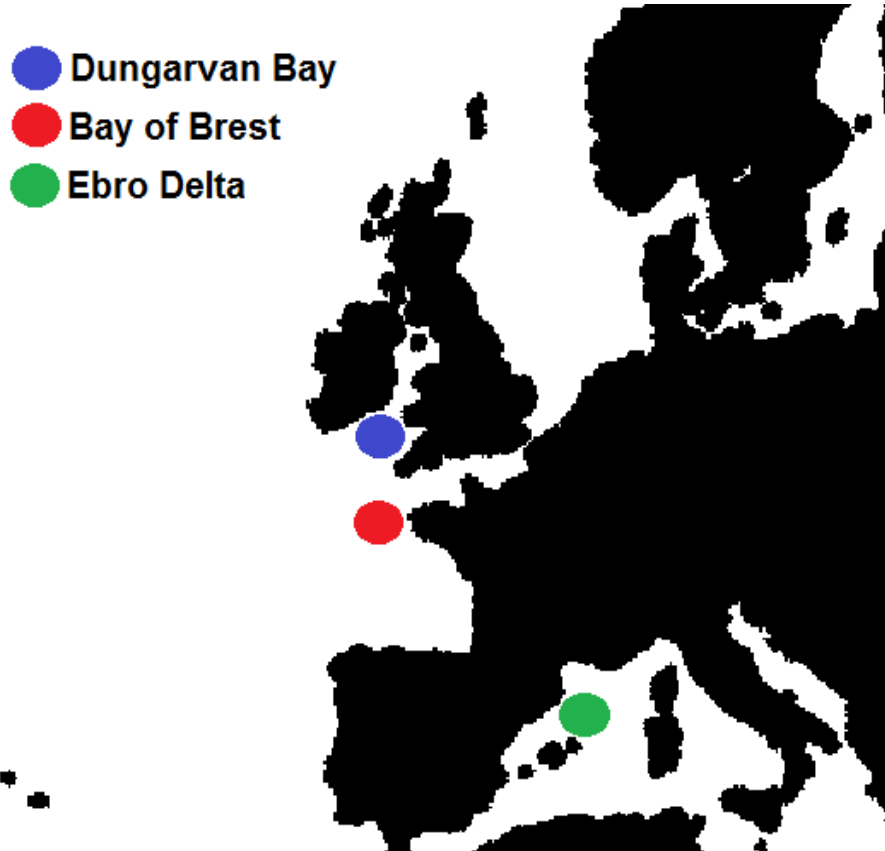


Not a single pathogen: microbial community analysis

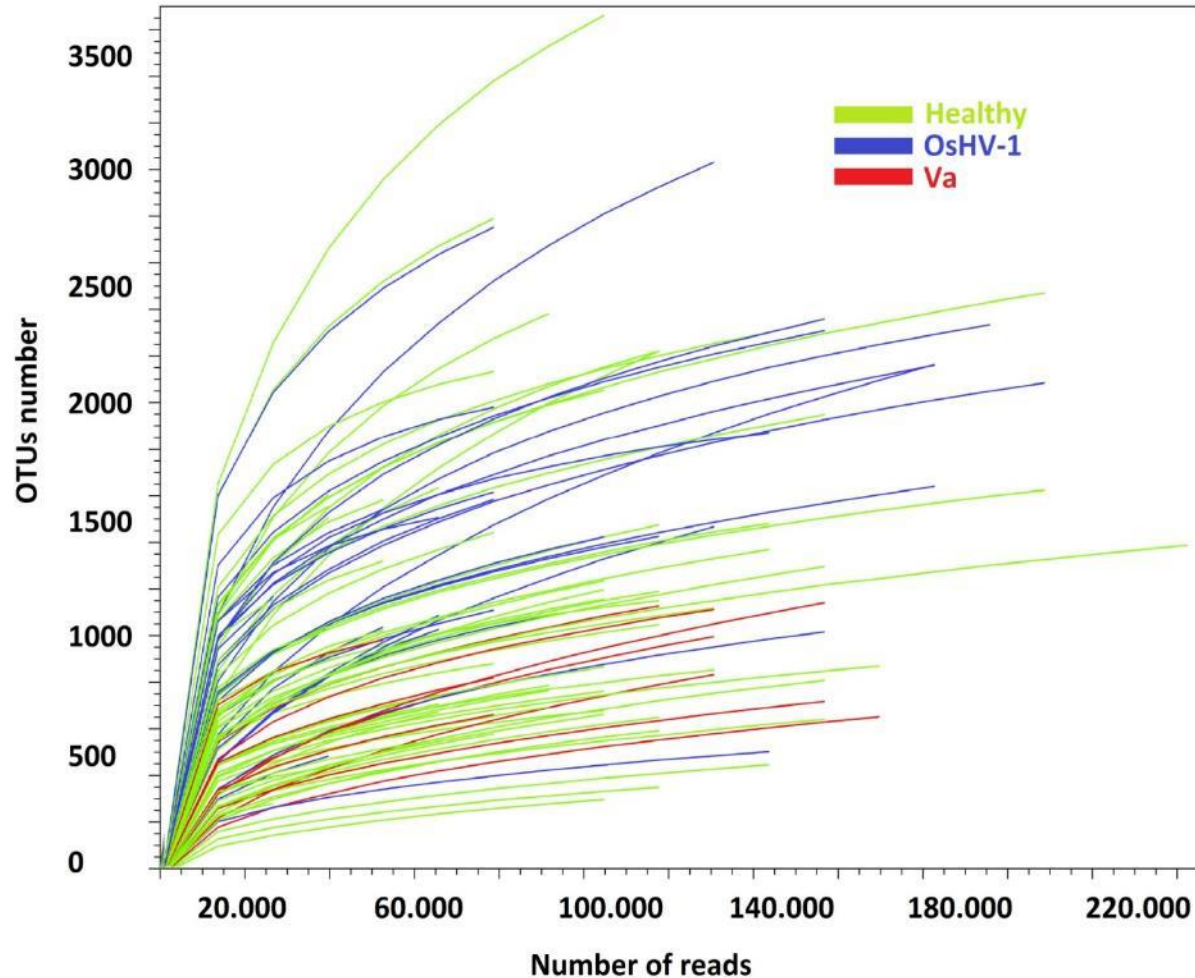


Very first encouraging results

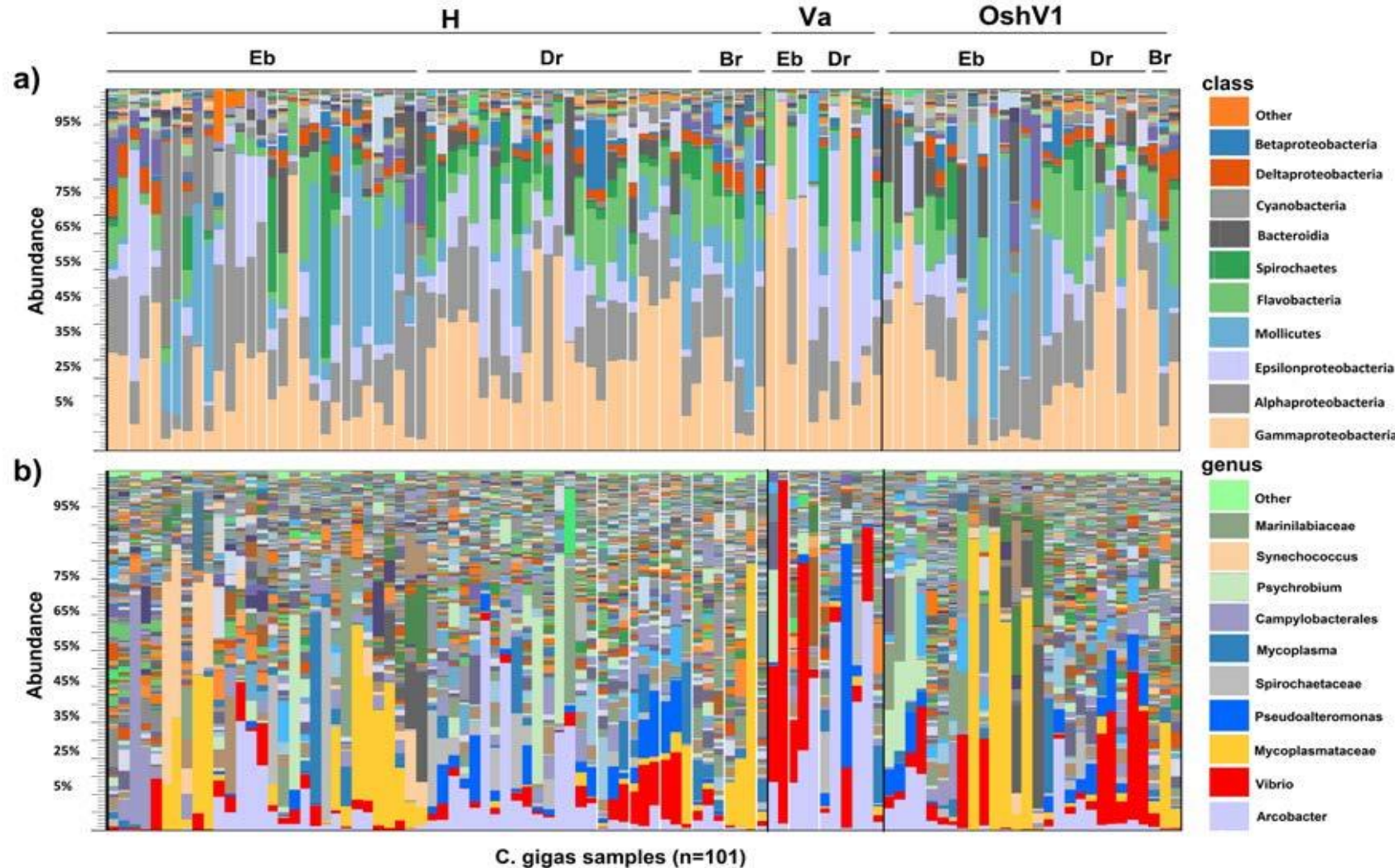
A total of 525 *C. gigas* samples collected from 2016 to 2017 in three European sites, i.e., Ebro delta, Dungarvan Bay and Bay of Brest were screened for the presence of OsHV-1 and *V. aestuarianus* using quantitative real-Time PCR



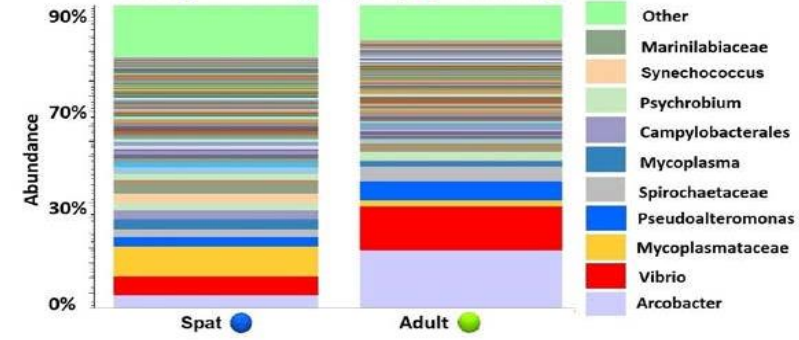
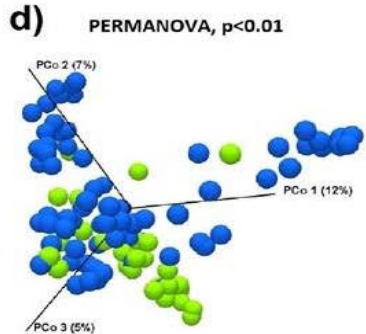
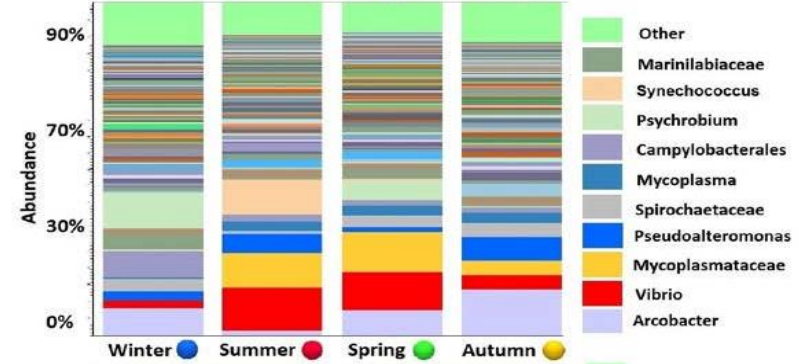
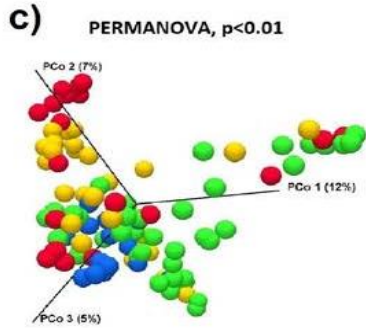
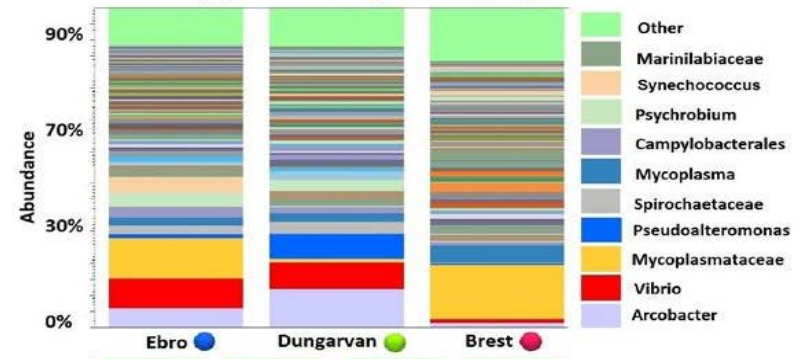
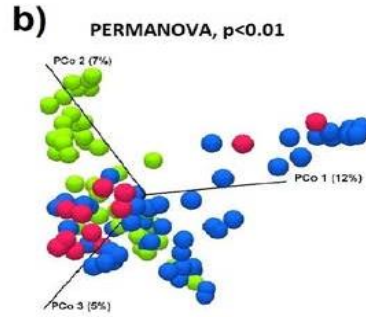
One hundred one contrasting *C. gigas* samples were selected in the Ebro Delta (n=50), Dungarvan bay (n=40) and the Bay of Brest (n=11) for microbiota analysis

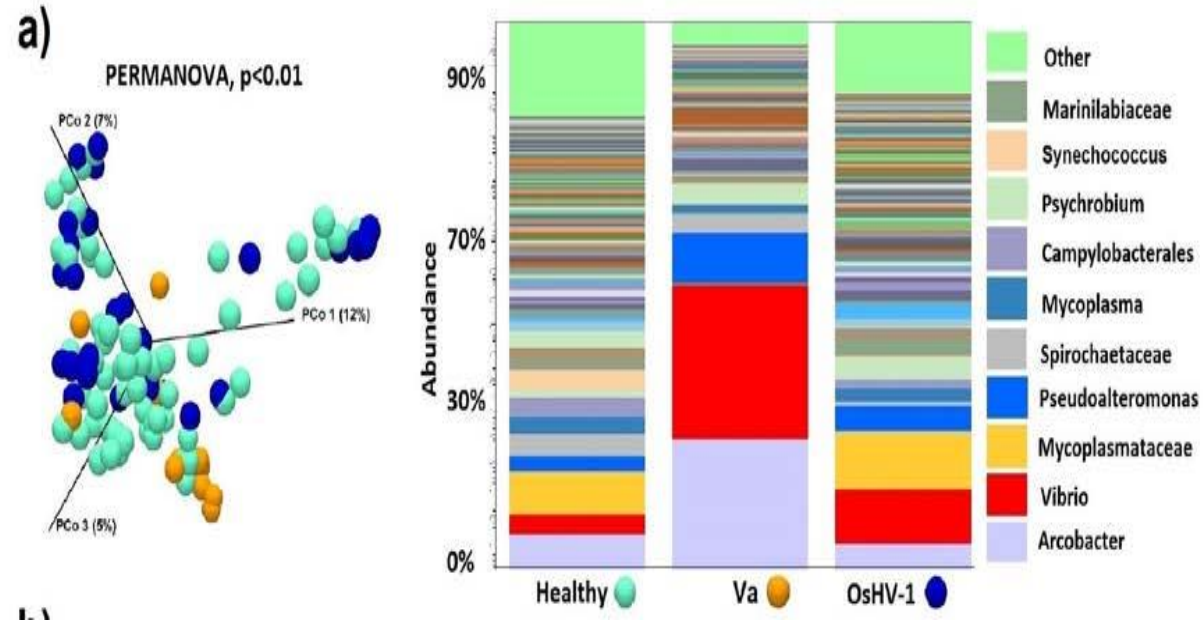


About 15M
reads
80k
reads/sample



Classes of Gamma and Alphaproteobacteria accounting on average for 28% and 15% of the total bacterial diversity followed by Epsilonproteobacteria (11%), Mollicutes (10%) and Flavobacteria (9%)



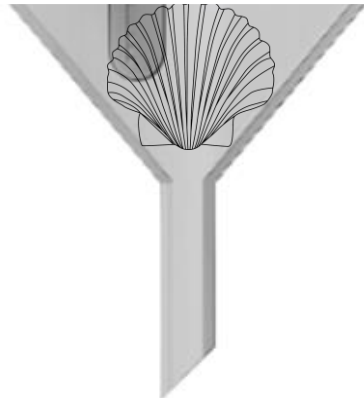


Comparative analysis of healthy vs infected *C. gigas* samples clearly showed that infected oysters displayed signs of community structure disruption and were characterized by a low diversity and proliferation of few bacterial taxa.

Metagenomic next-generation sequencing (mNGS) for pan-pathogen detection

Sample matrices

- PROS:
 - Hypothesis-free, or unbiased, testing
 - Discovery of new or unexpected organisms
 - Potential for quantitation
 - Ability to detect any portion of genome
- CONS:
 - Must also sequence host background
 - Expensive
 - Time consuming
 - Not all genomes are available
 - Prone to contamination



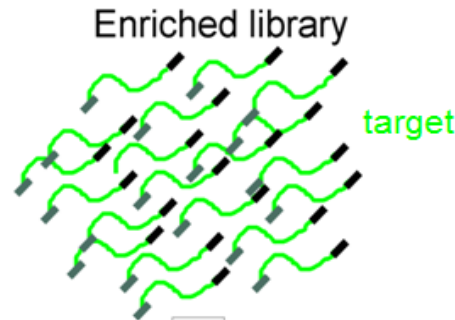
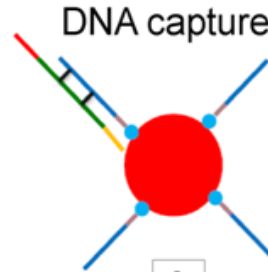
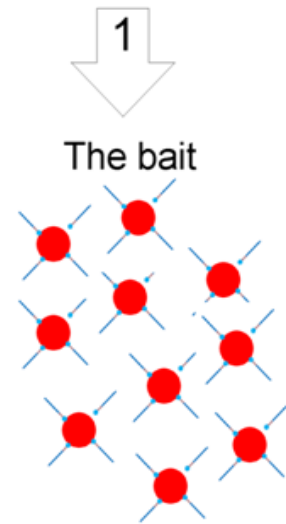
Metagenomics workflow



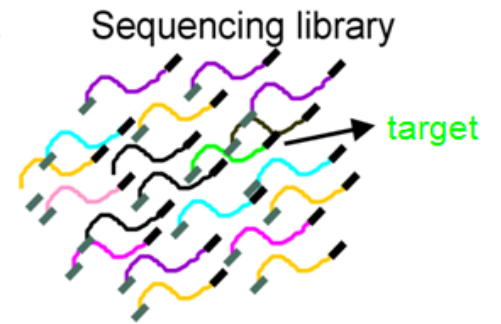
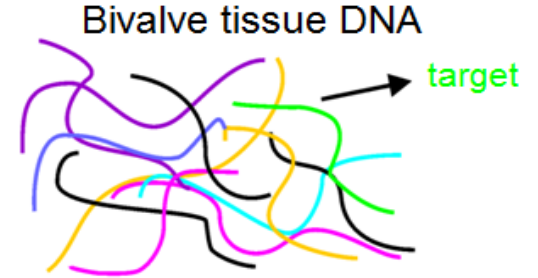
Detection of pathogen



884 phylogentic and virulence marker sequences (400nt) of the marine pathogenic microbial community



ILLUMINA SEQUENCING
MiSeq

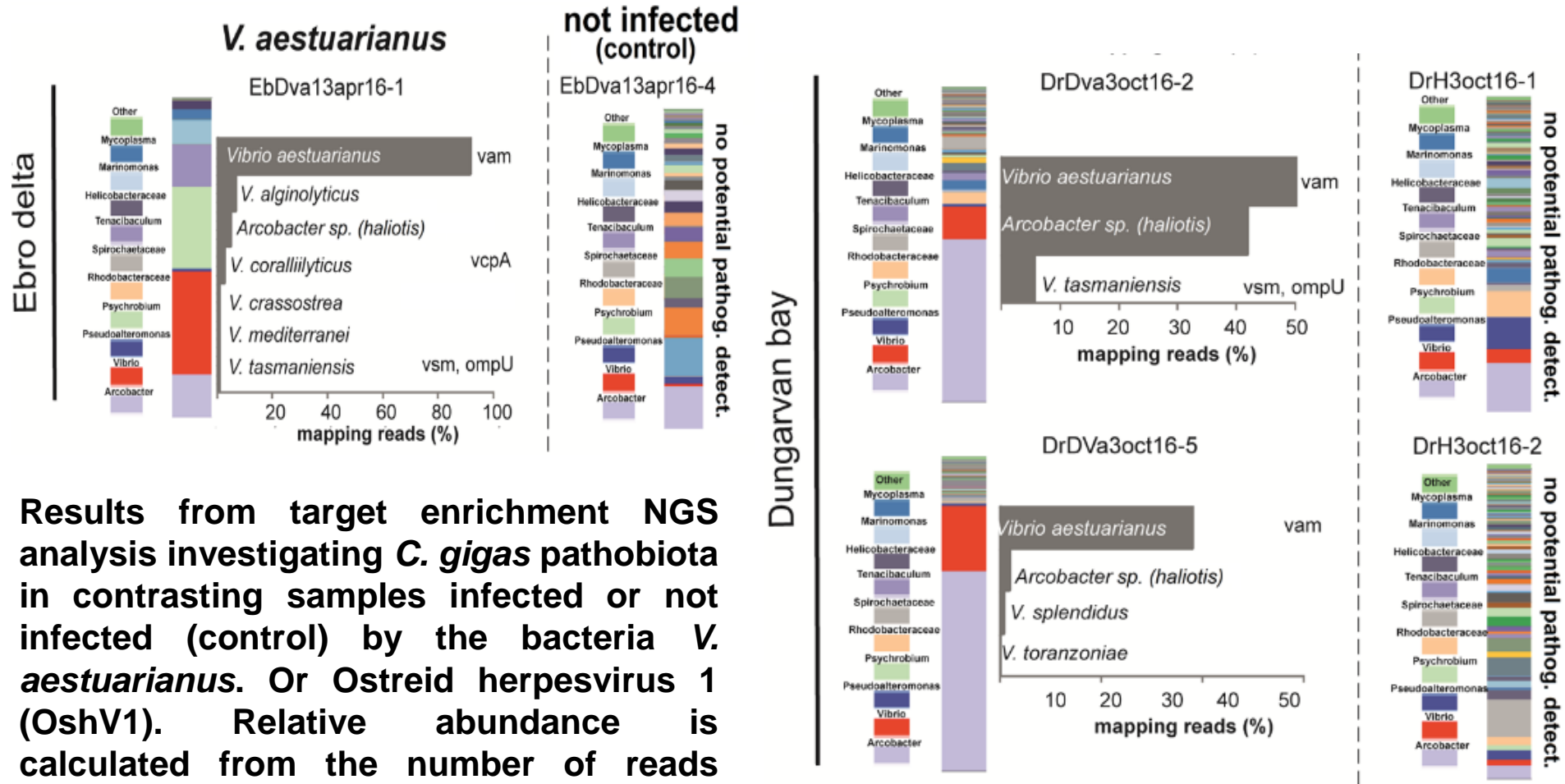


**Target enrichment
NGS sequencing of the
bivalve pathobiota**

Target Enrichment next generation sequencing for the analysis of the Bivalve Pathobiota




Target	Taxonomy	Main host	Marker gene	No. of allelic variants	Length (nt)
<i>Vibrio</i> spp.	<i>Vibrio</i> spp.	Human, Animal	<i>gyrB</i>	243	400
	<i>Vibrio</i> spp.	Human, Animal	<i>recA</i>	204	400
	<i>Vibrio</i> spp.	Human, Animal	<i>atpA</i>	133	400
	<i>Vibrio</i> spp.	Human, Animal	<i>dnaJ</i>	56	400
	<i>Vibrio</i> spp.	Human, Animal	<i>pyrH</i>	113	400
	<i>V. tasmaniensis</i>	<i>Crassostrea gigas</i>	LGP32 probes	10	400
	<i>V. cholerae</i> O1 El Tor	Human	<i>ctxA</i>	1	777
	<i>V. cholerae</i> O1 El Tor	Human	<i>ctxB</i>	1	375
	<i>V. cholerae</i> O139	Human	<i>ctxA-B</i>	1	938
	<i>V. cholerae</i> O1 el Tor	Human	<i>tcpA</i>	1	675
	<i>V. cholerae</i> O1 classical	Human	<i>tcpA</i>	1	675
	<i>V. cholerae</i> O1 el Tor	Human	<i>rstR</i>	1	339
	<i>V. cholerae</i> O1 classical	Human	<i>rstR</i>	1	336
	<i>V. cholerae</i> O139	Human	<i>wbf</i>	1	449
	<i>V. cholerae</i> O1 el Tor	Human	<i>gbpA</i>	1	400
	<i>V. parahaemolyticus</i>	Human	<i>toxR</i>	1	552
	<i>V. parahaemolyticus</i>	Human	<i>tdh, trh</i>	3	570
	<i>V. vulnificus</i>	Human	<i>vvhA</i>	1	1416
	<i>V. vulnificus</i>	Human	<i>rtxA1</i>	1	400
	<i>V. tasmaniensis</i>	<i>Crassostrea gigas</i>	<i>vsm</i> (LGP32 strain)	1	1824
	<i>V. tasmaniensis</i>	<i>Crassostrea gigas</i>	<i>ompU</i> (LGP32 strain)	1	400
	<i>V. aestuarianus</i>	<i>Crassostrea gigas</i>	<i>vam</i>	1	1836
	<i>V. tapetis</i>	<i>Ruditapes philippinarum</i>	<i>djlA</i>	1	1826
	<i>V. coralliilyticus</i>	<i>Paramuricea clavata</i>	<i>vcpA</i>	15	1824
	<i>V. harveyi</i>	Stony corals	<i>vhhA</i>	1	1260
	<i>V. crassostreae</i>	<i>Crassostrea gigas</i>	<i>R-5.7</i>	1	2397
	<i>V. tubiashii</i>	<i>Crassostrea gigas</i>	Metalloprotease	1	1821
	<i>Vibrio</i> spp.	Human, Animal	<i>MSHA</i>	12	400

<i>Arcobacter</i> spp.	<i>Arcobacter</i> spp.	Human, Animal	<i>gyrB</i>	12	400
<i>Nocardia crassostrea</i>	<i>Nocardia crassostrea</i>	Human	<i>gyrB</i>	27	400
		<i>Crassostrea gigas</i> , <i>Ostrea edulis</i>	<i>rpoB</i> , <i>hsp65</i> , <i>gyrB</i>	3	400
<i>Marteilia refringens</i>	<i>Marteilia refringens</i>	<i>Ostrea edulis</i> , <i>Mytilus edulis</i> , <i>M. galloprovincialis</i>	ITS10, ITS1M, probe	3	400
<i>Bonamia ostreae</i>	<i>Bonamia ostreae</i>	<i>Ostrea edulis</i>	5.8S-ITS rDNA, <i>hsp90</i> , <i>act1</i>	3	400
OsHV-1	Ostreid herpesvirus 1	<i>Crassostrea gigas</i>	C2/C6 (2), IA1-IA2, orf4, Hyp. Protein, RING finger protein gene	7	400
			ORF100	1	198
			C9-C10	1	197
			B3-B4	1	207
<i>Enterococcus</i> spp.	<i>E. faecalis</i> , <i>E. faecium</i> , <i>E. avium</i> , <i>E. gallinarum</i> , <i>E. casseliflavus</i> , <i>E. durans</i> , <i>E. raffinosus</i> , <i>E. mundtii</i>	Human	<i>atpA</i>	8	400
<i>Roseovarius crassostrea</i>	<i>Roseovarius crassostrea</i>	<i>Crassostrea virginica</i>	<i>dnaJ</i> , <i>pyrH</i>	6	400
<i>Escherichia coli</i>	<i>Escherichia coli</i>	Human	<i>dnaJ</i> , <i>pyrH</i> , <i>atpA</i> , <i>gyrB</i>	4	400
<i>Aspergillus sydowii</i>	<i>Aspergillus sydowii</i>	<i>Gorgonia ventalina</i> , Human	TUB2, <i>trpC</i> , ITS, calmodulin gene	4	400
<i>Aurantimonas coralicida</i>	<i>Aurantimonas coralicida</i>	Corals	<i>atpD</i> , <i>gyrB</i> , <i>recA</i> , <i>rpoB</i>	4	400
<i>Serratia marcescens</i>	<i>Serratia marcescens</i>	<i>Acropora palmata</i>	<i>gyrB</i> , <i>recA</i> , <i>dnaJ</i>	3	400
<i>Pseudoalteromonas</i> sp.	<i>Pseudoalteromonas</i> sp.	<i>Rhopaloeides odorabile</i>	<i>gyrB</i>	1	400
			Total	884	29,292



Results from target enrichment NGS analysis investigating *C. gigas* pathobiota in contrasting samples infected or not infected (control) by the bacteria *V. aestuarianus*. Or Ostreid herpesvirus 1 (OshV1). Relative abundance is calculated from the number of reads specifically mapping on target sequences and expressed as percentage

Dynamics of the Pacific oyster pathobiota during mortality episodes in Europe assessed by 16S rRNA gene profiling and a new target enrichment next-generation sequencing strategy

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environmental
microbiology



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