MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

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Deliverable D5.4

Assessment of improved culture efficiency by the addition of AHLs and exudates

Organisation name of lead contractor: UCC – partner 12

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Dissemination Level	
PU Public	
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	**X**

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Issue	Date	Implemented by
v.1		

The following document is related to this deliverable:

1.

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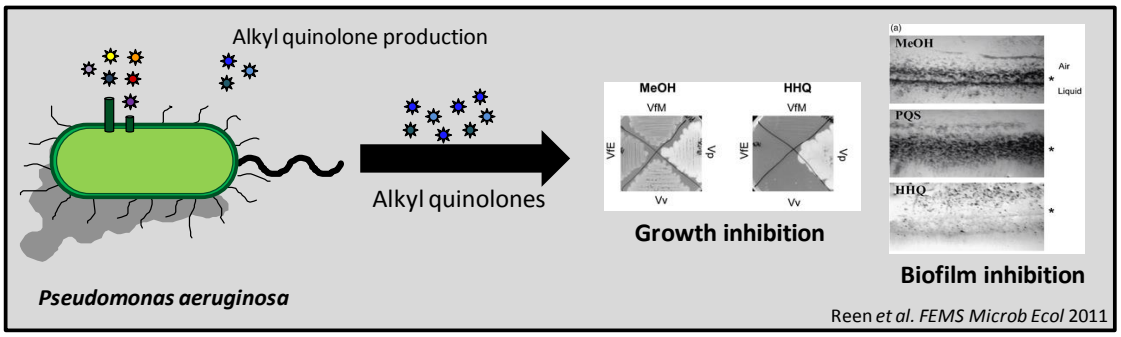
Summary

Objective(s): Assess the impact of QS compounds and QS producing marine isolates on the spectrum of culturability from marine sponges.

Rationale: The tasks associated with D5.4 focus on evaluating the impact of AHLs and other QS compounds on the culturability of marine sponge isolates. QS and QS-induced compounds have the ability to either promote or suppress the growth of other community isolates and therefore represent a key factor in improving the spectrum of culturability from the marine ecosystem. In this section of the proposal, the experimental focus is on establishing the growth effects of a range of QS compounds, including those identified in Task 5.1.



Results: Following on from the successful identification of QS producing isolates using a range of QS biosensor assays, partners have begun the process of establishing how these compounds are likely to impact on the growth of microbial communities and on the growth dynamics of individual marine isolates. **Partner 12 (UCC)** has shown that while addition of purified AHL compounds did not markedly influence the culturability or growth of an *Algoriphagus* sp. marine sponge isolate, the addition of exudates from QS producing isolates identified in Task 5.1 suppressed growth and pigment production in the same isolate. Growth of this isolate was also suppressed upon addition of the purified alkylquinolone compound HHQ. Addition of the same exudates to marine agar plates significantly reduced the diversity of organisms from marine sponge that were culturable on marine agar plates, while addition of pure C6-HSL appears to promote the growth of unique species. **Partner 14 (DTU)** has undertaken a screen of AHL producing Vibrionaceae from a global geographical collection and has characterised the range of compounds produced. This will inform the selection of the most relevant AHL compounds for further analysis on their impact on the culturability of marine isolates. **Partner 19 (PHM)** has had considerable success with the identification of abiotic factors that promote growth of pharmaceutically relevant marine species, particularly Actinobacteria. The convergence of all three experimental approaches has resulted in the experimental evidence for small molecules and abiotic factors that can influence the growth and culturability of marine sponge isolates. The next challenge is to exploit this data to design effective media compositions that will modulate QS signal production and thus promote the growth and culturability of previously unculturable marine isolates.

Partner(s) involved in Deliverable production:
[12] [14] [19]

Deliverable D5.4

***Assessment of improved culture efficiency by the addition of AHLs and exudates*.**

Since the first description of the cell-cell communication phenomenon termed quorum sensing in the marine symbiont *Vibrio fischeri*, there has been an explosion in the breadth and diversity of cell-cell communication systems discovered in microbes. This has been further enhanced by the advent of whole genome sequencing, allowing detection of signalling genes and receptors in a range of culturable and unculturable organisms. Given the prevalence of QS systems in the marine ecosystem, it is almost inconceivable that they would not play a significant role in moderating population dynamics within this niche. The question remains however as to whether the role of individual QS systems is antagonistic or agonistic to growth of co-existing organisms.

Partner [12] had previously profiled a large collection of marine sponge isolates for AHL and alkylquinolone producing strains, identifying marine exudates that were capable of activating biosensors from each class (**Table 1**; D5.1 and D5.2). BD129C30 (sample 95), BD129C31 (sample 214) and BD188M28 (sample 12) were included in this analysis as they activated the biosensor although no specific AHL was identified in the supernatants.

Table 1: Spectrum of AHL production in marine sponge isolates.

Sample ID	Sample No	Biosensor Activated	AHL	Isolate Classification
B24394A	273	*A.tumefaciens* NTL4	Long	*Brevibacterium sp. J3*
B213SK4	335	*A.tumefaciens* NTL4	Long	*Halomonas frigidi*
BD129C30	95	*A.tumefaciens* NTL4	Long	*Psychrobacter sp. P16*
BD129C6	211	*A.tumefaciens* NTL4	Long	*Psychrobacter sp. BSw20879*
BD129C23	230	*A.tumefaciens* NTL4	Long	*Psychrobacter sp. BSw20879*
BD213M10	342	*A.tumefaciens* NTL4	Long	*Psychrobacter nivimaris*
B130SK7	386	*S.marcescens* SP15	Short	*Psychrobacter sp. P16*
BD129C31	214	*A.tumefaciens* NTL4	Long	*Pseudoalteromonas sp. BSs20138*
B129C7	394	*A.tumefaciens* NTL4	Long	*Pseudoalteromonas sp. YASM-7*
B213SM5	419	*A.tumefaciens* NTL4	Long	*Pseudoalteromonadaceae*
BD188M28	12	*S.marcescens* SP15	Short	*Pseudomonas sp. p50*
BD226SM4	142	*S.marcescens* SP15	Short	*Pseudomonas sp. p50*
B213S5	411	*S.marcescens* SP15	Short	*Pseudomonas sp. P50*
BD188C13	163	*A.tumefaciens* NTL4	Long	*Vibrio splendidus isolate PB1-10rrnM*

Sample ID	C4	OH-C6	O-C6	C6	C8	OH-C10	O-C10	O-C12
B24394A							YES	
B213SK4							YES	
BD129C30								
BD129C6								YES
BD129C23								YES
BD213M10						YES	YES	
B130SK7								
BD129C31								
B129C7								YES
B213SM5		Trace				YES	YES	YES
BD188M28								
BD226SM4								YES
B213S5	Trace			Trace				
BD188C13							YES	YES

The cell-free exudates from these producing isolates were subsequently added to marine agar plates. To directly test whether QS isolates influenced the growth and culturability of individual marine isolates, an indicator organism (a marine sponge isolate *Algoriphagus* sp.) was used. In order to assess whether these exudates could influence the growth of communities within the sponge, a sponge sample was cultured in the presence and absence of exudates on these plates. As controls, DMSO and commercial AHL compounds C6-HSL and oxo-C8-HSL were used for comparison and to determine whether effects seen were due to the AHL compound itself or a QS controlled product.

Firstly, growth of the *Algoriphagus* sp. indicator organism (100 µl of a 10^{-6} dilution) was reduced in the presence of exudates from QS producing isolates, while production of the characteristic red pigment was also inhibited when compared to untreated plates (**Figure 1**). However, growth and pigment production were unaffected in the presence of purified AHL compounds C6-HSL and oxo-C8-HSL, suggesting the growth inhibitory effects may be due to QS-controlled products rather than the QS signal itself. Additional analysis with non-QS strains, and/or mutational analysis, will be required to establish fully the role of QS in this observed growth suppression.

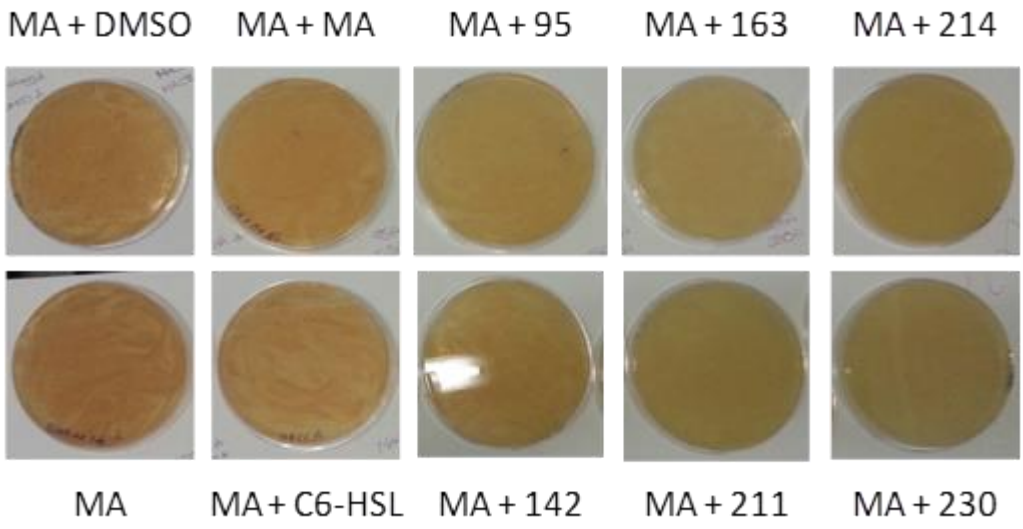


Figure 1. Growth modulation by bacterial exudates from QS producing marine sponge isolates. Top row, from left to right: Marine agar (MA) with DMSO, MA with MA, MA with 95 exudate, MA with

163 exudate, MA with 214 exudate. Bottom row, left to right: MA alone, MA with C6-HSL, MA with 142 exudate, MA with 211 exudate, MA with 230 exudate. Growth and red pigment production is suppressed in the plates to which exudates have been added, while control plates appear unaffected. The lack of suppression on the C6-HSL plate suggests that this molecule is not directly causing the suppression but may control production of the antagonistic factor.

Having established that QS producing isolates were capable of antagonising the growth of other marine isolates, **partner [12]** then investigated whether or not these exudates would influence the diversity of culturable organisms that could be isolated on marine agar from a marine sponge. A 2 g sponge sample was masticated using a scalpel in a sterile Petri dish. The homogenate was transferred into 10 ml of PBS and serial diluted to 10^{-4}. Aliquots (100 µl) were spread plated on MA control and test plates as outlined above. Plates were incubated at 23°C and visualised over a period of two weeks to look for differences between control and test plates (**Figure 2**).

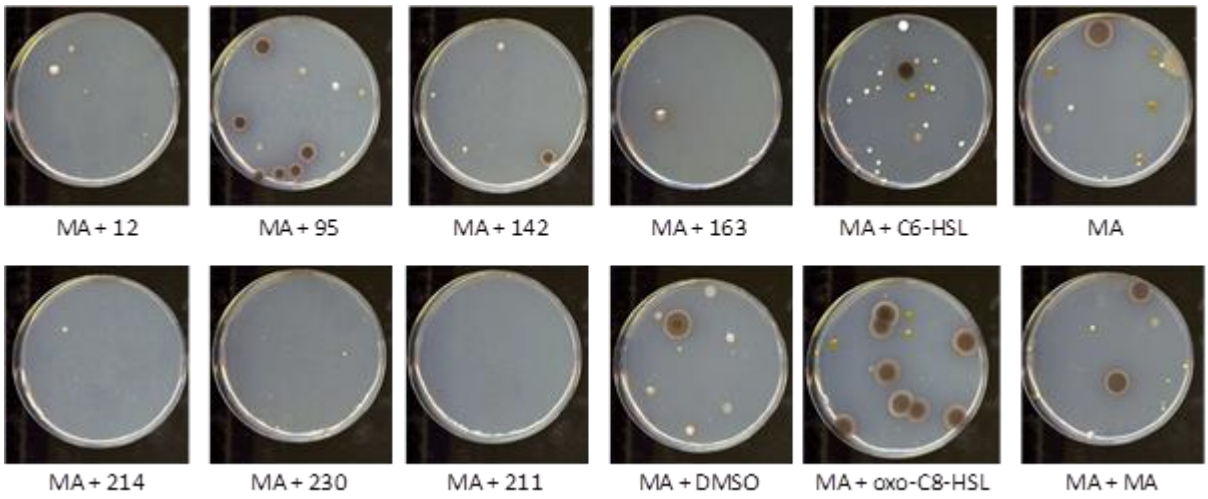


Figure 2. Marine sponge sample cultured on MA plates supplemented with AHLs and bacterial exudates. Top row, from left to right: MA with 12 exudate, MA with 95 exudate, MA with 142 exudate, MA with 163 exudate, MA with C6-HSL, and MA alone. Bottom row, from left to right: and MA with 214 exudate, MA with 230 exudate, MA with 211 exudate, MA with DMSO, MA with oxo-C8-HSL, and MA with MA. As above, addition of the exudates from QS producing isolates markedly influenced the spectrum of culturable organisms recovered from the marine sponge, while the effect of the AHL compounds themselves was also significant, particularly in the case of C6-HSL. Clearly this establishes the antagonistic role of QS in these marine isolates and highlights the bottleneck in culturability that exists as a consequence of QS signalling.

Several colony morphologies, both bacterial and fungal were evident on control plates. In contrast, several plates that were treated with exudate from QS producing isolates only

supported growth of few if any detectable organisms. Samples 12, 163, 214, 230 and 211 were particularly antagonistic towards growth, while most of the colony morphologies present in the control plates were also found in the plate treated with exudate from sample 95. It is worth noting that although this isolate did activate the Agrobacterium biosensor, no AHL was identified by LC-MS.

In considering the growth influence of AHLs and AHL-controlled toxins, there is likely to be specificity regarding species effects. This was observed where addition of C6-HSL to the marine agar led to the culturability of two distinct organisms that were not present in any other condition, including controls and oxo-C8-HSL (**Figure 3**). The absence of these morphologies from the exudate plates may be explained by the fact that none of the tested samples produced C6-HSL, with long-chain AHLs predominating.

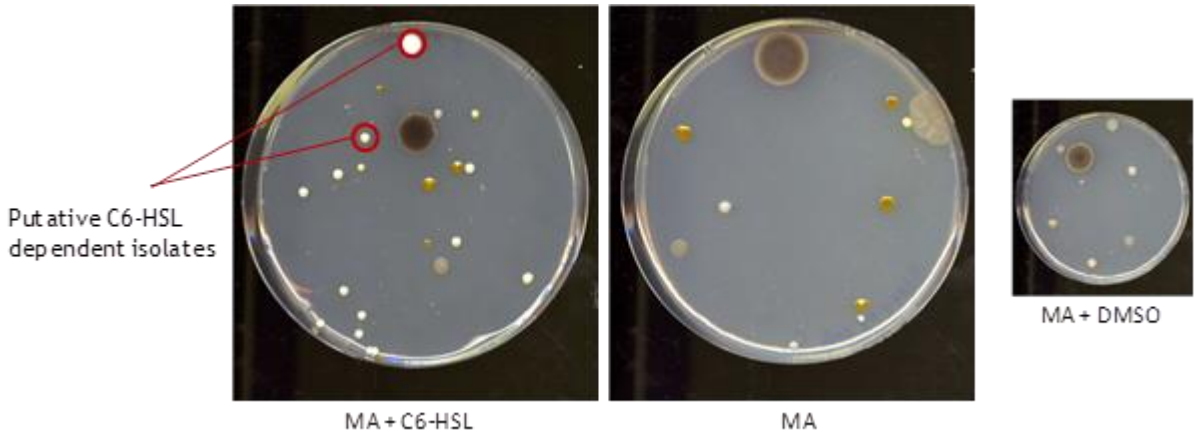


Figure 3. Presence of new colony morphologies on C6-HSL treated plates. This is the first evidence from our analysis that supports the view that QS signal molecules may be required for the growth of so-called non-culturable organisms in standard laboratory media.

In order to establish whether the agonistic effect of C6-HSL on the colony morphologies observed from the marine sponge sample was the result of direct or indirect effects, both colonies were sub-cultured onto fresh MA plates treated with and without C6-HSL. Surprisingly, both morphologies were observed on control and test plates after 2 days, which suggests that C6-HSL is not directly required for the growth of these organisms from pure culture. However, there are a number of factors that need to be considered when attempting to extrapolate findings from source biological material to pure cultures taken from supplemented plates. The single colonies that were replica plated were taken from a C6-HSL supplemented plate and may have assimilated sufficient levels of C6-HSL to enable growth on untreated MA plates. Alternatively, C6-HSL may be required at low cell-densities, or to recover cells from a viable but non-culturable state, and therefore its effect would not

be observed when using pure colonies. These isolates are currently undergoing further characterisation, including identification and dilution plating, to establish the extent to which C6-HSL (and perhaps other AHLs) is required for their culturability.

The specificity of the AHL influence on culturability suggested by the data from **partner [12]** supports the need for comprehensive screening and identification of the specific factors that are prevalent in the marine ecosystem. While previous work described in earlier reports described the activation of long and short chain QS biosensors, the identification of the AHLs themselves and their abundance needs to be established. To this end, **partner [14]** has conducted a study of AHLs in Vibrionaceae isolated from a global research sampling (Gram et al. 2010 Mar Biotechnol). The strains were, as mentioned collected on a global research cruise and the map (**Figure 4**) and a tentative phylogeny was based on 16 S rRNA gene sequences.

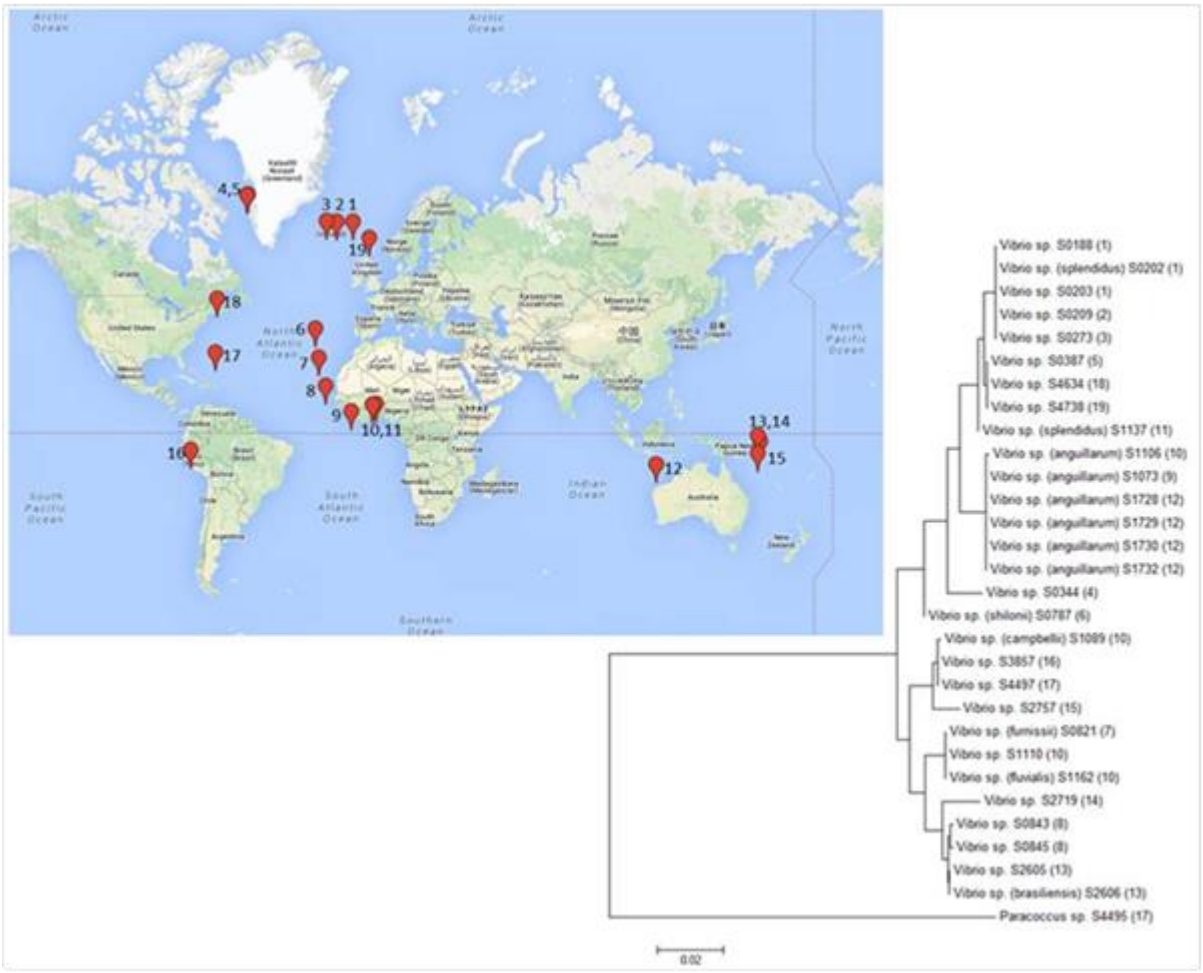
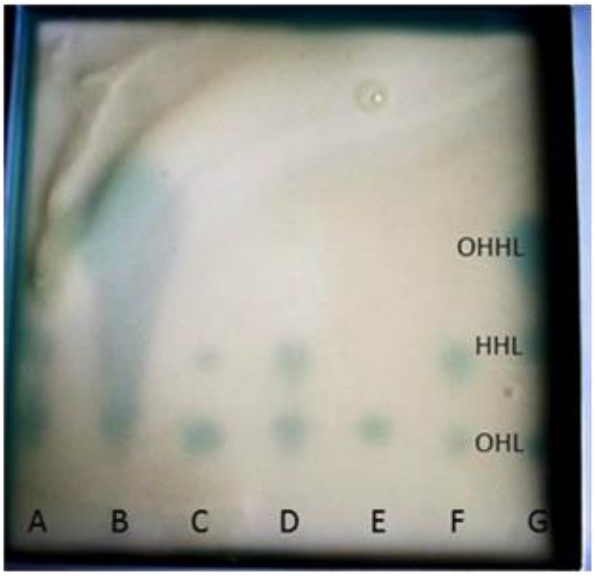


Figure 4. A) World map showing sampling site from where the AHL positive Vibrionaceae strains were isolated. B) Neighbour joining maximum likelihood tree of partial 16s rRNA gene sequences (Default settings), of active strains. The x in (x) indicates site of isolation.

Of 308 strains tested using biosensors (*Chromobacterium violaceum* and *Agrobacterium tumefaciens*), 37 were positive. Selected positive strains that activated the biosensors were profiled using thin-layer chromatography (TLC) and ultra-high performance liquid chromatography-diode array detection-quadrupole time of flight mass spectrometry (UHPLC-DAD-QTOFMS). This indicated that many strains produced several compounds (**Figure 5**).


OHHL
HHL
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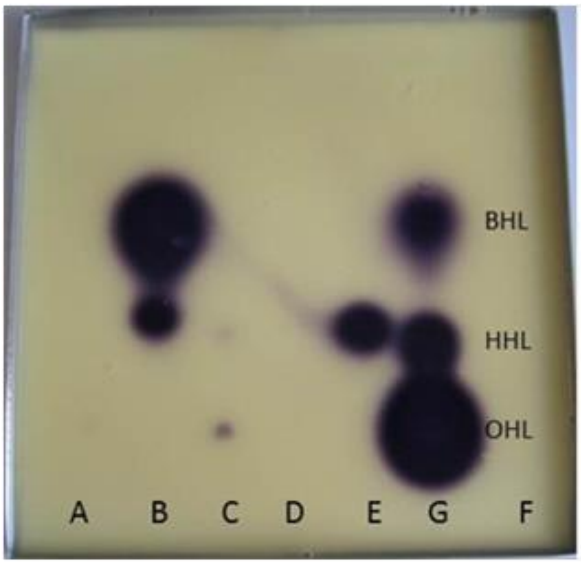


Figure 5. Separation of AHLs on TLC plates, *A. tumefaciens* TLC plate on the left and *C. violaceum* plate on the right. Six strains were extracted and tested, the first five (S-strains) from the global collection; the latter being *V. anguillarum* control Samples abbreviations: A=S0821, B=S0245, C=S4495, D=S1729, E=S3857, F=90.11.287, G=AHL standards.

Chemical profiling demonstrated that OH-C6 and OH-C10 were the most prevalent compounds being produced (jointly) by several strains (**Table 2**)

Table 2. Overview of the AHLs identified in 37 Vibrionaceae strains

				No of strains producing							
BHL	HHL	OHL	DHL	OH-C6	OH-C10	OH-C12	OH-C14	OHHL	OOHL	ODHL	OdDHL
5	4	2	1	15	7	5	1	1	1	10	2

These data are being further analysed and will be submitted as an article to Marine Drugs (Rasmussen *et al*., In Preparation). In light of the influence of C6-HSL described by **partner [12]**, the high prevalence of C6-HSL among Vibrionaceae may have a significant impact on the community dynamics where these producing isolates are present.

Partner [19] has taken the complementary approach of focusing on the effect of selective isolation media exploring biotic/abiotic factors for culturing bacteria (mainly actinobacteria)

associated with deep-sea sediments. Three independent expeditions have led to the collection of diverse banks of species which are currently undergoing analysis (**Table 3**).

Description of the three deep-sea expeditions:

1. IG2 in Cadiz Gulf and Gibraltar Strait (39 samples, -500m to -2600m) by dreges.
2. MUR in Costa Rica by submarine (24 samples, -80 to -383m) by submarine.
3. IG4 in Canary Islands (6 samples, -4.600 to -4.900m) by dreges (IN PROCESS)

Table 3. Number of strains isolated from difference media and expeditions.

Expedition	Isolation media	#Petri dishes	#Unique strain isolated	Unique strains/plates
IG2	BEN	195	431	2,21
IG2	Induction_1	195	451	2,31
IG2	Induction_2	195	330	1,69
MUR	BEN	120	135	1,13
MUR	Induction_1	120	96	0,80
MUR	Induction_3	120	187	1,56
IG4(*)	BEN	49	14	0,29
IG4	Induction_2	49	16	0,33
IG4	Induction_4	49	14	0,29

(*)IG4 without nalidixic in all cases

Some "rare" strains, belonging to *Kitasatospora* and *Georgenia* deep-sea actinobacteria have been isolated using addition of humic acid and vitamins group B (Induction_2) to a standard isolation medium (BEN) composed of glycerol, L-Asn and marine salts. Changing the medium to Induction_2 isolation medium, BEN complemented with SiO2, trace elements, vitamins B and L-Trp+ L-Tyr led to the isolation of *Pseudonocardia, Micrococcus, Isoptericola and Streptosporangium* species. The majority of other deep-sea actinobacteria genera have been isolated using two or more media compositions and they do not appear to depend on special growth factors. The conclusions arising from this work are that Induction_1 composition is the best medium to isolate strains with low homologies (<98,5%) respect to the control (BEN).

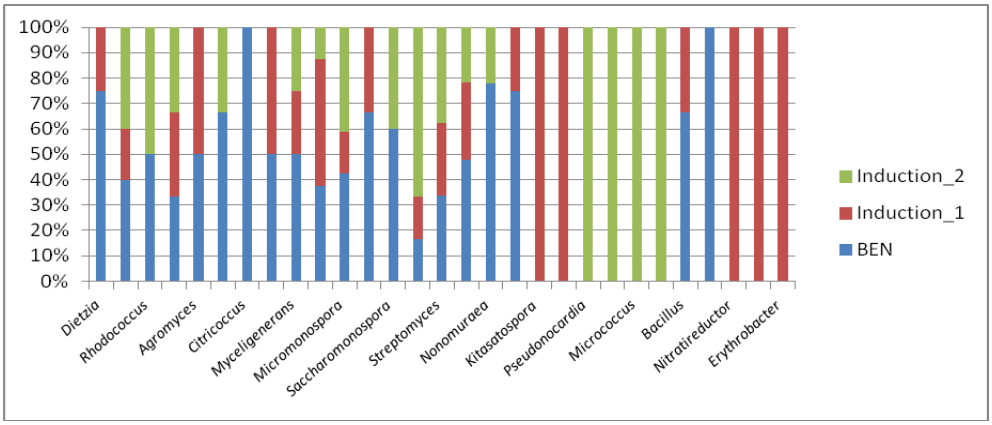
Table 4. 16S rRNA analysis of marine isolates

Expedition	Isolation media	# strains	# Genera	# Species	# Possible new species*
IG2	BEN	397	20	184	19 (10%)
IG2	Induction_1	308	19	153	28 (18%)
IG2	Induction_2	412	16	175	18 (10%)
MUR	BEN	100	9	54	2 (4%)
MUR	Induction_1	103	6	50	5 (10%)

MUR	Induction_3	143	9	66	4 (6%)
IG4	BEN	11 (**)	7	8	0
IG4	Induction_2	14 (**)	9	11	0
IG4	Induction_4	12 (**)	7	8	0

(**) In process, not finished.

(A)



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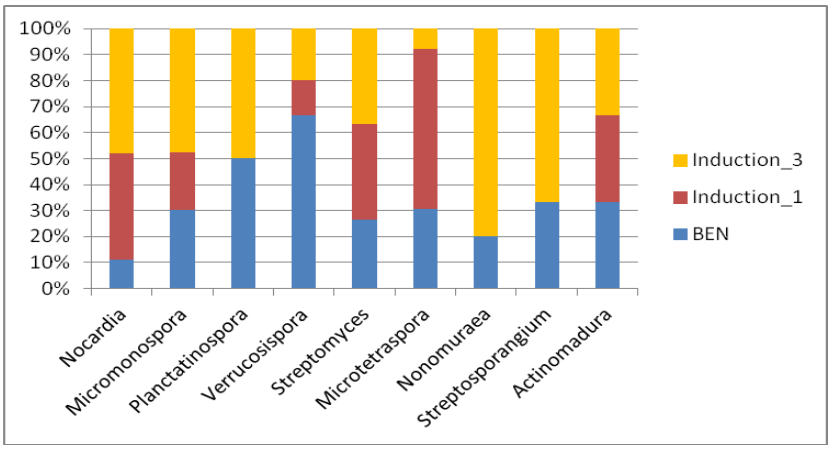

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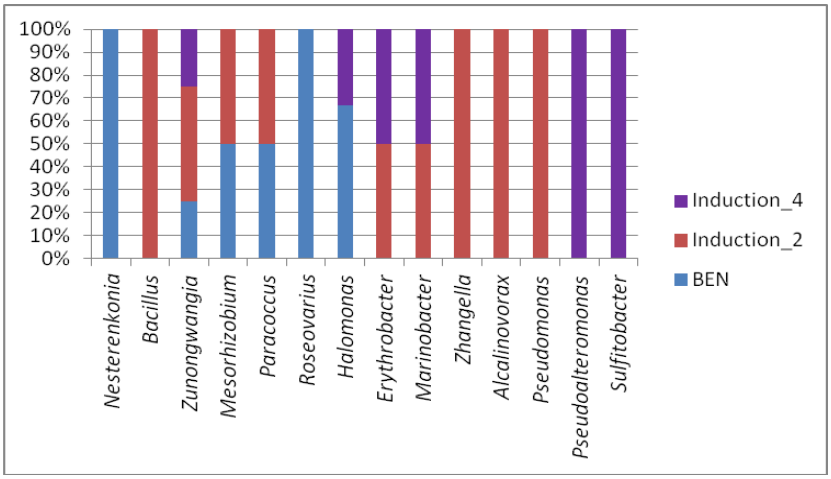

Figure 6. Distribution of genera of deep-sea bacteria (mainly actinomycetes) according to the induction isolation media. (A) Expedition **IG2** by Instituto Geológico Minero de España, IGME. (Cádiz and Gibraltar Strait, -500 -2.600m); **(B)** Expedition **MUR** by PharmaMar (Costa Rica, by submarine -80 -383m); **(C)** Expedition **IG4** by Instituto Geológico Minero de España, IGME. (Canary Islands, -4.500 -5.000m).

In general, the abundance of deep-sea actinobacteria depends on the depth or the habitat. For instance, a very low abundance of actibacteria have been isolated from ultradeep-sea sediments (5 km below surface!), from where only strains belonging to Nesterenkonia have been isolated. Independent of the growth factors used into the isolation medium, the abundance of *Micromonospora/Streptomyces* was found to be higher than habitual in the sediments collected in Costa Rica.

References

MaCuMBA publication; names of MaCuMBA principal investigators in boldface letters

Gram, L., Melchiorsen, J. and J.B. Bruhn. (2010). Antibacterial Activity of Marine Culturable Bacteria Collected from a Global Sampling of Ocean Surface Waters and Surface Swabs of Marine Organisms. *Marine Biotechnology*. **12**(4): 439-451.

Rasmussen, B.B., K.F. Nielsen, H. Machado, **L. Gram** and E.C. Sonnenschein 2014. Global and phylogenetic distribution of quorum sensing signals, acyl homoserine lactones, in the family of Vibrionaceae. (In preparation for submission to Marine Drugs).