"Parthenope" University of Naples

International PhD Course in

"Environment, Resources, and Sustainable Development"

Department of Environmental Sciences (DiSAm)

Joint PhD with

Hasselt University

universiteit hasselt

The role of Quorum Sensing in marine bacteria, *Archaea* and inter-kingdom communication

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XXV Cycle, Year 2013

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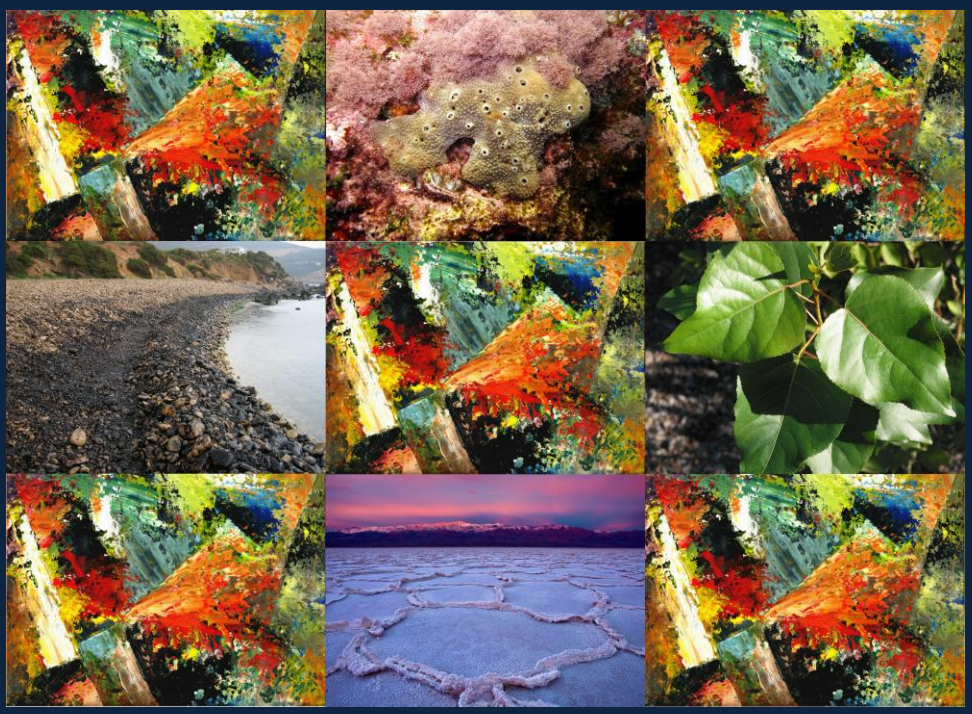

Gennaro Roberto Abbamondi

PhD Thesis

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Chapter 1

Introduction

INTRODUCTION

Quorum Sensing (QS) links the concentration of signal molecules to the expression of multiple genes in many diverse bacteria. The most well characterised are the *N*-acyl homoserine lactone signal molecules (AHLs) utilised by many Proteobacteria, however several other chemical compounds are employed as QS signal molecules by Gram negative bacteria including 2-alkyl-4-quinolones (AQs), long-chain fatty acids and fatty acid methyl esters and also furanone compounds derived from dihydroxypentanedione (DPD). Gram positive bacteria tend to use linear, modified or cyclic peptide molecules [Williams P. 2007]. These compounds regulate bacterial gene expression in response to external stimuli, mediating the adaptation of bacterial communities to changing environmental conditions and nutrient resources. Signal molecules may not only be utilised by producer-populations, but may also be detected by neighbouring bacteria of differing species, a process called 'cross-talk'. For example, using Gfp-based bioreporter strains, Steidle et al. were able to directly visualise AHL communication between bacterial populations in the rhizosphere of axenically grown tomato plants [Steidle A. et al. 2001]. Similarly, AHLs produced by *Pseudomonas aeruginosa* can activate siderophore, lipase and protease activity within *Burkholderia* [Riedel K. et al. 2001]. Other compounds, such as the diketopiperazine compounds produced by *P. aeruginosa*, can also activate AHL-mediated QS. Holden et al. demonstrated that DKPs antagonized the 3-oxo-C6-HSL-mediated induction of bioluminescence, suggesting that these compounds could compete for the same LuxR-binding site [Holden M.T.G. et al. 1999]. Conversely, AHLs have also been shown to inhibit the signalling system of Gram positive bacteria [Qazi S. et al. 2006].

Consequently, signal molecules potentially serve as a means of communication between cells of different species, for co-ordination of community behaviour or for the inhibition of competing species.

This research focused on four different models:

- ***Bacteria-sponge association***
- ***Archaea***
- ***Bacteria-plant association***
- ***Consortium of diesel-degrading strains***

→ ***Bacteria involved in bioremediation processes***

Bacteria-sponge association

The bactericidal property of seawater was recognize about 50 years ago, and it was suspected that this property was due to the production of antibiotics by phytoplanktonic algae [Steemann-Nielsen E. 1955] and/or bacteria [Baslow M.H. 1969].

Despite these early observation, relatively little attention has been directed towards the study of natural products from marine microorganisms, which are difficult to isolate and cultivate, because only a small percentage of bacterial cells in marine samples ultimately grow under standard culture conditions. Marine microorganisms are uniquely adapted to marine environment and they require salt, and specific media for growth. Up to day result that few marine microorganisms have been the subject of comprehensive chemical study

[Egan S. et al. 2008]. The surfaces and internal spaces of marine sponges are a unique microhabitat in which microorganisms are regularly observed. These environments are more nutrient rich than seawater and most sediments, thus they would likely be a unique niche for the isolation of diverse bacteria and fungi. In addition to the realization of high microbial diversity in sponges, there is also the characterization of an intriguing and specific distribution "host-symbiont" of these bacterial population [Taylor M.W. et al. 2004b].

It appears that a given species of sponge contains a mixture of generalist and specialist microorganisms and that the associated microbial communities are fairly stable in both space and time. For example, there is a widespread existence of sponge-specific bacterial clusters which are found only in sponges [Vacelet J. and Donadey C. 1977]. Based on electron microscopy and bacterial cultivation studies, these researchers proposed three types of microbial associates in sponges:

° Abundant population of sponge-specific microbes in the sponge mesohyl;

° Small population of specific bacteria occurring intracellularly;

° Population of nonspecific bacteria resembling those in the surrounding seawater.

One type of bacterial isolate, regarded as single species, was recovered from 35 taxonomically diverse sponges from several geographic regions, but never from seawater. Immunological experiments showed that these same isolates cross-reacted with other "sponge-specific" bacteria but not with seawater isolates [Wilkinson C.R. 1984]. These experiments were taken as further evidence of sponge specificity.

Generally, little is known about the mechanisms that regulate the association and communication between sponges and associated microorganisms. It has been

demonstrated that multispecies bacterial communities can communicate with each other via small secreted molecules, such as N-acyl homoserine lactones (AHLs) and diketopiperazines (DKPs), in a process called quorum sensing (QS) [Shiner E.K. et al. 2005; Tommonaro G. et al. 2012].

The production of AHLs by bacteria associate with marine sponges was already reported [Taylor M.W. et al. 2004a]. There are also recent reports about the role that these signal-molecules could have in the producing strain.

Recently, researchers performed a detailed analysis of AHL quorum sensing in sponge-associated bacteria, in particular *Ruegeria* sp. KLH11, isolated from the marine sponge *Mycale laxissima*.

Flagellar biosynthesis resulted under strict SsaRI (luxR and luxI homologues) control, and active motility was expressed only at high culture density. SsaI produced predominantly long-chain 3-oxo-AHLs.

An SsaI mutant was generated, with complete loss of AHLs production. Addition of 2 μM 3-oxo-C16:1 Δ11-HSL into swim agar could partially restore motility in the SsaI mutant, therefore swarming ability resulted regulated by long-chain 3-oxo-AHLs [Zan J. et al. 2012].

Several DKPs were isolated from an α-*Proteobacterium* of the genus *Ruegeria* associated with marine sponge *Suberites domuncula* and from a strain of genus *Staphylococcus* and *Bacillus* associated with *Ircinia variabilis*, and it was supposed a role of these cyclic dipeptides in quorum sensing mechanism that could regulate bacterial-sponge interaction [Mitova M. et al. 2004; De Rosa S. et al. 2003].

Marine microorganisms are of considerable current interest as a new and promising source of biologically active compounds [Penesyan A. et al. 2010]. They produce a variety of active metabolites with potential application in medicine and in the field of crop protection [Pietra F. 1997]. It is also shown that some bioactive compounds isolated from marine invertebrates originate from symbiotic microorganisms, e.g. swinholide [Piel J. 2006], okadaic acid [Dickey R.W. et al. 1990], chlorocarolides [Abrell L.M. et al. 1996].

It is not known enough about the specific nutrients and growth factors for cultivation of marine microorganisms. Really, it is estimated that less than 0.1% of bacteria observed by microscopic analysis in marine samples could be cultured using standard media [Chandramohan D. 2005]. This fact restrict our ability to isolate and culture the majority of the new microorganisms present.

In this PhD thesis is reported the isolation and identification of four bacterial strains associated with marine sponges. A screening of signalling molecules was performed in order to detect active compounds involved in quorum sensing mechanism, that could regulate the interaction between microbial community and its host (sponge). Furthermore it is reported that cyclic dipeptides produced by two marine strains in different bacteria-sponge association models, *Vibrio sp.* associated with marine sponge *Dysidea avara* and *Bacillus* sp. associated with marine sponge *Ircinia muscarum*, could be involved in quorum sensing mechanism.

Archaea

The *Archaea* are a phylogenetic separate group of microorganisms, differing from eubacteria and eukaryotes in genetic and molecular aspects. These microorganisms often

inhabit harsh environments, such as hot springs and salt lakes, given rise to the term "extremophiles". The physiology and the adaptation mechanisms *Archaea* use to survive in these extreme conditions has attracted many studies [Averhoff B. et al. 2010, Oren A. et al. 2008], both for their biotechnological applications and for their lifestyle.

Despite the large number of studies on QS, very few papers have addressed QS communication in *Archaea*.

Only one study has reported the detection of autoinducer molecules, most likely belonging to the AHL class in the haloalkaliphilic archaeon *Natronococcus occultus*. They also demonstrated correlation between these molecules and the production/activation of an extracellular protease produced by cells of *N. occultus* [Paggi R.A. et al. 2003].

Then, AHLs-based quorum sensing system was detected in a methanogenic archaeon; this suggests the existence of a possible universal mechanism of communication among prokaryotes [Zhang G. et al. 2012].

Halophilic bacteria are a group of microorganisms that need of an high salt concentration for growth. Bacterial cells respond to high salinity by structural changes in cell walls and in cellular protein composition, and in the accumulation in the cytoplasm of small molecules (osmolytes) to preserve osmotic balance [Oren A. et al. 2005, Roberts M.F. 2005]. This behaviour could be due to the activation of the different pathways depending on salt concentrations and this in turn might lead to a modulation of gene expression and protein activation [Steil L. et al. 2003, Weber A. et al. 2002]. For example, the Gram positive soil bacterium *Bacillus subtilis* evolved a physiological adaption reaction, by induction and repression of different genes, when exposed in hypersaline habitat [Steil L. et al. 2003].

The halophilic bacterium *Halobacillus halophilus* carries a homologue of LuxS, a protein involved in the production of autoinducer-2, which mediates quorum-sensing mechanism in some bacteria. Expression of *luxS* was growth phase dependent, and its transcription was strictly salt dependent. Western blot analyses showed a growth phase- and salinity-dependent production of LuxS. Then, cellular LuxS levels were strictly chloride dependent [Sewald X. et al. 2007].

According to the 16S rRNA classification, halophilic archaea (together with hyperthermophiles) belong to Euryarchaeota kingdom, wich is part of *Archaea* domain [Fendrihan S. et al. 2006].

Halophilic archaea have special properties which are not (or only rarely) present in other organisms; in particular, glycolipids and phosphatidyl glycerol sulfate (PGS) are the taxonomic markers of these microorganisms (polar lipids derive from sn 2, 3 substituted glycerol) [Chakravorty D. et al. 2012].

The aim of this PhD thesis has been to screen for the production of signal molecules in the extreme halophile archaeon *Haloterrigena hispanica*.

Halobacteriaceae are the prevalent microorganisms in extreme habitats such as saline and hypersaline environments [Averhoff B. et al. 2010, Oren A. 2008].

H. hispanica was isolated from Fuente de Piedra saline lake, Spain. This strain, designated FP1^T, was a pleomorphic coccoid and required for growth at least 15% (w/v) NaCl, pH 7.0 and optimal temperature of 50 °C [Romano I. et al. 2007]. While supernatant extracts of *H. hispanica* activated AHL bioreporters, the molecule responsible was found to be a diketopiperazine, cyclo-[(L-prolyl–L-valine)], not an AHL signal molecule.

Consortium of diesel degrading strains

Bioremediation is the term used to describe purification processes of different kind of polluted environments (marine, soil, aqueous environments, groundwater, etc.) by means biotechnological systems.

Pollutants can be divided in different class such as pesticides, heavy metals, waste matters, fertilizers and radioactive compounds. The isolation of strains from samples collected from contaminated sites is an important source of microorganisms with promising degrading ability, that could contribute to bioremediation systems.

For example actinomycetes isolated from an illegal storage of organochlorine pesticide in Argentina, showed capacity to growth, remove and use different organochlorine pesticide [Fuentesa M.S. et al. 2010].

Two microbial consortia isolated by selective enrichment from a soil sample of a nitroaromatic-contaminated site, resulted able to degrade 2,4-DNT as their sole nitrogen source [Snellinx et al. 2003].

Bacteria exhibiting multiple heavy-metal resistance characteristics were isolated from the Zn hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria* grown in a soil collected from an abandoned Zn-Pb mine and smelter in Plombières, Belgium [Lodewyckx C. et al. 2002].

The microbiological approach to remediate polluted environments involves in fact supplying microorganisms able to degrade target compounds.

This strategy can include the selection of bacteria-plant associations. For example it is well known that poplars have a natural ability to sequester heavy metals in their biomass; thus planting poplar trees in polluted sites is useful to extract these substances from the soil (phytoremediation).

This bioremediation system can be improved by the inoculum of bacteria which can produce siderophores and/or organic acids, that can enhance the bioavailability of heavy metals in the rhizosphere (contribution to root-to-shoot translocation).

Although the availability of microorganisms with these abilities, inoculation is one of the crucial steps of a bioremediation system. Soil indigenous predators and competitors were both involved in the regulation of the inoculated strain population density. Competitiveness and survival of bacteria in soil and rhizosphere may be influenced by the expression of traits controlled by quorum sensing.

For this reason it is interesting to better investigate communication mechanisms of pollutants-degrading bacteria, in order to solve problems correlated to inoculation step.

Petroleum hydrocarbons are ones of the most common global environmental pollutants. Because of the increasing global energy demand (both for industry and daily life), diesel fuel, crude oil and petroleum distillates have to be stored, transported, or transferred, with the potential risk of accidents and leakages.

The use of Hydrocarbon-degrading microorganisms is one of the most investigated strategy to remediate areas contaminated with petroleum pollutants, since it is cost-effective and will lead to complete mineralization [Holliger C. et al. 1997, Ganesh A. and Lin J. 2009].

Actually there are two main approaches which can be considered in this specific bioremediation technology [Das N. and Chandran P. 2011]:

- Bioaugmentation: oil degrading bacteria are inoculated in addition to the existing microbial population.

- Biostimulation: the polluted site is supplemented with nutrients or other growth-limiting cosubstrates, in order to stimulate the growth of indigenous oil degraders.

The model analyzed in this research is a consortium of bacteria with the metabolic capability of degrading a particular product of petroleum industry: diesel fuel.

Diesel fuel is a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from the middle-distillate, gas-oil fraction during petroleum separation.

One of the limiting factors in diesel-degrading process is the bioavailability of the oil. A strategy adopted by bacteria to access water-insoluble carbon source is biosurfactant production. Bioemulsifiers increase the surface area of this oil substrates (contact between bacteria and water-insoluble hydrocarbons) and consequently their bioavailability enhance [Ganesh A. and Lin J. 2009, Ron E.Z. and Rosenberg E. 2001 and 2002].

Another ability useful to contrast stress caused by environments contaminated with Petroleum is extracellular polymeric substances (EPS) synthesis. Actually, exposure to toxic chemicals (not only organic pollutants, but also heavy metals) could induce increased EPS production in bacterial cells [Kang Y. S. and Park W. 2010a].

Some of the activities regulated by QS mechanism are deeply implicated in hydrocarbon degrading capability; in particular motility, EPS synthesis and biofilm formation, that are known as crucial factors in the adherence to hydrocarbons and the effective degradation rates.

Recently, researchers deeply investigated diesel-degrading ability of *Acinetobacter* sp. strain DR1, but also explored the implication of quorum sensing in the activities that regulate Petroleum Hydrocarbon degradation.

They showed that biofilm formation in DR1 might depend on QS signals, and the formation of biofilms on hydrocarbons plays an important in the hexadecane-degradation abilities of DR1 [Kang Y.S. and Park W. 2010b].

In this research, it was performed a preliminary screening of signal molecules produced by a consortium of 15 diesel degrading strains, with the aim to improve the effectiveness of systems for remediation of soils and groundwater contaminated with Petroleum Hydrocarbons.

Bacteria-plant association

Bacteria-plant association has been extensively studied for different aspects and applications.

A clear distinction should be drawn between bacteria resident in the rhizosphere or phyllosphere and endophytes. Endophytic bacteria reside in specific tissues of the plant, such as root cortex or xylem.

These organisms develop a closer association with the plant, with mutual exchange of nutrients, enzymes (lipase, catalase, oxidase, etc.), functional agents (siderophores, biosurfactant, etc.), but also "signals".

Endophytes deeply colonize plant hosts tissues in which persist at high levels, without developing the negative effects of an infection (disruption of respiration, photosynthesis, translocation of nutrients, transpiration, and other aspects of growth and development).

On the contrary, endophytic bacteria permanence in the host plant produces beneficial effect on its health and/or growth.

We can also consider "beneficial health effects" a series of mechanisms that make possible the survival of the host in hostile environments, these include contaminated soils and groundwater, for instance allowing it to obtain nutrition from sources they were unable to feed from before, or to avoid the action of destructive chemicals, or to produce chemicals that protect them from pathogens.

Exposed for long periods to pollutants, some organisms are capable of genetically altering their ecotype with specific tolerances to specific contaminants, adapted through microevolutionary processes.

For example, plants living in man-made metalliferous habitats located in the surroundings of mines can develop tolerance to heavy metals such as cadmium, copper, lead, nickel, zinc, etc.

This particular ability could be employed in phytoremediation, a particular type of bioremediation in which polluted environments are treated by means plant-based clean-up technologies. It includes various applications: phytofiltration, phytostabilization, phytoextraction, and phytodegradation [Ahmadpour P. et al. 2012] :

- Phytofiltration or rhizofiltration is the remediation of waste water, surface water, or extracted ground water employing free floating or wetland plants.
- In phytostabilization approach, plant roots are used to stabilize and reduce the bioavailability of contaminants in the treated area.
- Phytovolatilization is a techinque used to extract volatile contaminants (Hg, Se, etc.) from the soil; pollutants are taken up into plant tissue and volatilized into the atmosphere.

- Phytodegradation or phytotransformation refers to the use of plants to uptake, metabolize, and degrade contaminants with the subsequent breakdown, mineralization, or metabolization by various metabolic processes.
- Phytoextraction is a particular type of bioremediation process which refers to the uptake of contaminants from soil or water by plant roots and their translocation to any harvestable plant part.

Poplars have a natural ability to adapt to different environments and to accumulate metals, thus different phytoremediation models make use of them. Different poplar clones have been selected for phytoremediation purposes, based on their strong metal tolerance and accumulation abilities [Di Lonardo S. et al. 2011].

Particularly interesting is the case of hyperaccumulator, term adopted to describe a number of plants able to accumulate extraordinarily high amounts of heavy metals in the aerial organs, far in excess of the levels found in the majority of species, without suffering phytotoxic effects [Rascio N. et al. 2011].

A recent analysis showed that *Nopalea cochenillifera* could be qualified as a potential Cr hyperaccumulator and therefore a promising candidate for phytoremediation purposes [Adki V.S. et al. 2013].

Bacteria can enhance host health, producing plant-growth regulator such as auxins, cytokinins and gibberellins, but also with the suppression of stress ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, nitrogen fixation and the mobilization of unavailable nutrients such as phosphorus and other mineral nutrients [Weyens N. et al. 2009].

Eight Cu-tolerant and ACC deaminase-producing endophytic bacteria were selected from one hundred isolates originated from a Cu mine wasteland located in Nanjing, China. The positive results concerning plant-growth promotion and improving uptake of copper, suggested a potential use of these strains in the design of phytoremediation systems of Cu-contaminated environments [Zhang Y.F. et al. 2011].

This research aim to investigate Quorum Sensing mechanism in bacteria involved in bioremediation processes, with special attention to inter-kingdom communication strategies.

The synthesis of some of mentioned growth regulators is under the control of quorum sensing, and some of them also act as signal molecules for some bacteria, regulating gene expression.

The indole-3-acedic acid (IAA), which belongs to the class of auxin, it's known to be a phytohormone, but can act as a reciprocal signaling molecule in microbe-plant interactions.

Recent reports indicate that IAA can also be a signaling molecule in microorganisms, in both IAA-producing and IAA-nonproducing species [Spaepen S. et al. 2007].

It has been shown that treatment with IAA induces changes in gene expression, enzymatic activity and levels of metabolites involved in central metabolic pathways in *Escherichia coli* [Bianco C. et al. 2006].

The existence of this mutual exchange of "information" between different organisms opens new chapters in the study of inter-species and especially inter-kingdom communication processes. Concerning environmental research, a better understanding of

these communication mechanisms, can help to improve systems for remediation of contaminated soils, based on bacteria-plant partnership.

Chapter 2

Methods

METHODS

1ST MODEL OF STUDY: *Bacteria-sponge association*

- ***Isolation of Strains.***

Samples of *Dysidea avara*, *Geodia cynodium* were collected in the bay of Naples at a depth of 20-25 m, and kept in fresh seawater during transport in the laboratory. Sponges were rinsed in sterile seawater twice, then transferred into new fresh sterile seawater, and the surface carefully removed. The mesohyl tissues were once more rinsed with sterile seawater and cut in small pieces. The pieces were inoculated in 20 mL Bactomarine (bactopeptone 5 grL^{-1}; yeast extract 1 grL^{-1} in artificial sea water) and in 20 mL Bactomarine supplemented with a pool of antibiotics (ampicillin 100 $\mu g\ mL^{-1}$, kanamycin monosulfate 100 $\mu g\ mL^{-1}$, tylosin tartrate 100 $\mu g\ mL^{-1}$, tetracycline 100 $\mu g\ mL^{-1}$, gentamicin sulfate 10 $\mu g\ mL^{-1}$). After 72 h of incubation at 20 °C, 100 µL aliquot of each culture was spread onto fresh solid Bactomarine. After 72 h incubation at 20 °C, single colonies were transferred on solid fresh medium.

- ***Identification of Strains.***

Isolated strains were identified by PCR analysis. Complete 16S sequences for each bacteria were obtained by amplification with the primers 9bfm (5' GAGTTTGATYHTGGCTCAG 3') and 1512uR (5' ACGGHTACCTTGTTACGACTT 3') (Mühling et al. 2008). A single colony was added to 50 µL sterile water, heated for 5 min at 100 °C and 1 µL used in a 50 µl PCR reaction containing 5X PCR buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U of GoTaq Flexi DNA polymerase (Promega) and 0.2 µM of each primer. Following an

initial denaturation at 95 °C for 4 min, 35 cycles of PCR were performed (94 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min) followed by a final extension step at 72 °C for 5 min. rRNA sequences were submitted to GenBank.

- ***Antibiotic Resistance Test.***

The isolated marine bacteria were exposed to the same pool of antibiotics, and at the same concentration. Briefly, bacteria were grown in 10 mL of Bactomarine supplemented with ampicillin, kanamycin monosulfate, tylosin tartrate, tetracycline (at concentration 100 μg mL^{-1}) and gentamicin sulfate (10 μg mL^{-1}) at T = 30 °C on rotary shaker overnight. The growth was monitored by O.D. measurement at λ = 540 nm.

- ***Isolation and Chemical Characterization of Diketopiperazines from Bacillus sp. and Vibrio sp.***

The isolation and chemical characterization of cyclic dipeptides were performed as previously described [De Rosa et al. 2003]. Briefly, the spent medium (10L) of *Vibrio* sp. and *Bacillus* sp. were extracted twice with dichloromethane. After dried in vacuum, the dichloromethane extracts were purified by Lobar RP-18 columns (water/methanol gradient). All recovered fractions were assayed for activation of quorum sensing by TLC-overlay test by using *Agrobacterium tumefaciens* NTL4 and CV026 bioreporters. The active fractions were characterized by NMR (^{1}H, ^{13}C) analysis with the aim to characterize chemically active compounds. ^{1}H- NMR spectra were recorded on a Bruker AMX-400 spectrometer in CD_3OD; ^{13}C spectra were recorded on a Bruker AMX-300 spectrometer in CD_3OD.

- ***Bioassays for Putative AHL Signal Molecules.***

Lux screen Assay. The presence of putative AHL(s) signal molecules was initially detected using the *Escherichia coli* lux-based biosensors pSB536, pSB401 and pSB1075 [Swift S. et al. 1997; Winson M.K. et al. 1998]. These allow a range of AHL detection from C4 (pSB536), C6–C8 (pSB401) and C10–C14 (pSB1075) acyl chain lengths signal molecules, thus covering a wide range of AHL signal molecules. Spent medium (10 L) from stationary phase cultures of *Vibrio* sp. was centrifuged at 10.000 rpm for 40 min. Pellets were stored at -20 °C for further investigation, while supernatants were extracted with dichloromethane (1:1 v/v; twice). Dichloromethane extracts were dried in vacuum at T < 40°C to avoid the alteration of metabolites and resuspended in acetonitrile. Aliquots of the supernatant extract were then applied to white/clear bottom 96-well microtitre plate (Corning), and 200 µL of overnight cultures of biosensors diluted 1/25 were added to each well. The microplates were incubated at 37°C and the luminescence and absorbance (620 nm) measured using a Berthold MITHRAS microplate reader after 3 h incubation for pSB536 and 6 h for pSB401 and pSB1075.

TLC-overlay. Supernatant extracts were also applied to C-18 RP-TLC plates (20 cm x 20 cm; VWR International) and a mobile phase of 60% (v/v) aqueous methanol used to separate the extracts. The TLC plates were overlaid with 100 mL of ATGN (Tempé *et al*. 1977) soft agar (0.6% w/v) supplemented with 0.5% glucose, 40 µg mL^{-1} X-Gal (Bromo-chloroindolyl beta-D- galactopyranoside), the antibiotics (streptomycin 50 µg mL^{-1}; tetracycline 4 µg mL^{-1}) and the biosensor *Agrobacterium tumefaciens* NTL4 (pCF218; pCF372) (Fuqua and Winans 1996). The NTL4 AHL bioreporter expresses a *lacZ* fusion in

response to medium-chain length AHLs. The TLC plates were kept in a sterile container and incubated at 30 °C for 24-48 h.

This test was performed as spot test on purified compounds. Different amounts (from 0.5 mg up to 2 mg) of isolated active diketopiperazines were applied to Silica gel TLC plates. Plates were overlaid with 100 mL of Luria Bertani (LB) medium soft agar supplemented with chloramphenicol 30 μg mL^{-1} and kanamicin 25 μg mL^{-1} and inoculated with bioreporter CV026. The TLC plates were kept in a sterile container and incubated at 30 °C for 24-48 h.

Plate T-streak. All strains were tested by T-streak assay with *Agrobacterium tumefaciens* NTL4 biosensor to detect the production of AHLs molecules. In this test, the tester strains are streaked on solid media near biosensor strain to form a "T". The appearance of blue colour in biosensor streak due the activation of AHL gene indicate the presence of exogenous AHLs produced by the test strain. Because marine bacteria did not grow on ATGN media (used for biosensor strain), it was utilized a new method to test the production of AHLs molecules. Plates with two solid medium were prepared, Bactomarine and ATGN, in way to fit together. Marine bacteria were streaked onto Bactomarine and *A. tumefaciens* NTL4 was inoculated into ATGN medium soft agar; than plates were incubated at 30 °C for 24-48 h. All strains were also tested by T-streak assay with *Chromobacterium violaceum* to detect inhibition or degrading activity of AHLs molecules. Both *C. violaceum* and marine bacteria were streaked on same plate with LB solid media to form a "T". An inhibition or degrading activity is detected by a visible negative gradient of violacein production.

2ND MODEL OF STUDY: *Archaea*

- ***Bacterial strain and growth media***

H. hispanica (type strain FP1^T DSM=18328 ^{T} ATCC=BAA-1310 ^{T}) was grown with shaking in 372 DSM medium containing the following components (per litre): 5.0 g yeast extract (Oxoid), 5.0 g Casamino acids (Oxoid), 3.0 g trisodium citrate (Applichem), 2.0 g KCl (Applichem), 20 g $MgSO_4.7H_2O$ (Carlo Erba), 200 g NaCl (Applichem), 0.36 mg $MnCl_2.4H_2O$ (J. T. Baker) and 0.05 g $FeSO_4.7H_2O$ (Carlo Erba). The pH of medium was 7.0 ± 0.2and the temperature of growth was 50 °C.

- ***Bioassays for putative AHL signal molecules***

Lux screen assay. The presence of putative AHL signal molecules was initially detected using the *Escherichia coli* lux-based biosensors pSB536, pSB401 and pSB1075 [Swift S. et al. 1997; Winson M.K. et al. 1998]. These allow a range of AHL detection from C4 (pSB536), C6–C8 (pSB401) and C10–C14 (pSB1075) acyl chain length signal molecules, thus covering a wide range of AHL signal molecules. Spent medium (10 L) from stationary phase cultures of *H. hispanica* was centrifuged at 10.000 rpm for 40 min. Pellets were stored at -20 °C for further investigation, while supernatants were extracted with dichloromethane (1:1 v/v; twice). Dichloromethane extracts were dried in vacuum at T < 40°C to avoid the alteration of metabolites and resuspended in acetonitrile. Aliquots of the supernatant extract were then applied to white/clear bottom microtitre plate wells (Corning), overnight cultures of biosensor diluted 1/25 and 200 µL added to each well. The microplates were incubated at 37°C and the luminescence and absorbance (620 nm) measured using a Berthold MITHRAS microplate reader after 3 h incubation for pSB536 and 6 h for pSB401 and pSB1075.

TLC-overlay. Supernatant extracts and standards (3-oxo-C6-HSL 10 µM and 3-oxo-C10-HSL 400 µM) were also applied to C-18 RP-TLC plates (20 cm × 20 cm; VWR International) and a mobile phase of 60% (v/v) aqueous methanol used to separate the extracts. The TLC plates were overlaid with 100 mL of ATGN [Tempé J. et al. 1977] soft agar (0.5% w/v) supplemented with 0.5% glucose, 40 µg mL^{-1} X-Gal (Bromo-chloroindolyl beta-D-galactopyranoside), the antibiotics (streptomycin 50 µg mL^{-1}; tetracycline 4 µg mL^{-1}) and the biosensor *Agrobacterium tumefaciens* NTL4 (pCF218; pCF372) [Fuqua C. and Winans S.C. 1996]. The NTL4 AHL bioreporter expresses a *lacZ* fusion in response to medium-chain length AHLs. The TLC plates were kept in a sterile container and incubated at 30 °C for 24-48 h.

Detection of signal-molecules by means Vibrio anguillarum mutant. Crude extract and purified compound were also tested by using *Vibrio anguillarum* strain (DM27/pDM42) that has an in frame deletion of the *vanM*-gene, so does not able to produce AHLs. It also contains an *luxR*-P_{luxI}-RBSII::*gfp*mut3*-T_0 insertion on the chromosome, then it respond to exogenous AHL source by expressing the green fluorescent protein (*gfp)* [Tait K. et al. 2005]. Crude extract and purified fraction were dissolved in 1 mL and 0,1 mL of acetonitrile, respectively, and 10 µL of each sample were used in the assay. Samples were added to the wells of black 96 well plates, evaporated to dryness and 200 µL of biosensor culture (*V. anguillarum*, diluted 1/10 from an overnight culture) were added. The standard used was C6-HSL at different concentration (form 15 nM up to 1000 nM). The plate was incubated for 6 hours then took a measurement of *gfp* ($\lambda_{excitation}$ 485 nm, $\lambda_{emission}$ 535 nm) and absorbance (OD 600nm) using a Berthold MITHRAS plate reader. The units were

expressed as *gfp*/OD $_{600}$ nm to take into account the density of the bacteria producing the *gfp* signal.

- ***Isolation and purification of diketopiperazines***

To identify the AHL bioreporter activating signal, the acetonitrile soluble part of *H. hispanica* supernatant extract was also chromatographed on a Lobar RP-18 column eluted with a gradient of H_2O/CH_3OH, starting from 9:1 up to CH_3OH (with steps of 100 mL); fractions of 100 mL were collected. All fractions were analyzed by TLC-overlay assay using the NTL4 AHL bioreporter. Fractions showing activity in the TLC-overlay bioassay, were further chromatographed on a Lobar RP-18 column (gradient of H_2O/CH_3OH, starting from 9:1 up to CH_3OH; each step of 100 ml; flow 2 mL/min, fractions of 5 mL were collected) and five compounds were isolated and chemically characterized by NMR analysis. (^1H, ^{13} C). ^1H- NMR spectra were recorded on a Bruker AMX-400 spectrometer in CD_3OD; ^{13}C spectra were recorded on a Bruker AMX-300 spectrometer in CD_3OD.

3RD MODEL OF STUDY: ***Consortium of diesel degrading strains***

- ***Selection of the consortium***

This research was performed in collaboration with Hasselt University, Diepenbeek, BELGIUM.

The isolates originate from the site of the Ford Motor Company in Genk (Belgium). 15 strains were selected from a pool of 380 based on their ability to degrade diesel fuel [Gkorezis P. et al. 2012].

- ***Extraction of cultural medium***

The 15 strains were grown with shaking (100 rpm) in Luria Bertani (LB) medium (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}) [Bertani G. 1952] for 48 hours, until stationary phase. The pH of medium was 7.0, and the temperature of growth was 30°C. Bacterial broths were centrifuged in 50 mL tubes at 4.000 rpm for 55 min, and supernatants were extracted twice with dichloromethane 1:2 (v/v).

Dichloromethane extracts were dried under vacuum at T < 40°C to avoid the alteration of metabolites.

- ***Bioassay for detection of QS molecules***

Lux screen assay. The presence of putative AHL signal molecules was initially detected using the *Escherichia coli* lux-based bioreporter pSB536, pSB401 and pSB1075, thus covering a wide range of AHL signal molecules [Swift S. et al. 1997; Winson M.K. et al. 1998].

The strain *E.coli* pSB536, grown in LB 0,5% NaCl [Lennox E.S. 1955] supplemented with ampicillin 50 μg mL^{-1}, was used to detect AHL with short chain (C4-HSLs); the strain *E. coli* pSB401, grown in LB 0,5% NaCl supplemented with tetracycline 10 μg mL^{-1}, was used to detect AHL with mid chain (C6-C8); the strain *E. coli* pSB1075, grown in LB 0,5% NaCl

supplemented with tetracyclin 10 µg mL^{-1}, was used to detect AHL with long chain (C10-C14).

30 mL of all bacterial broths were centrifuged in 50 mL tubes at 4.000 rpm for 55 min. pH of supernatant was adjusted to pH 2 with 1M HCl and incubate overnight at 37°C. Then samples were extracted as previously described. Extracts were solubilised in 350 µL acetonitrile, and aliquots of 50 µL were then applied to black 96-well microtitre plates. Plates were left to dry in fume hood 1 hour, then 200 µL of overnight cultures of bioreporters diluted 1/10 were added to each well. 2 µL of homoserine lactones standard solutions (20 µg mL^{-1}) were added to selected wells as control: C4-HSL for pSB536, C6-HSL for pSB401 and OC10-HSL for pSB1075. The microplates were incubated at 37°C and luminescence was measured using a microplate reader after 3 h incubation for pSB536 and 6 h for pSB401 and pSB1075.

TLC-overlay. Dichloromethane extracts were tested for the detection of QS molecules by using TLC-overlay test with *Agrobacterium tumefaciens* NTL4 bioreporter, which detects a broad range of AHLs and also displays the greatest sensitivity towards these compounds [Steindler L. et al. 2007].

200 mL of all bacterial broths were extracted using previously described technique. Aliquots of the extracts were dissolved in 50 µL of acetonitrile and loaded onto a C-18 RP-TLC plate. Thin-layer chromatography plates were developed in a 60% methanol/water mobile phase, dried, and overlaid with 100 mL of 0.6% ATGN (minimal salts supplemented with glucose) media supplemented with 40 µg mL^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and antibiotics (streptomycin 50 µg mL^{-1};

tetracycline 5 µg mL^{-1}) and inoculated with *A. tumefaciens* NTL4 bioreporter (grown overnight at 30°C). TLC plates were kept in a sterile container and incubated at 30 °C for 24-48 h. The appearance of light blue spot indicated activation of quorum-sensing mechanism.

Plate T-streak. All strains were tested by T-streak assay with CV026 as biosensor [Steindler L. et al. 2007]. In this test, the tester strains are streaked on LB 0,5% NaCl agar plates near biosensor strain to form a "T". A violet colour (violacein) at the interface between the two strains is an indication of signal molecules production.

- ***Evaluation of enzymatic and physiological activities of the consortium***

Swimming and swarming motility. Bacterial swimming motility was assessed on semi-solid (0,2% agar) nutrient agar plates (Peptone 5 g L^{-1}, Yeast extract 3 g L^{-1}, NaCl 5 g L^{-1}) [Kang Y. S. et al. 2010c].

The 15 strains were grown with shaking (100 rpm) in rich medium (D(+)glucose 1 g L^{-1}, Peptone 15 g L^{-1}, NaCl 6 g L^{-1}, Yeast extract 3 g L^{-1}) for 48 hours, until stationary phase. The pH of medium was 7.5 ± 0.2, and the temperature of growth was 30°C.

Bacterial broths were centrifuged at 4000 rpm for 20 min. Pellets were washed with PBS buffer and centrifuged again, then O.D. was normalized to 0.2 with the same buffer.

Then, 5 µL of each solution was spotted on semi-solid plates and incubated for 24h at 30°C.

The diameter (mm) of the bacterial zone was measured as the average of 5 replicates performed.

The swarming motility was evaluated under the same conditions, except for agar percentage (0,5%). Swarming motility was observed after 72h.

Oxidase activity. Oxidase activity was investigated adding 10 µL of 1% (w/v) N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride onto culture on a glass slide [Kang Y.S. et al. 2011]. The reaction is positive if color change to violet or purple within 20 sec.

Catalase activity. 50 µL of hydrogen peroxide 3% (v/v) solution was dropped onto culture on a glass slide. The production of bubbles in presence of hydrogen peroxide is an indication of catalase activity [Kang Y.S. et al. 2011].

Lipase activity. In order to assess lipase activity, agar plates composed of 2% Tween 80, 2,5% agar and 0,5% Methylred were prepared. One well per plate was cut (diameter ≈ 0,6 cm). Aliquots (20 µL) of cell free supernatant were added to the wells and plates were incubated overnight at room temperature. The ones that showed a clear zone around well were considered lipase producer. MilliQ water was used as negative control [Sriram M.I. et al. 2011].

- ***Selection of the strains***

A selection of the strains was performed, based on the evaluation of their diesel-degrading ability and the results of previous assays.

Therefore the most promising strains concerning their potential use in bioremediation systems and the most interesting strains concerning quorum sensing research were selected for further investigation.

- ***Antibiotic sensitivity test***

Susceptibility to antibiotics was evaluated by means Kirby-Bauer method [Bauer A.W. et al. 1966].

Cultures were grown with shaking (100 rpm) in LB medium (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}) for 48 hours, until stationary phase. The pH of medium was 7.0, and the temperature of growth was 30°C.

Amikacin (10 µg/disk), Bacitracin (10 µg/disk), Gentamycin (10 µg/disk) and Neomycin (30 µg/disk) were used as disk formula; Ampicillin (10 µg/disk), Ciprofloxacin (20 µg/disk), Chloroamphenicol (30 µg/disk), Erythromycin (15 µg/disk), Tetracyclin (10 µg/disk), Kanamycin (25 µg/disk) and Rifampicin (10 µg/disk) were prepared.

Then cultures were spread on Muller-Hinton agar plates (Beef Extract 2 g L^{-1}, Casein 17,5g L^{-1}, Starch 1,5 g L^{-1}, Agar 17 g L^{-1}; Final pH 7.3 ± 0.1, incubation at 25°C) using sterile cotton swabs [Sriram M.I. et al. 2011].

The antibiotic disks were then placed over the bacterial lawn and incubated overnight at 37°C.

After incubation, antibiotic sensitivity was shown by a zone of clearance around the well.

- ***Heavy metal tolerance assay***

Tolerance to Cd, Fe, Zn, Mn, Cu and Ni of selected strains was evaluated both on liquid (*Tube method*) and on solid (*Agar diffusion method*) medium.

Tube method. Strains were cultivated in M-284 medium + C_{mix} (Lactate 0,35 g L^{-1}, Glucose 0,52 g L^{-1}, Gluconate 0,66 g L^{-1}, Fructose 0,54 g L^{-1}, Succinate 0,81 g L^{-1}) until stationary phase (48h, 30°C, 100 rpm). Heavy metal salts were dissolved in phosphate saline buffer (pH=7) and prepared for 1 M, 500 mM, 100 mM, 50 mM and 10 mM concentrations.

Then 50 µL of corresponding metal salt solutions were added to each test tube (5 mL cultures), in order to reach a final concentration of 0.1 mM, 0.5 mM, 1 mM, 5 mM and 10 mM, and incubated at 30°C for 7 days.

After incubation, qualitative turbidity was measured as an indication of heavy metal resistance. The ones that showed growth were considered as resistant to the corresponding heavy metal and those that lacked growth were considered as sensitive or non resistant.

Agar diffusion method. Strains were cultivated in M-284 medium + C_{mix} until stationary phase (48h, 30°C, 100 rpm). The lawn culture was grown over M-284 + C_{mix} plates and then two wells per plate were added (diameter ≈ 0,6 cm; 2 cm from the border). Heavy metal salts were dissolved in phosphate saline buffer (pH=7) and prepared for 1 M, 500 mM, 200 mM, 50 mM and 10 mM concentrations.

Then 50 µL of corresponding metal salt solutions were added to each well and incubated for 7 days at 30°C. The areas of inhibition around the wells were measured.

4TH MODEL OF STUDY: *Bacteria plant association*

- ***Selection of samples***

Eight different samples were selected for the screening of molecules involved in QS mechanism (bacterial cultures, plant cultures, bacteria-plant cultures and sterile media): Hoagland's nutrient solution [Barac T. et al. 2004], 869 (rich) medium [Mergeay M. et al. 1985], Bacteria + plant with roots culture (in Hoagland's nutrient solution), Bacteria + plant cuttings culture (in Hoagland's nutrient solution), Bacteria inoculated in Hoagland's nutrient solution, Bacteria inoculated in 869 (rich) medium, Plant with roots culture (in Hoagland's nutrient solution), Plant cuttings culture (in Hoagland's nutrient solution).

The bacterial strain was *Pseudomonas putida* W619, a natural root endophyte of the poplar cultivar *Hoogvorst* (*Populus trichocarpa* x *Populus deltoides*) [Taghavi S. et al.2005].

The isolated strain was cultivated with another poplar cultivar, namely *Grimminge* [*P. deltoides* x (*P. trichocarpa* x *P. deltoides*)]. This cultivar is more resistant to poplar rust and it is very similar to *Hoogvorst* [Weyens N. et al. 2011].

- ***Extraction of cultural medium***

An aliquot of 200 mL of each culture was centrifuged in 50 mL tubes at 4.000 rpm for 55 min, and supernatants were extracted twice with dichloromethane 1:2 (v/v).

Dichloromethane extracts were dried under vacuum at T < 40°C to avoid the alteration of metabolites.

- ***Bioassays for Putative AHL Signal Molecules.***

Lux screen Assay. The presence of putative AHL signal molecules was initially detected using the *Escherichia coli* lux-based bioreporter pSB536, pSB401 and pSB1075, thus covering a wide range of AHL signal molecules [Swift S. et al. 1997; Winson M.K. et al. 1998].

The strain *E.coli* pSB536, grown in LB 0,5% NaCl supplemented with ampicillin 50 μg mL^{-1}, was used to detect AHL with short chain (C4-HSLs); the strain *E. coli* pSB401, grown in LB 0,5% NaCl supplemented with tetracycline 10 μg mL^{-1}, was used to detect AHL with mid chain (C6-C8); the strain *E. coli* pSB1075, grown in LB 0,5% NaCl supplemented with tetracyclin 10 μg mL^{-1}, was used to detect AHL with long chain (C10-C14).

200 mL of all bacterial broths were extracted as previously described. Extracts were solubilised in acetonitrile, and aliquots of 50 μL were then applied to white 96-well microtitre plates.

Plates were left to dry in fume hood 1 hour, then 200 μL of overnight cultures of bioreporters diluted 1/10 were added to each well. Specific homoserine lactones (sol. 20 μg mL^{-1}) were added to selected wells as control: 2 μL C4-HSL for pSB536, 4 μL C6-HSL for pSB401 and 2 μL OC12-HSL for pSB1075. The microplates were incubated at 37°C and luminescence was measured using an image viewer after 3 h incubation for pSB536 and 6 h for pSB401 and pSB1075.

TLC-overlay. Dichloromethane extracts were tested for the detection of QS molecules by using TLC-overlay test with *Agrobacterium tumefaciens* NTL4 bioreporter, which detects a broad range of AHLs and also displays the greatest sensitivity towards these compounds [Steindler L. et al. 2007].

Aliquots of supernatant extracts were also applied to C-18 RP-TLC plates (20 cm x 20 cm; MERCK) and a mobile phase of 60% (v/v) aqueous methanol used to separate the extracts. The TLC plates were overlaid with 100 mL of ATGN (minimal salts supplemented with glucose) [Tempé J. et al. 1977] soft agar (0.6% w/v) supplemented with 0.5% glucose, 40 µg mL^{-1} X-Gal (Bromo-chloroindolyl beta-D-galactopyranoside), the antibiotics (streptomycin 50 µg mL^{-1}; tetracycline 5 µg mL^{-1}) and the biosensor *Agrobacterium tumefaciens* NTL4 (pCF218; pCF372) [Fuqua C. and Winans S.C. 1996]. The NTL4 AHL bioreporter expresses a *lacZ* fusion in response to medium-chain length AHLs. The TLC plates were kept in a sterile container and incubated at 30 °C for 24-48 h. The appearance of light blue spot indicated activation of quorum-sensing mechanism.

Chapter 3

Results

RESULTS

1ST MODEL OF STUDY: *Bacteria-sponge association*

- ***Isolation and Identification of Strains***

Specimens of sponge *Dysidea avara* and *Geodia cynodium*, collected in the gulf of Naples, were used for the isolation of bacteria. Strains were identified by PCR analysis as belonging to genus *Vibrio* and *Pseudoateromonas* for those associated to *D. avara* and genus *Vibrio* and *Photobacterium* for those associated to *G. cynodium*. The strain belonging to genus *Bacillus* sp. associated with marine sponge *Ircinia variabilis*, has been previously isolated as described in De Rosa et al. 2003 [Fig. 1].

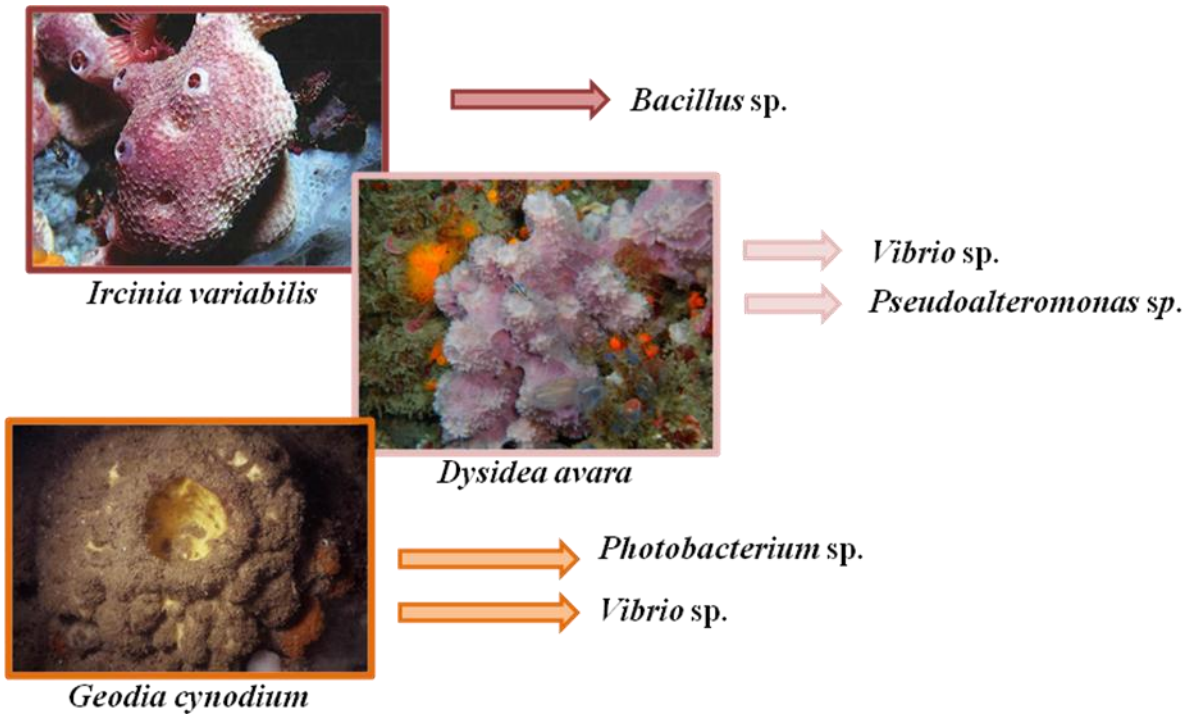


Fig. 1 Bacterial strains associated with marine sponges

Pseudoalteromonas sp. strain associated with *D. avara* was isolated from sponge previously treated with Bactomarine supplemented with a pool of antibiotic.

- ***Growth Condition and Antibiotic Resistance Test of Marine Bacteria***

All isolated strains were grown in Bactomarine medium at 30 °C on shaking overnight. The growth was monitored by O.D. measurement at 540 nm. After 24 h all bacteria reached logarithmic phase. Despite *Pseudoalteromonas* sp. was isolated by using Bactomarine supplemented with a pool of antibiotics, it was susceptible to all antibiotics used in the isolation step [Fig.2].

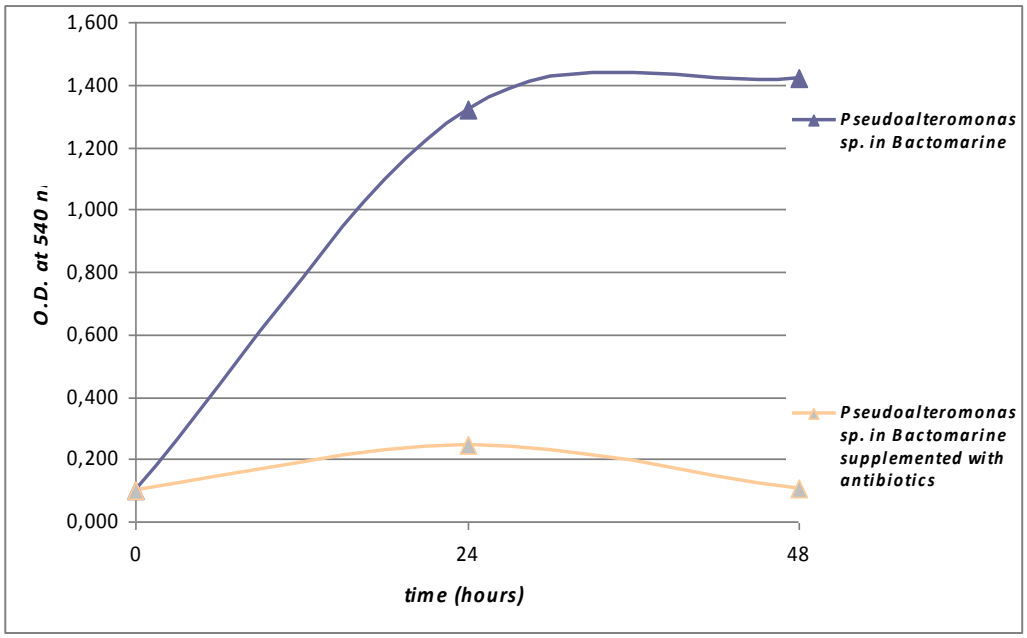


Fig. 2 Evaluation of *Pseudoalteromonas* sp. susceptibility to antibiotics

This could be an evidence that this bacteria had an intracellular localization in sponges so that it was not exposed to pool of antibiotics used in the isolation step.

- ***Isolation and Chemical Characterization of Diketopiperazine from Vibrio sp.***

To identify the AHL bioreporter activating signal, the dichloromethane extract (635 mg) of cell-free supernatant of *Vibrio* sp. was chromatographed on a Lobar RP-18 column eluted with a gradient of H_2O/CH_3OH, starting from 9:1 up to CH_3OH (with steps of 100 mL); fractions of 100 mL were collected. All fractions were analysed by TLC-overlay assay using the NTL4 bioreporter. Fraction (DAMBF5, 260 mg, eluted with H_2O/MeOH 6:4) showing activity in the TLC-overlay bioassay, was further chromatographed on a Lobar RP-18 column (gradient of H_2O/CH_3OH, starting from 9:1 up to CH_3OH; each step of 100 mL; flow 2 mL/min, fractions of 5 mL were collected) and four compounds were isolated and chemically characterized by NMR analysis. (^1H, ^{13} C). ^1H- NMR spectra were recorded on a Bruker AMX-400 spectrometer in CD_3OD; ^{13}C spectra were recorded on a Bruker AMX-300 spectrometer in CD_3OD.

The isolated compounds showed a typical spectrum of cyclic dipeptides and were identified as cyclo-(cis-4-hydroxy-D-prolyl-L-leucine), cyclo-(L-prolyl-L-leucine), cyclo-(L-prolyl-L-phenylalanine), cyclo-(trans-4-hydroxy-L-prolyl-L-phenylalanine) [Fig. 3].

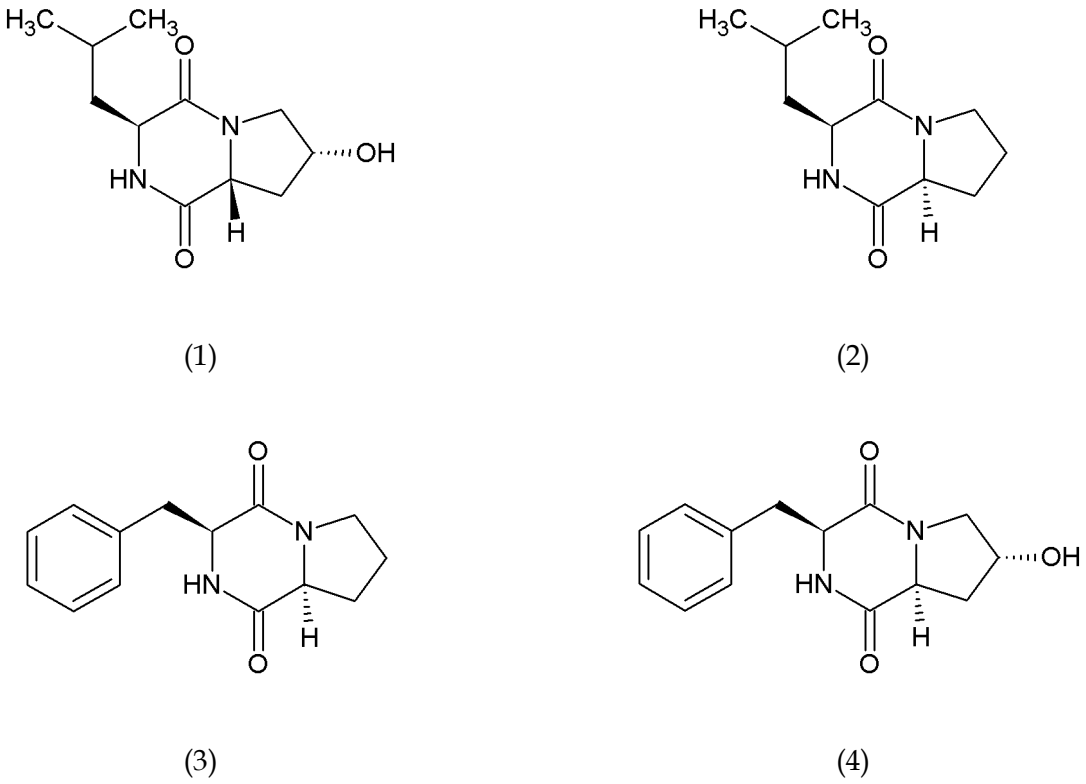


Fig. 3 Diketopiperazine isolated from *Vibrio* sp. (from *Dysidea avara*): **(1)** cyclo-(cis-4-hydroxy-D-prolyl-L-leucine) ; (**2**) cyclo-(L-prolyl-L-leucine) ; (**3**) cyclo-(L-prolyl-L-phenylalanine) ; (**4**) cyclo-(trans-4-hydroxy-L-prolyl-L-phenylalanine).

Spectroscopic data were in accordance with data present in literature [De Rosa et al. 2003, Degrassi G. et al. 2002]. (see *Supplementary Data*)

- ***Isolation and Chemical Characterization of Diketopiperazine from Bacillus sp.***

Dichloromethane extract (216,4 mg) of cell-free medium of *Bacillus* sp. was purified by Lobar RP-18 column eluted with a gradient of H_2O/CH_3OH, starting from 9:1 up to CH_3OH (with steps of 100 mL); fractions of 100 mL were collected.

Seven compounds were isolated by NMR analysis (^1H, ^{13}C), and identified as cyclo-(cis-4-hydroxy-D-prolyl-L-leucine), cyclo-(trans-4-hydroxy-L-prolyl-L-leucine), cyclo-(glycyl-L-

leucine), cyclo-(cis-4-hydroxy-D-prolyl-L-phenylalanine), cyclo-(trans-4-hydroxy-L-prolyl-L-phenylalanine), cyclo-(D-prolyl-L-tyrosine), cyclo-(L-prolyl-L-tyrosine) [Fig. 3].

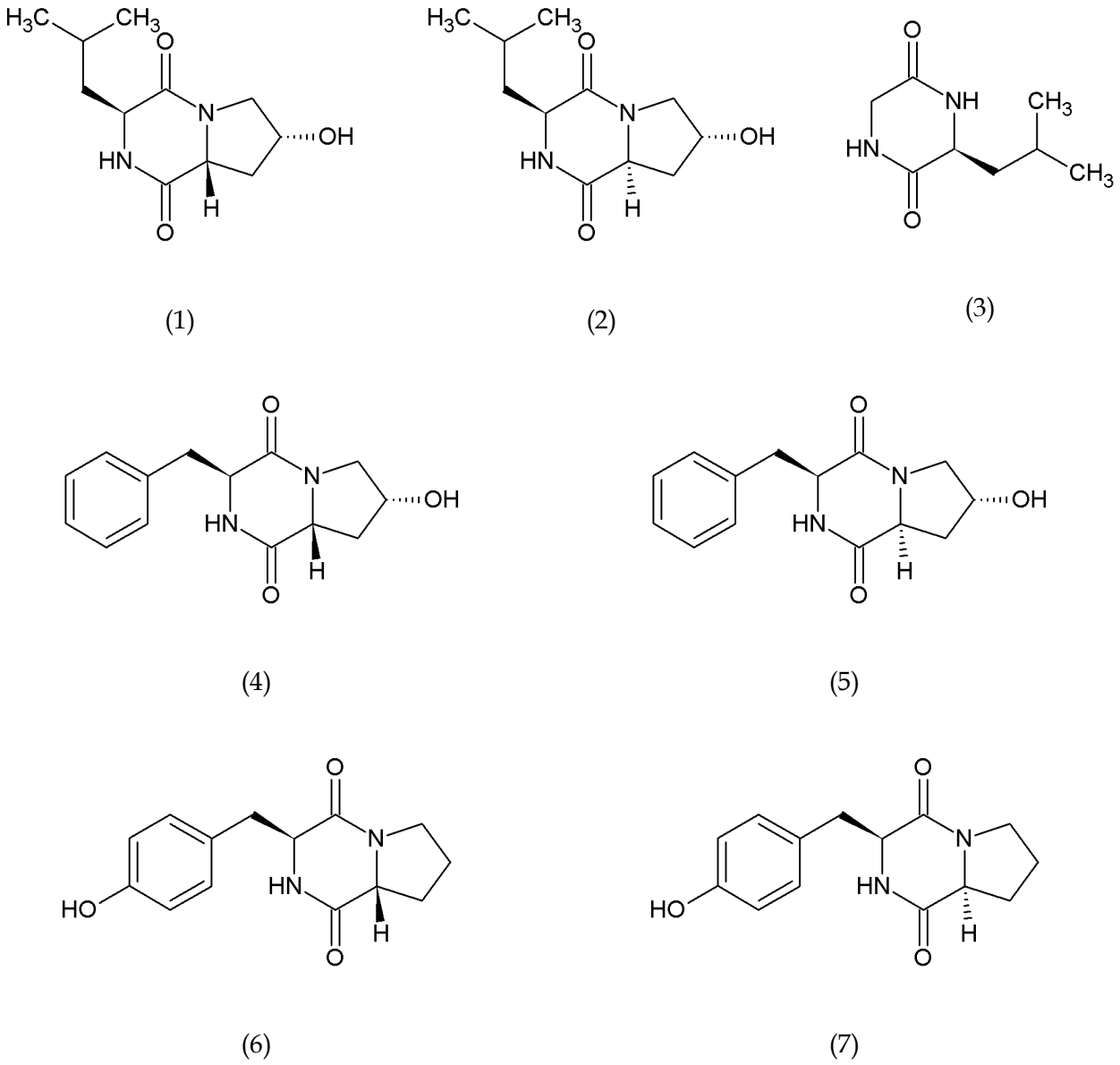


Fig. 3 Diketopiperazine isolated from *Bacillus* sp. (from *Ircinia variabilis*) (**1**) cyclo-(cis-4-hydroxy-D-prolyl-L-leucine), (**2**) cyclo-(trans-4-hydroxy-L-prolyl-L-leucine), (**3**) cyclo-(glycyl-L-leucine), (**4**) cyclo-(cis-4-hydroxy-D-prolyl-L-phenylalanine), (**5**) cyclo-(trans-4-hydroxy-L-prolyl-L-phenylalanine), (**6**) cyclo-(D-prolyl-L-tyrosine), (**7**) cyclo-(L-prolyl-L-tyrosine).

Spectroscopic data were in accordance with data present in literature [De Rosa et al. 2003].

(see *Supplementary Data*)

Among the seven diketopiperazines isolated, only cyclo-(cis-4-hydroxy-D-prolyl-L-leucine), isolated from the active fraction (BACF4, 66,7 mg, eluted with H_2O/MeOH 6:4) and already isolated [De Rosa S. et al. 2003], showed positive activity in bioassays for putative AHL signal molecules (activation of NTL4 bioreporter).

- ***AHLs Bioassays***

All isolated strains were tested for production of AHLs by means of "T" streak assay using *Chromobacterium violaceum* for QS inhibition and *Agrobacterium tumefaciens* NTL4 for QS activation. Dichloromethane extract of cell-free medium of *Vibrio* sp. isolated from *D. avara* showed activation of QS both in TLC overlay test, by using CV026 bioreporter, and Lux screen assay. Then the isolation of compounds was performed following the positive response in QS bioassay. Among purified cyclic dipeptides, just two of them showed activity in performed bioassay. In fact, cyclo-(L-prolyl-L-leucine) and cyclo-(L-prolyl-L-phenylalanine) did not activate NTL4 bioreporter in TLC overlay assay, but activated CV026 bioreporter starting at an amount of 0.5 mg [Fig. 4].

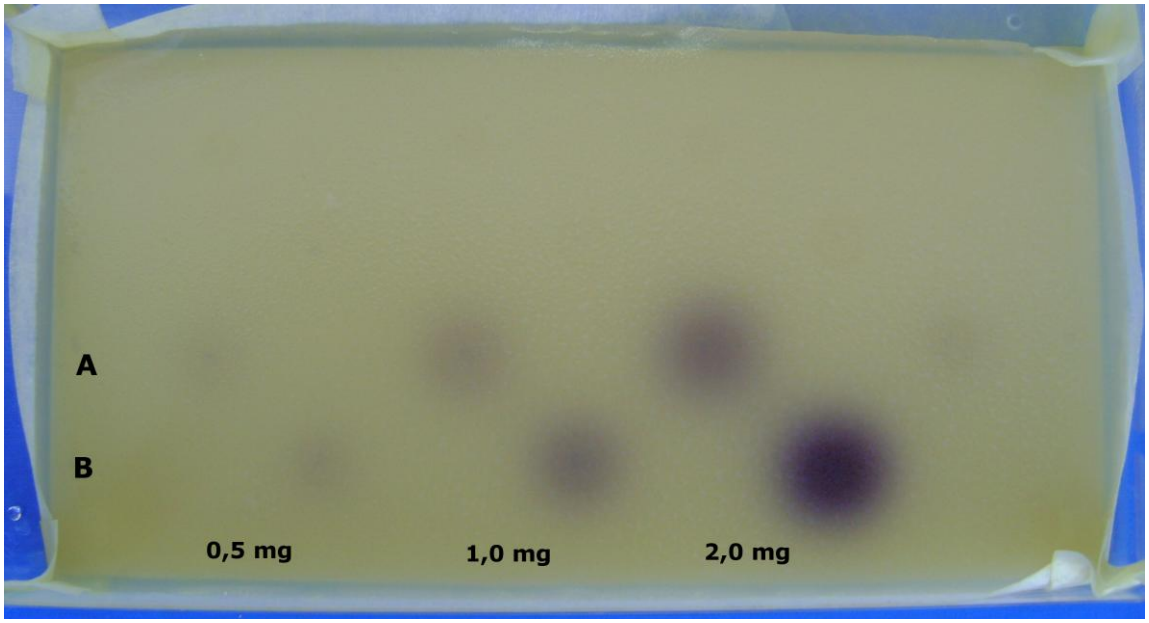


Fig. 4 TLC-overlay performed as spot test, biosensor CV026. Positive activities
[**A**] Cyclo-(L-prolyl-L-leucine): 0,5 mg [+/-], 1 mg [+], 2 mg [++]
[**B**] Cyclo-(L-prolyl-L-phenylalanine): 0,5 mg [+], 1 mg [++], 2 mg [+++]

Pseudoalteromonas sp isolated from *D. avara* showed inhibition of QS in "T" streak assay with *C. violaceum*, and a negative gradient of violacein production was observed, that is under control of AHL molecules [Fig. 5]. This means an inhibition or degrading activity of AHLs molecules by *Pseudolateromonas* sp.

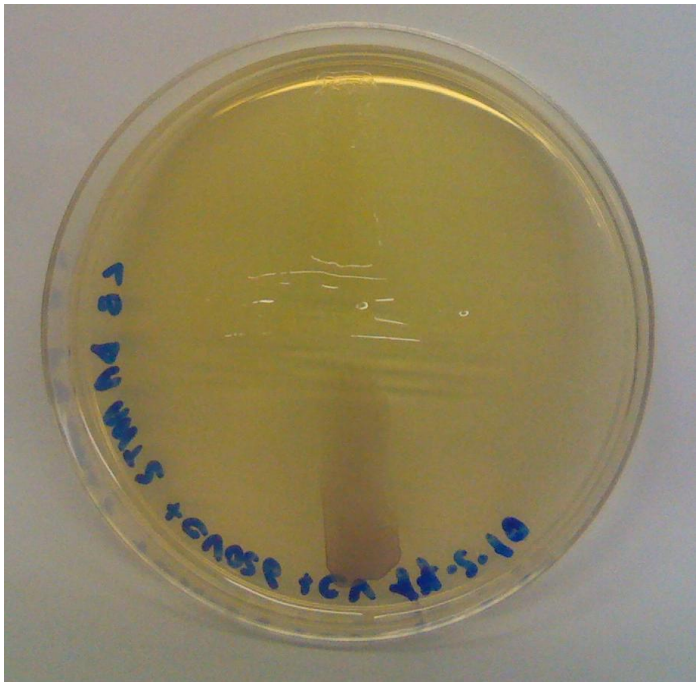


Fig. 5 T-streak assay; inhibition or degrading activity of AHLs molecules by *Pseudolateromonas* sp.

In De Rosa et al. 2003 it was supposed a role of cyclo-(cis-4-hydroxy-D-prolyl-L-leucine) in the quorum-sensing mechanism. Really the dichloromethane extract of culture of *Bacillus* sp. showed activation of QS both in TLC overlay test and Lux screen assay. Then the isolation of compounds was performed following the positive response in QS bioassay. The isolated cyclic dipeptide activated the QS mechanism in *A. tumefaciens* NTL4 bioreporter starting at a concentration of 0.5 mg.

The strains belonging to genus *Vibrio* showed positive response in TLC-overlay [Fig. 6] and in Lux screen assay [Tab. 1].

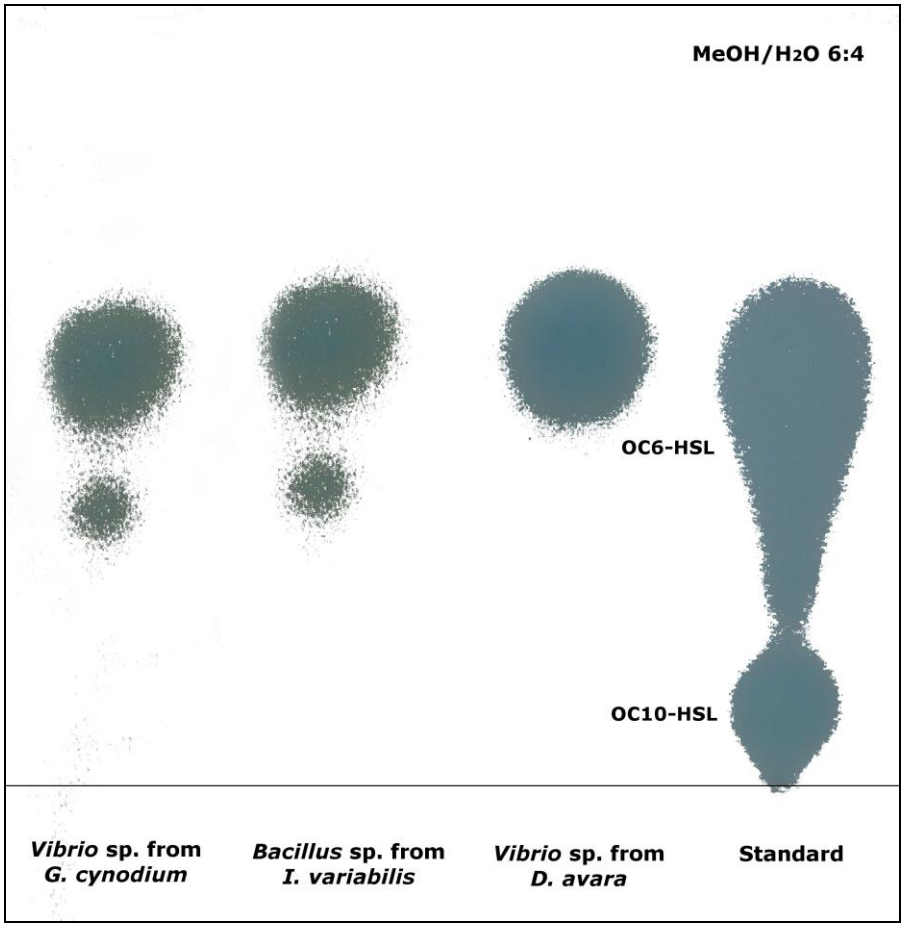
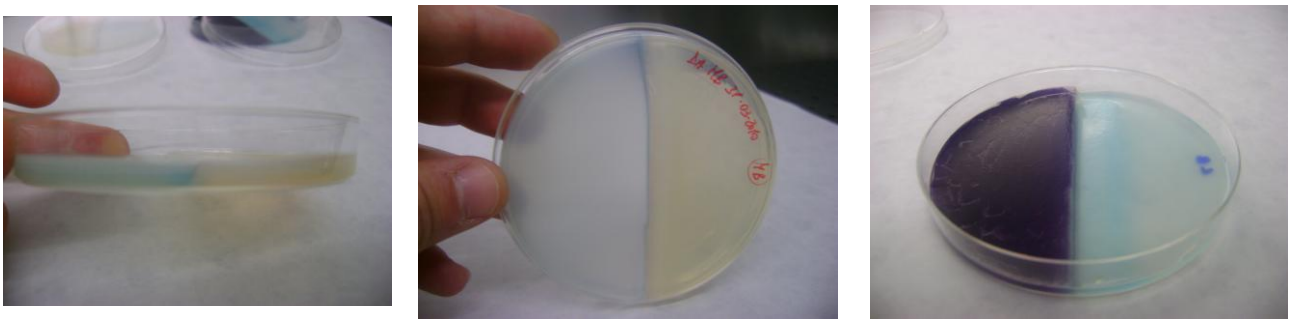


Fig. 6 TLC-overlay test (image reconstruction) performed on *Vibrio* sp. from *G. cynodium*, *Bacillus* sp. from *I. variabilis* and *Vibrio* sp. from *D. avara* DCM extracts.

Tab. 1 Lux - screen assay for AHL production. [a] AHL standard (C4-HSLs for pSB536; C6-HSLs for pSB401; C10-HSLs for pSB1075); [b] dichloromethane extracts of blank medium (without inoculum); [c] dichloromethane extracts of cultural media of marine strains.

	Bioreporters		
	pSB1075	**pSB401**	**pSB536**
AHL standard[a]	++	++	++
DCM Ex. blank medium[b]	-	-	-
DCM Ex. Vibrio sp. by D. avara[c]	-	+	-
DCM Ex. Pseudoalteromonas sp. by D. avara[c]	-	-	-
DCM Ex. Vibrio sp. by G. cynodium[c]	-	+	-
DCM Ex. Photobacterium sp. by G. Cynodium[c]	-	+	-
DCM Ex. Bacillus sp. by I. variabilis[c]	-	+	-

The positive response was also evident in the new "T" streak assay by using double medium. The appearance of a bleu strip in touch point of both media containing test and bioreporter strain (*A. tumefaciens* NTL4) was observed [Fig. 7].



(1) (2) (3)

Fig. 7 New "T" streak assay (double medium) results: (1) *Vibrio* sp. from *G. cynodium* ; (2) *Vibrio* sp. from *D. avara* ; (3) CV-NTL4 (control)

2ND *MODEL OF STUDY*: ***Archaea***

- ***AHLs Bioassays - Preliminary results***

H. hispanica supernatant activates AHL bioreporters. Preliminary results using the *E. coli lux*-based AHL bioreporters indicated that *H. hispanica* supernatant activated pSB401 (results not shown), and so the supernatant extract was further analysed using the *A. tumefaciens* NTL4 (pCF218; pCF372) AHL bioreporter [Fuqua C. and Winans S.C. 1996]. Two spots were present on the TLC, however, only one stronger spot was analysed further due to poor recovery of the weaker spot (results not shown).

- ***Isolation and chemical characterization of diketopiperazines***

To determine the nature of the signal activating the AHL bioreporters, the acetonitrile soluble part (393 mg) of dichloromethane extract (567 mg) of cell-free medium of *H. hispanica*, was repeatedly purified by Lobar RP-18 column to obtain pure compounds. The compounds were identified by means of spectroscopic data (NMR, EIMS), which were in accordance with data present in literature [De Rosa S. et al. 2003, Degrassi G. et al. 2002]. (see *Supplementary Data*)

While no AHL compounds were found, five diketopiperazines were isolated and structurally determined: cyclo-(D-prolyl-L-tyrosine) (**1**) (3.2 mg); cyclo-(L-prolyl-L-tyrosine) (3.3 mg) (**2**); cyclo-(L-prolyl–L-valine) (12 mg) (**3**); cyclo-(L-prolyl- L-phenylalanine) (1.7 mg) (**4**); cyclo-(L-prolyl-L-isoleucine) (7.1 mg) (**5**) [Fig. 8].

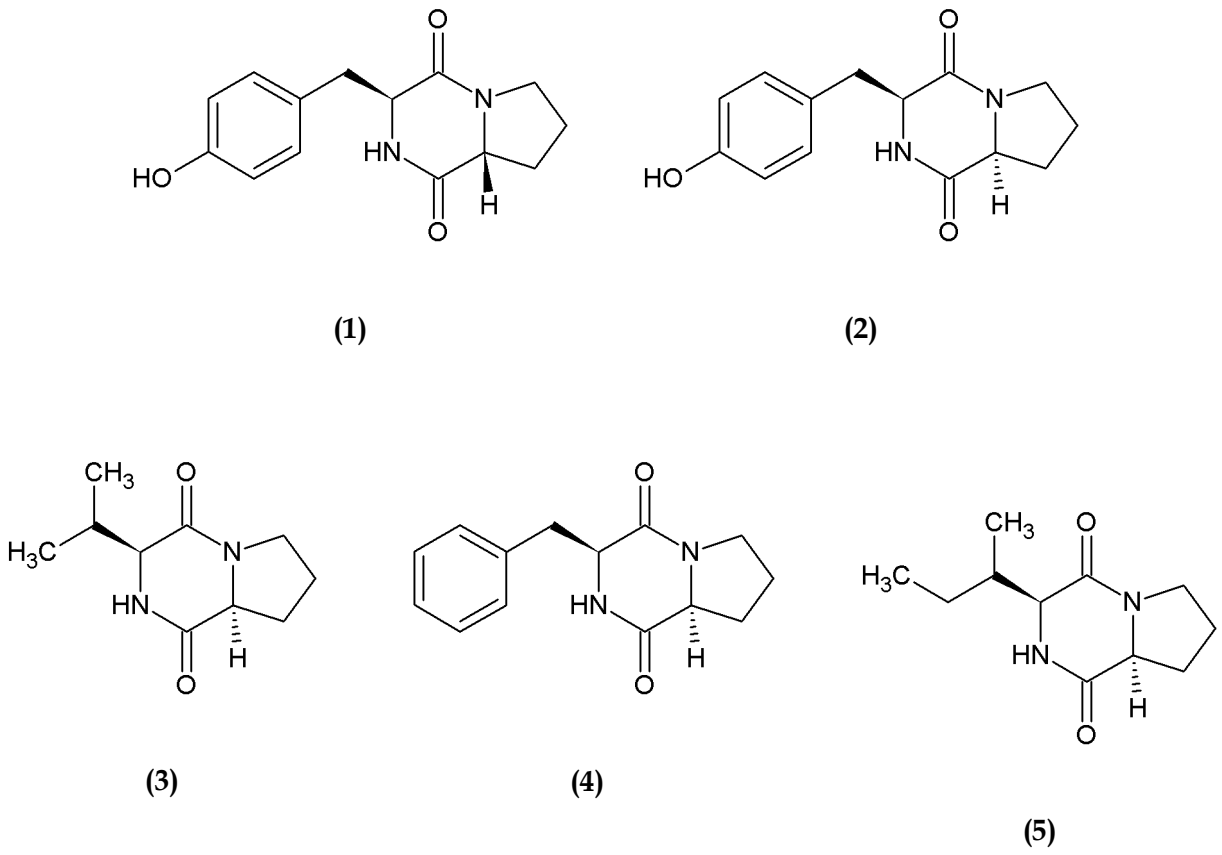


Fig. 8 Chemical structures of compounds (**1**) cyclo-(D-prolyl-L-tyrosine), (**2**) cyclo-(L-prolyl-L-tyrosine), (**3**) cyclo-(L-prolyl-L-valine), (**4**) cyclo-(Lprolyl-L-phenylalanine) and (**5**) cyclo-(L-prolyl-L-isoleucine)

Fractions obtained from chromatography were tested with the NTL4 AHL bioreporter. Fractions F4 (93 mg) eluted with H_2O/CH_3OH 7:3 (v/v), and F5 (121 mg) eluted with H_2O/CH_3OH 6:4 (v/v) obtained from the first column showed positive response in the TLC assay [Fig. 9].

Figure 9 TLC-overlay assay by using *A. tumefaciens* NTL4 biosensor. Fractions F4 and F5 recovered from purified dichloromethane extract of H. hispanica showed a positive response (light blue spots). Standards used were 3-oxo-C6-HSL 10 µM and 3-oxo-C10-HSL 400 µM

These fractions contained compounds **1**, **2**, **3** from F4, and compounds **3**, **4**, **5** from F5. Only compound **3**, [cyclo-(L-prolyl–L-valine)], found within both fractions, showed activity in TLC-overlay assay.

In Fig. 10 and Fig. 11 are respectively reported ^{1}H and ^{13}C NMR spectra of **(3)** cyclo-(L-prolyl–L-valine), which activated NTL4 bioreporter.

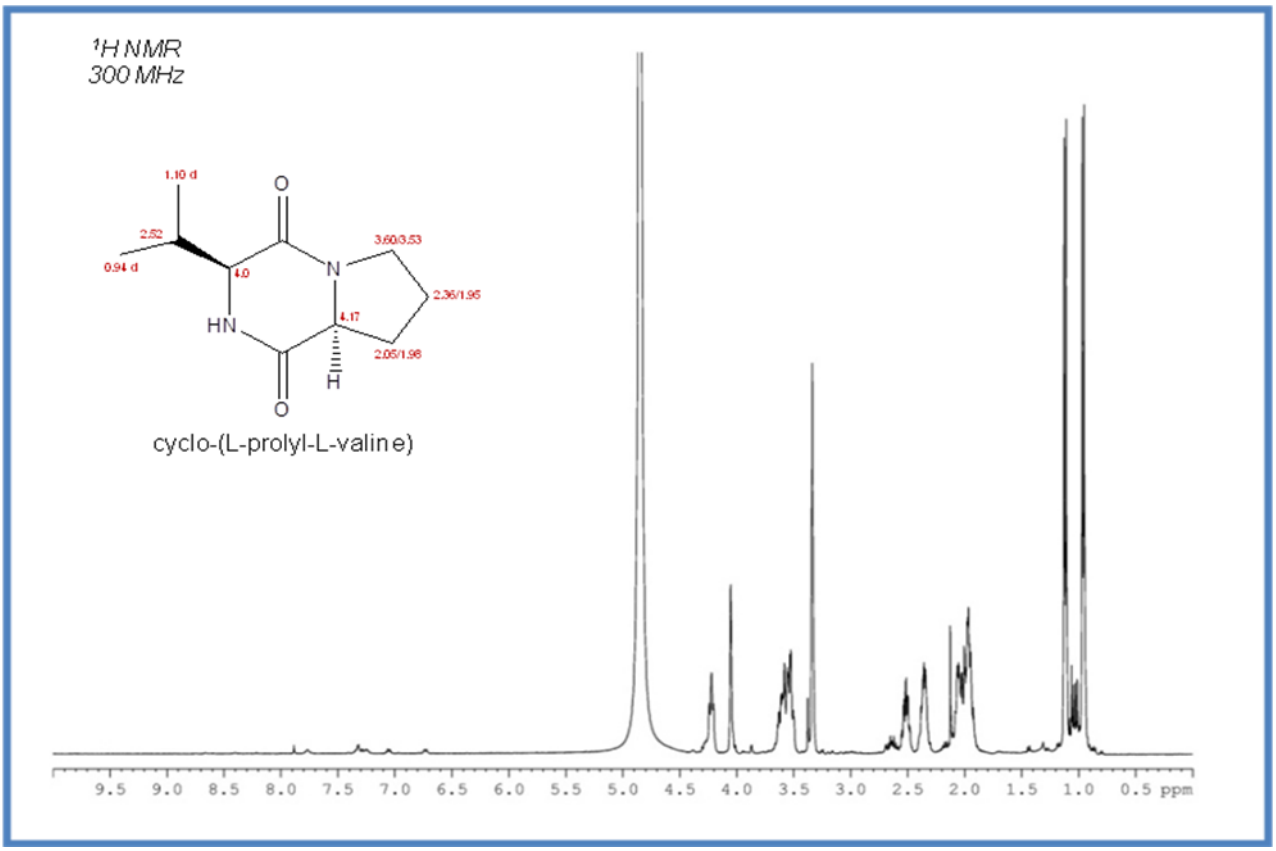


Fig. 10 ^{1}H NMR spectrum of **(3)** cyclo-(L-prolyl–L-valine)

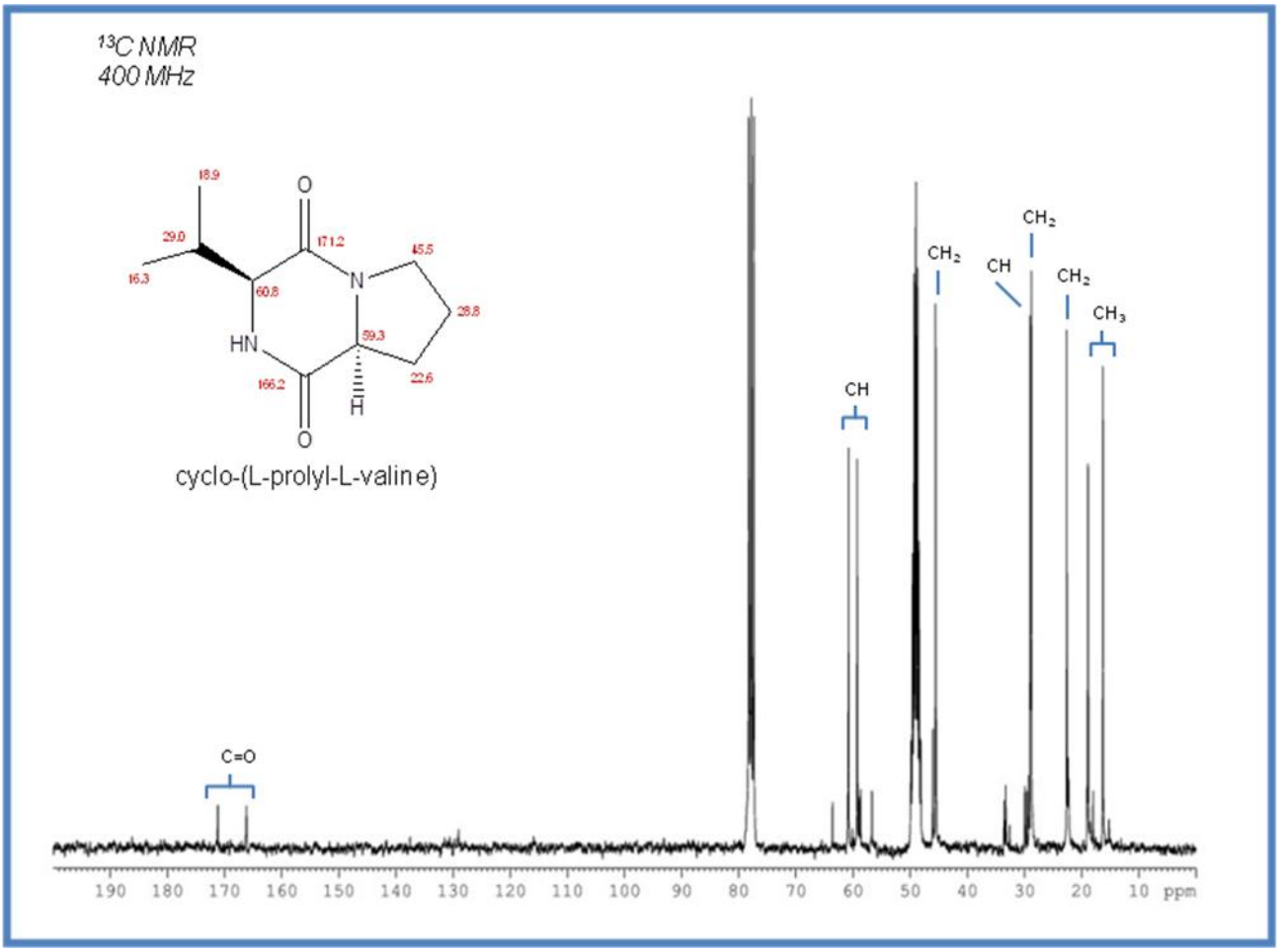
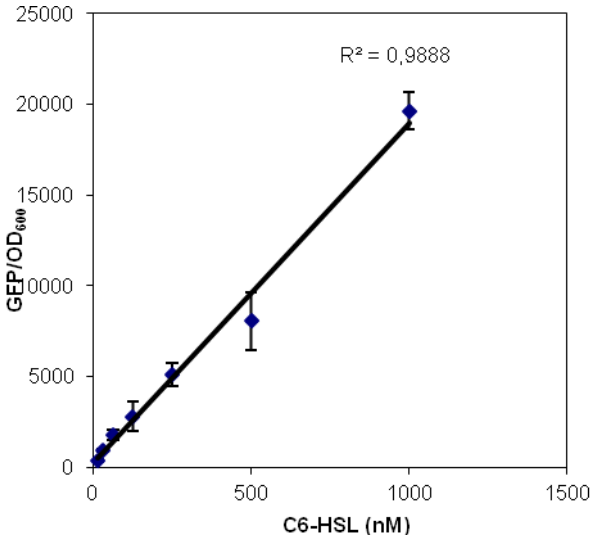


Fig. 11 ^{13}C NMR spectrum of **(3)** cyclo-(L-prolyl–L-valine)

Compound **3** also activated the expression of *gfp* by *Vibrio anguillarum* DM27 strain [Fig. 12] in a concentration-dependent manner, although at much higher concentration than the standard used C6-HSL. The minimum concentration of compound 3 needed to activate the AHL bioreporter strain was 2.5 mM.

A



B

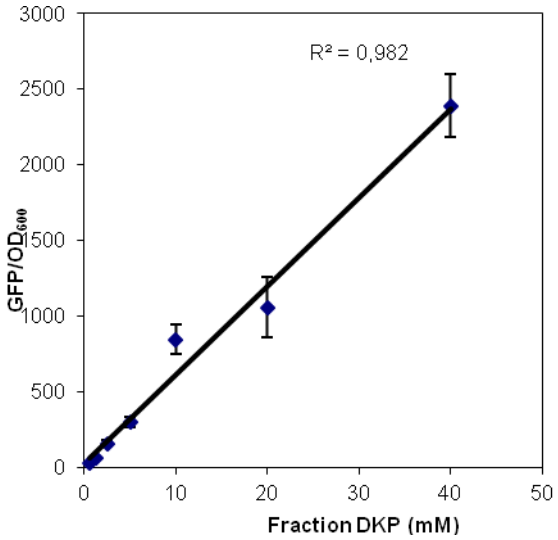


Figure 12 Expression of *Gfp* by *V. anguillarum* vanM mutant carrying a *gfp*-based AHL biosensor luxR-PluxI-RBSII:: gfpmut3*-T0, in response to different concentrations of standard C6-HSL (a) and cyclo (Lprolyl-L-valine) by *Haloterrigena hispanica* (b). The units are expressed as *Gfp* per OD600 to consider the density of the bacteria producing the *Gfp* signal

3RD MODEL OF STUDY: ***Consortium of diesel degrading strains***

- ***Selection of the consortium***

The isolates originate from the site of the Ford Motor Company in Genk (Belgium). Genotypic analysis revealed the existence of 17 different taxa, with *Pseudomonas* and *Promicromonospora* as the most abundant genera [Gkorezis P. et a. 2012, Fig. 13].

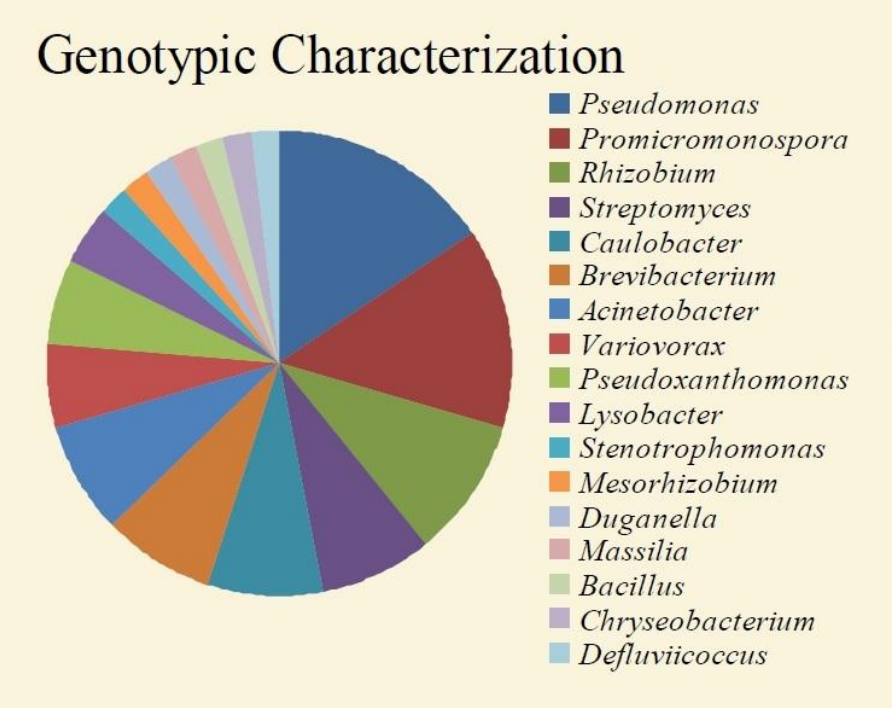


Fig. 13 Genotypic characterization of the consortium of diesel-degrading strains

15 strains were selected from a pool of 380 based on their ability to degrade diesel fuel.

This study was undertaken to investigate the role of quorum sensing in bioremediation processes, with the aim to increase the effectivity of systems for remediation of soils and groundwater contaminated with Petroleum Hydrocarbons by means of Plant-Microbe partnerships.

- ***Extraction of cultural medium***

Bacterial broths were centrifuged in 50 mL tubes at 4.000 rpm for 55 min, and supernatants were extracted twice with dichloromethane 1:2 (v/v).

Extractions were performed on 30 mL bacterial broth to carry out Lux screen assay; 200 mL were extracted to perform TLC-overlay test [Tab. 2].

Dichloromethane extracts were dried under vacuum at T < 40°C to avoid the alteration of metabolites.

Tab. 2 DCM extraction yield on 200 mL bacterial broth.

Sample	EX DCM	Sample	EX DCM
1	20,8 mg	9	40,9 mg
2	78,0 mg	10	42,8 mg
3	53,1 mg	11	12,8 mg
4	37,3 mg	12	34,9 mg
5	14,3 mg	13	69,3 mg
6	11,3 mg	14	12,5 mg
7	25,8 mg	15	32,9 mg
8	11,8 mg		

- ***Bioassay for detection of QS molecules***

Lux screen assay. The presence of putative AHL signal molecules was initially detected using the *Escherichia coli* lux-based bioreporter pSB536, pSB401 and pSB1075.

Extracts of 30 mL bacterial broth were solubilised in 350 µL acetonitrile, and aliquots of 50 µL were then applied to black 96-well microtitre plates.

Preliminary results using the *E. coli* lux-based AHL bioreporters indicated that supernatant extracts of all strains activated pSB536 (short chain AHLs). Extracts of strains n. 9 and 10 also activated pSB1075 (long chain AHLs), while extracts of strains n. 12 and 15 also activated pSB401 (mid chain AHLs) [Tab. 3].

Tab. 3 Lux screen assay results

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
pSB536	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pSB401												+			+
pSB1075									+	+					

TLC-overlay. Dichloromethane extracts were tested for the detection of QS molecules by using TLC-overlay test with *Agrobacterium tumefaciens* NTL4 bioreporter, which detects a broad range of AHLs and also displays the greatest sensitivity towards these compounds [Steindler L. et al. 2007].

200 mL of all bacterial broths were extracted using previously described technique. Aliquots of the extracts were dissolved in 50 µL of acetonitrile and loaded onto a C-18 RP-TLC plate.

The appearance of light blue spot indicated activation of quorum-sensing mechanism.

Eight extracts showed positive activity in TLC overlay assay, in particular strains n. 5 (+/--, 1 spot), 6 (+, 2 spots), 7 (+/-, 1 spot), 8 (+, 2 spots), 11 (+, 1 spot), 12 (++, 2 spots) and 14 (+, 1 spot) [Fig. 14].

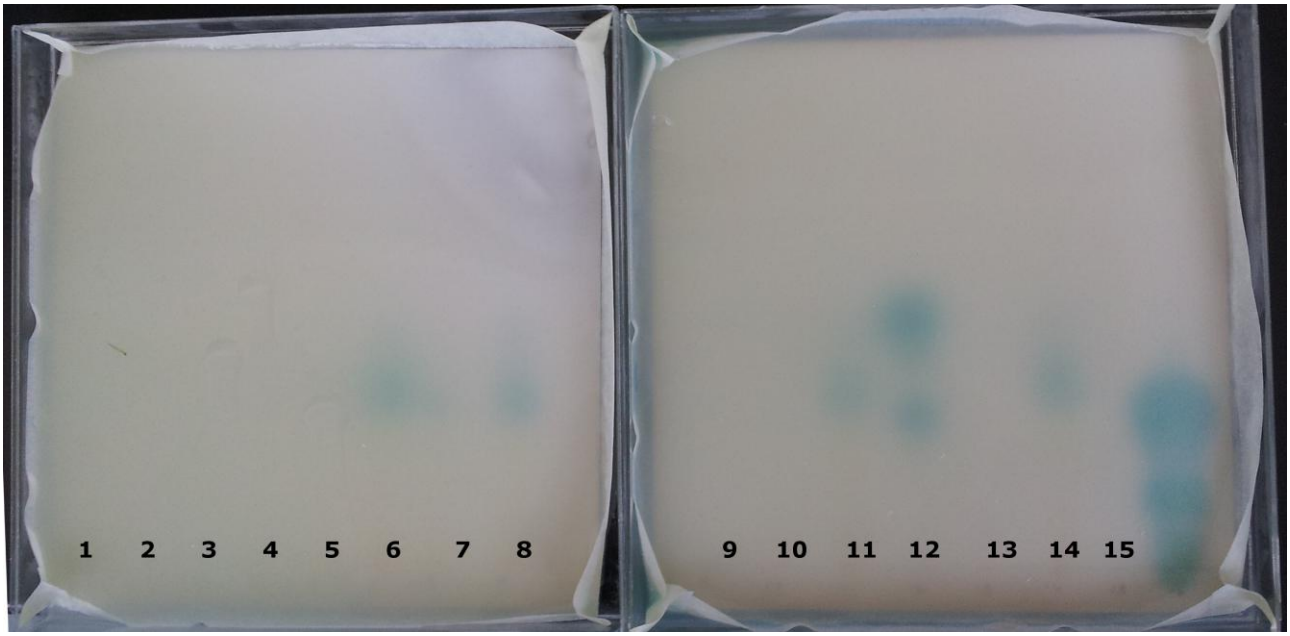


Fig. 14 TLC overlay assay results.
Positive activities: strains n. 5 (+/--, 1 spot), 6 (+, 2 spots), 7 (+/-, 1 spot), 8 (+, 2 spots), 11 (+, 1 spot), 12 (++, 2 spots) and 14 (+, 1 spot)

Plate T-streak. All strains were tested by T-streak assay with CV026 as biosensor [Steindler L. et al. 2007]. Activation of QS mechanism of CV026 was visible for strains n. 4, 12 and 13 [Fig. 15].

(A) (B) (C)

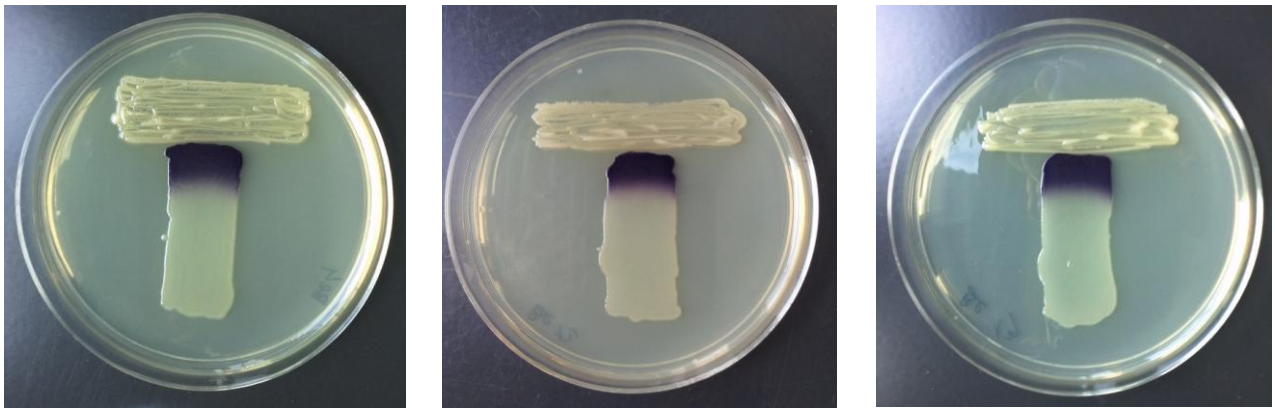


Fig. 15 T-streak assay results.
Positive activities: strains n. 4 (A), 12 (B) and 13 (C).

- ***Evaluation of enzymatic and physiological activities of the consortium***

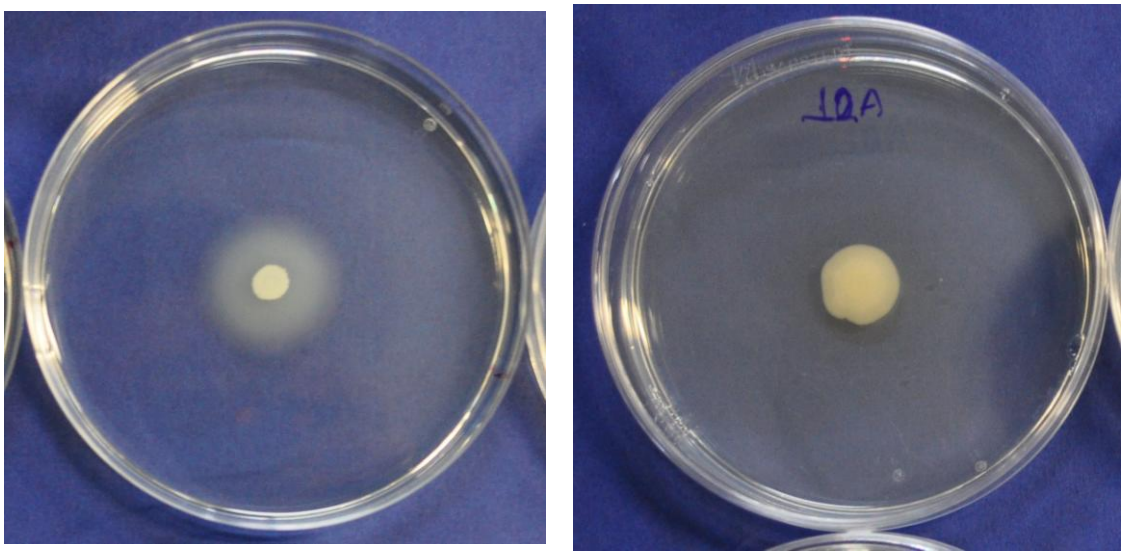
Swimming and swarming motility. Bacterial swimming motility was assessed on semi-solid (0,2% agar) nutrient agar plates [Kang Y.S. et al. 2010c].

The diameter (mm) of the bacterial zone was measured as the average of 5 replicates performed [Tab. 4, Fig. 16].

The swarming motility was evaluated under the same conditions, except for agar percentage (0,5%). Swarming motility was observed after 72h [Tab. 4, Fig. 16].

Tab. 4 Swimming and swarming motility results

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Swimming motility (mm)	7,4	6,2	7,2	26,6	8,0	7,4	6,8	7,4	7,2	7,6	7,6	24,2	21,4	20,6	7,4
Swarming motility (mm)	7,0	7,2	6,4	7,8	7,0	10,0	7,8	5,2	7,8	7,8	5,4	9,6	10,6	7,2	8,6



(A) (B)

Fig. 16 Evaluation of swimming (A) and swarming (B) motility of strain n. 12

Oxidase activity. Oxidase activity was investigated by means a rapid assay on a glass slide [Kang Y.S. et al. 2011]. None of analyzed strains showed positive activity to this assay.

Catalase activity. The production of bubbles in presence of hydrogen peroxide is an indication of catalase activity [Kang Y.S. et al. 2011]. None of analyzed strains showed catalase ability.

Lipase activity. Lipase activity was assessed on agar plates composed of 2% Tween 80, 2,5% agar and 0,5% methylred [Sriram M.I. et al. 2011]. None of analyzed strains produced lipase in the presence of diesel as a sole carbon source [Fig. 17].

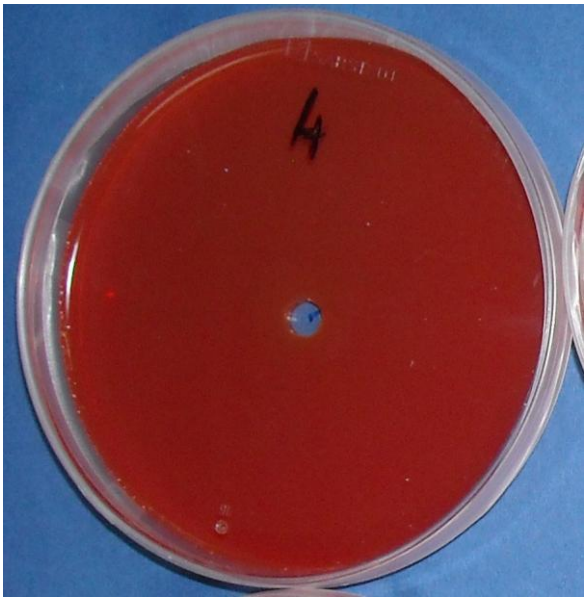


Fig. 17 Lipase activity test on strain n. 4.

- ***Selection of the strains***

Strains n. 4, 12 and 15 were selected based on their strong ability to degrade diesel fuel and, consequently, their potential use in systems for remediation of soils and groundwater contaminated with Petroleum Hydrocarbons.

These strains also showed positive results in most of performed assays [Tab. 5].

Tab.5 Selection of the strains. Positive activities in Lux-screen, Plate T-streak and Swimming motility assays

	Lux-screen assay	TLC-overlay (NTL4)	Plate T-streak (CV026)	Swimming motility
Strain 4	+	-	+	+
Strain 12	+	+	+	+
Strain 15	+	-	-	-

These strains were identified as:

- strain 4 : *Acinetobacter oleovorans*

- strain 12: *Acinetobacter calcoaceticus*

- strain 15: *Brevibacterim sp.*

- ***Antibiotic sensitivity test***

Susceptibility to antibiotics was evaluated by means Kirby-Bauer method [Bauer A.W. et al. 1966]. The antibiotic disks were then placed over the bacterial lawn and incubated overnight at 37°C. After incubation, antibiotic sensitivity was shown by a zone of clearance around the well [Tab. 6].

Tab. 6 Antibiotic sensitivity test results

	Acinetobacter oleovorans			*Acinetobacter calcoaceticus*			*Brevibacterim sp.*		
Antibiotic	**Resistance**	**Quantity per disk (µg)**	**Halo radii**	**Resistance**	**Quantity per disk (µg)**	**Halo radii**	**Resistance**	**Quantity per disk (µg)**	**Halo radii**
Ampicillin	+	10		+	10		+	10	
Amikacin	-	30	7 mm	-	30	7 mm	-	30	10 mm
Bacitracin	+	10		+	10		+	10	
Ciprofloxacin	-	20	5 mm	-	20	6 mm	-	20	7 mm
Chloroamphenicol	-	30	5 mm	+	30		+	30	
Erythromycin	-	15	4 mm	-	15	3 mm	-	15	2 mm
Gentamycin	-	10	7 mm	-	10	7 mm	-	10	7 mm
Tetracyclin	-	10	4 mm	-	10	2 mm	-	10	6 mm
Kanamycin	-	25	4 mm	-	25	4 mm	-	25	7 mm
Neomycin	-	30	5 mm	-	30	5 mm	-	30	5 mm
Rifampicin	-	10	3 mm	-	10	4 mm	-	10	5 mm
Streptomycin	-	50	3 mm	-	50	6 mm	-	50	7 mm

Antibiotic resistance results are summarized in Tab. 7.

Tab. 7 Antibiotic resistance results of *Acinetobacter oleovorans*, *Acinetobacter calcoaceticus* and *Brevibacterim* sp.

Antibiotic	***Acinetobacter oleovorans***	***Acinetobacter calcoaceticus***	***Brevibacterim* sp.**
Ampicillin (10 µg/disk)	+	+	+
Bacitracin (10 µg/disk)	+	+	+
Chloroamphenicol (30 µg/disk)	-	+	+

- ***Heavy metal tolerance assay***

Tolerance to Cd, Fe, Zn, Mn, Cu and Ni of selected strains was evaluated both on liquid (*Tube method*) and on solid (*Agar diffusion method*) medium.

Tube method. Heavy metal salts were dissolved in phosphate saline buffer (pH=7) and prepared for 1 M, 500 mM, 100 mM, 50 mM and 10 mM concentrations.

Then 50 µL of corresponding metal salt solutions were added to each test tube (5 mL cultures), in order to reach a final concentration of 0.1 mM, 0.5 mM, 1 mM, 5 mM and 10 mM, and incubated at 30°C for 7 days.

All strains showed a multiple heavy metal resistence [Tab. 8].

Tab. 8 Heavy metal tolerance by *Liquid medium assessment*

	Heavy metal final concentrations					
Strain	0,1 mM	0,5 mM	1 mM	5 mM	10 mM	**Heavy metal salt**
Acinetobacter oleovorans	++	++	-	-	-	Cadmium chloride $\cdot H_2O$
	++	++	++	-	-	Ferrous sulphate $\cdot 7H_2O$
	++	++	++	-	-	Zinc sulphate $\cdot 7H_2O$
	++	++	-	-	-	Manganese sulphate $\cdot H_2O$
	++	++	-	-	-	Copper sulphate $\cdot 5H_2O$
	++	++	++	-	-	Nickel sulphate $\cdot 6H_2O$
Acinetobacter calcoaceticus	++	++	-	-	-	Cadmium chloride $\cdot H_2O$
	++	++	++	-	-	Ferrous sulphate $\cdot 7H_2O$
	++	++	-	-	-	Zinc sulphate $\cdot 7H_2O$
	++	++	-	-	-	Manganese sulphate $\cdot H_2O$
	++	++	-	-	-	Copper sulphate $\cdot 5H_2O$
	++	-	-	-	-	Nickel sulphate $\cdot 6H_2O$
Brevibacterim sp.	-	-	-	-	-	Cadmium chloride $\cdot H_2O$
	++	++	++	-	-	Ferrous sulphate $\cdot 7H_2O$
	+	+	+	-	-	Zinc sulphate $\cdot 7H_2O$
	+	-	-	-	-	Manganese sulphate $\cdot H_2O$
	-	-	-	-	-	Copper sulphate $\cdot 5H_2O$
	+	-	-	-	-	Nickel sulphate $\cdot 6H_2O$

In Tab. 9 results are reported as the maximum concentration (ppm) at which bacteria showed growth, thereby they were considered as resistant to the corresponding heavy metal.

Tab. 9 Heavy metal tolerance by *Liquid medium assessment*. Maximum concentration (ppm) tolerated by analyzed strains

Heavy metal tolerance (final concentration)			
Acinetobacter oleovorans	***Acinetobacter calcoaceticus***	***Brevibacterim sp.***	**Heavy metal salt**
56,21 ppm	56,21 ppm	-	**Cadmium chloride $\cdot H_2O$**
55,85 ppm	55,85 ppm	55,85 ppm	**Ferrous sulphate $7H_2O$**
65,38 ppm	32,69 ppm	65,38 ppm	**Zinc sulphate $7H_2O$**
27,47 ppm	27,47 ppm	5,49 ppm	**Manganese sulphate $\cdot H_2O$**
31,77 ppm	31,77 ppm	-	**Copper sulphate $5H_2O$**
58,69 ppm	5,87 ppm	5,87 ppm	**Nickel sulphate $6H_2O$**

Agar diffusion method. Heavy metal salts were dissolved in phosphate saline buffer (pH=7) and prepared for 1 M, 500 mM, 200 mM, 50 mM and 10 mM concentrations. Then 50 µL of corresponding metal salt solutions were added to each well and incubated for 7 days at 30°C. The areas of inhibition around the wells were measured [Tab. 10, Fig. 18 a,b].

Tab. 10 Heavy metal tolerance by *Agar diffusion method*

	Heavy metal solution					
Strain	10 mM	50 mM	100 mM	500 mM	1 M	**Heavy metal salt**
Acinetobacter oleovorans	+	+	17 mm	20 mm	24 mm	Cadmium chloride $\cdot H_2O$
	+	+	5 mm	10 mm	13 mm	Ferrous sulphate $\cdot 7\ H_2O$
	+	+	10 mm	13 mm	17 mm	Zinc sulphate $\cdot 7H_2O$
	+	+	+	+	8 mm	Manganese sulphate $\cdot H_2O$
	+	+	7 mm	15 mm	19 mm	Copper sulphate $\cdot 5H_2O$
	5mm	8 mm	12 mm	19 mm	22 mm	Nickel sulphate $\cdot 6H_2O$
Acinetobacter calcoaceticus	+	+	15 mm	18 mm	23 mm	Cadmium chloride $\cdot H_2O$
	+	+	6 mm	11 mm	14 mm	Ferrous sulphate $\cdot 7\ H_2O$
	+	+	8 mm	13 mm	15 mm	Zinc sulphate $\cdot 7H_2O$
	+	+	+	2 mm	5 mm	Manganese sulphate $\cdot H_2O$
	+	+	13 mm	15 mm	18 mm	Copper sulphate $\cdot 5H_2O$
	4 mm	6 mm	14 mm	16 mm	17 mm	Nickel sulphate $\cdot 6H_2O$
Brevibacterim sp.	+	3 mm	15 mm	20 mm	20 mm	Cadmium chloride $\cdot H_2O$
	+	1 mm	6 mm	12 mm	14 mm	Ferrous sulphate $\cdot 7\ H_2O$
	+	+	10 mm	13 mm	15 mm	Zinc sulphate $\cdot 7H_2O$
	+	+	2 mm	3 mm	3 mm	Manganese sulphate $\cdot H_2O$
	+	+	12 mm	15 mm	18 mm	Copper sulphate $\cdot 5H_2O$
	5 mm	8 mm	13 mm	18 mm	19 mm	Nickel sulphate $\cdot 6H_2O$

The *Agar diffusion method* confirmed a multiple heavy metals resistance for all analyzed strains. In Tab. 11 results are reported as the maximum concentration (ppm) at which bacteria showed growth (areas of inhibition), thereby they were considered as resistant to the corresponding heavy metal.

Tab. 11 Heavy metal tolerance by *Agar diffusion method* expressed in ppm

Heavy metal tolerance (final concentrations)			
Acinetobacter oleovorans	***Acinetobacter calcoaceticus***	***Brevibacterim sp.***	**Heavy metal salts**
5620,55 ppm	5620,55 ppm	1124,11 ppm	**Cadmium chloride $\cdot H_2O$**
2792,25 ppm	2792,25 ppm	558,45 ppm	**Ferrous sulphate $7H_2O$**
3269,00 ppm	3269,00 ppm	3269,00 ppm	**Zinc sulphate $7H_2O$**
27469,02 ppm	5493,80 ppm	2746,90 ppm	**Manganese sulphate $\cdot H_2O$**
3177,30 ppm	3177,30 ppm	3177,30 ppm	**Copper sulphate $5H_2O$**
-	-	-	**Nickel sulphate $6H_2O$**

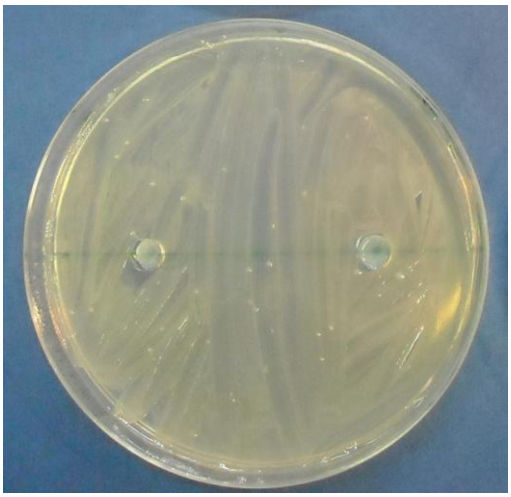


Fig. 18a

Acinetobacter oleovorans
50 mM Zn/Mn

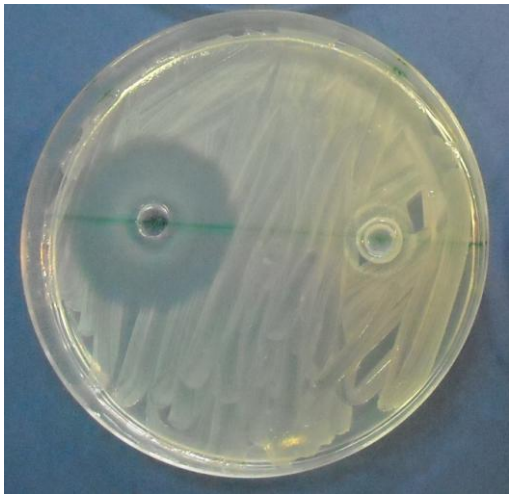


Fig. 18b

Acinetobacter oleovorans
500 mM Zn/Mn

4TH MODEL OF STUDY: *Bacteria plant association*

- ***Selection of samples***

Eight different samples were selected for the screening of molecules involved in QS mechanism (bacterial cultures, plant cultures, bacteria-plant cultures and sterile media) [Tab. 12].

Tab.12 Selection of samples: Index

	Sample	**Sample lab. name**
1	Bacteria + plant with roots culture (in Hoagland's nutrient solution)	Cuttings 10^8
2	Bacteria + plant cuttings culture (in Hoagland's nutrient solution)	Roots 10^8
3	Bacteria inoculated in Hoagland's nutrient solution	Hoagland 10^8
4	Bacteria inoculated in 869 (rich) medium	Cuttings 0
5	Plant with roots culture (in Hoagland's nutrient solution)	Roots No Inoculum
6	Plant cuttings culture (in Hoagland's nutrient solution)	869 + Bact
7	Hoagland's nutrient solution	Hoagland 0
8	869 (rich) medium	869

The bacterial strain was *Pseudomonas putida* W619, a natural root endophyte of the poplar cultivar *Hoogvorst* (*Populus trichocarpa* x *Populus deltoides*) [Taghavi S. et al.2005].

The isolated strain was cultivated with another poplar cultivar, namely *Grimminge* [*P. deltoides* x (*P. trichocarpa* x *P. deltoides*)]. This cultivar is more resistant to poplar rust and it is very similar to *Hoogvorst* [Weyens N. et al. 2011].

- ***Extraction of cultural medium***

Supernatants were extracted twice with dichloromethane 1:2 (v/v).

Dichloromethane extracts were dried under vacuum at T < 40°C to avoid the alteration of metabolites [Tab. 13].

Tab.13 Dichloromethane extracts index

	Sample	**DCM**	**EX DCM**
- 1 --> Cuttings 10^8	200 mL	2 x 200mL	1,4 mg
- 2 --> Roots 10^8	200 mL	2 x 200mL	0,4 mg
- 3 --> Hoagland 10^8	200 mL	2 x 200mL	1,2 mg
- 4 --> Cuttings 0	200 mL	2 x 200mL	1,5 mg
- 5 --> Roots No Inoculum	200 mL	2 x 200mL	0,5 mg
- 6 --> 869 + Bact	200 mL	2 x 200mL	13,4 mg
- 7 --> Hoagland 0	200 mL	2 x 200mL	1,4 mg
- 8 --> 869	200 mL	2 x 200mL	13,6 mg

- ***Bioassays for Putative AHL Signal Molecule.***

Lux screen Assay. The presence of putative AHL signal molecules was initially detected using the *Escherichia coli* lux-based bioreporter pSB536, pSB401 and pSB1075.

Extracts were solubilised in 350 µL acetonitrile, and aliquots of 50 µL were then applied to black 96-well microtitre plates, in order to reach quantities reported in Tab. 14.

Tab.14 Lux screen Assay. Quantity of extract applied to microtitre plates.

Sample	**EX DCM**	**Loaded (in 50 µl Acetonitrile)**
- 1 --> Cuttings 10^8	1,4 mg	31,11 µg
- 2 --> Roots 10^8	0,4 mg	8,89 µg
- 3 --> Hoagland 10^8	1,2 mg	26,67 µg
- 4 --> Cuttings 0	1,5 mg	33,33 µg
- 5 --> Roots No Inoculum	0,5 mg	11,11 µg
- 6 --> 869 + Bact	13,4 mg	297,78 µg
- 7 --> Hoagland 0	1,4 mg	31,11 µg
- 8 --> 869	13,6 mg	302,22 µg

Because no relevant activities were detected at after 3 h incubation for pSB536 and 6 h for pSB401 and pSB1075, luminescence was measured again at 24h using an image viewer.

After 24h, only "Bacteria + plant with roots culture (in Hoagland's nutrient solution)" (sample n.1, Cuttings 10^8), activated *E. coli* 536, which is used to detect short chain AHLs.

TLC-overlay. Dichloromethane extracts were tested for the detection of QS molecules by using TLC-overlay test with *Agrobacterium tumefaciens* NTL4 bioreporter, which detects a

broad range of AHLs and also displays the greatest sensitivity towards these compounds [Steindler L. et al. 2007].

Aliquots of supernatant extracts were also applied to C-18 RP-TLC plates in order to reach concentration reported in Tab. 15.

Tab.15 TLC-overlay. Quantity of extract applied to C-18 RP-TLC plates.

Sample	**Loaded**
- 1 --> Cuttings 10^8	0,56 mg
- 2 --> Roots 10^8	0,16 mg
- 3 --> Hoagland 10^8	0,48 mg
- 4 --> Cuttings 0	0,60 mg
- 5 --> Roots No Inoculum	0,20 mg
- 6 --> 869 + Bact	2,68 mg
- 7 --> Hoagland 0	0,56 mg
- 8 --> 869	2,72 mg

The appearance of light blue spot indicated activation of quorum-sensing mechanism.

All samples, excluding Hoagland's nutrient solution extract (sample n. 7, Hoagland 0), activated NTL4 AHL bioreporter. In particular, the highest activity was found in the extract of "Bacteria + plant with roots culture (in Hoagland's nutrient solution)" (sample n. 1, Cuttings 10^8).

Results are showed in Fig. 19 and an evaluation of detected activities is reported in Tab. 16.

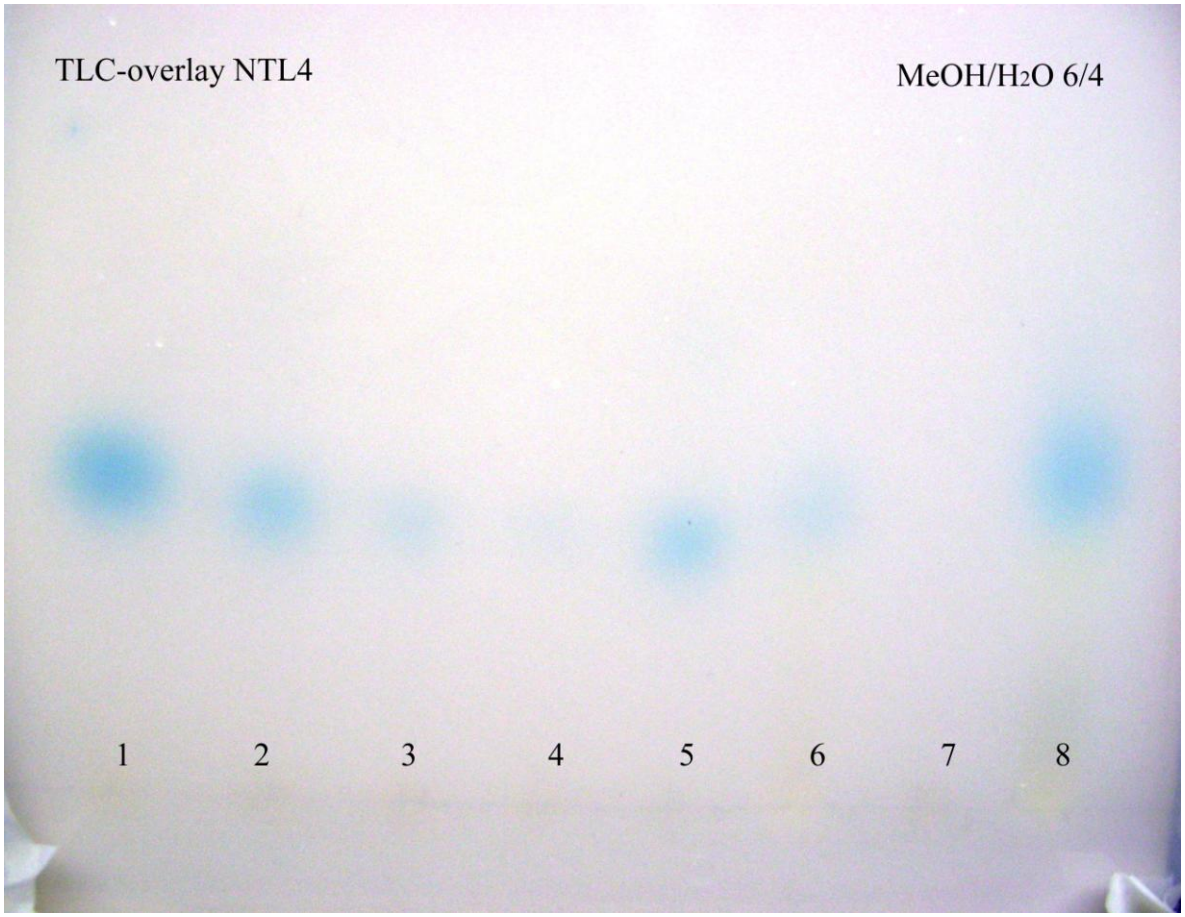


Fig. 19 TLC-overlay results.

Positive activities: (1) Cuttings 10^8, (2) Roots 10^8, (3) Hoagland 10^8, (4) Cuttings 0, (5) Roots No Inoculum, (6) 869 + Bact, (8) 869.

Tab.16 Evaluation of TLC-overlay results

Sample	Positive 24h	Positive 48h
- 1 --> Cuttings 10^8	+/- (+)	++
- 2 --> Roots 10^8	+/-	+
- 3 --> Hoagland 10^8		+/-
- 4 --> Cuttings 0		+/--
- 5 --> Roots No Inoculum		+
- 6 --> 869 + Bact		+/-
- 7 --> Hoagland 0		
- 8 --> 869		+

DISCUSSION

Species-specific cell-cell signalling is involved in pathogenic or symbiotic interactions between a variety of bacteria and their plant and animal hosts [Parsek M.R. and Greenberg E.P. 2000]. It has been demonstrated that QS molecules are involved in attraction of zoospores of green seaweed *Ulva* and the detection of AHLs results in calcium influx into the zoospore. That was the first example of a calcium signalling event in a eukaryote in response to bacterial QS molecules [Joint I. et al. 2007]. Moreover, it has been showed that the interdial surfaces colonized by *Ulva* are dominated by *a-Proteobacteria*, and that this diverse assemblage both produces and degrades AHLs. These results suggested that AHL-degrading strains can affect bacterial community behaviour by interfering with QS between neighbouring bacteria [Tait K. et al. 2009]. It may therefore be the case that some kind of "communication" also exists between marine sponges and microorganisms that are specifically associated with them. From *a-* and *γ-Proteobacteria* isolated from marine sponges *Mycale laxissima* and *Ircinia strobilina* was detected a range of AHLs molecules, and among the bacteria tested, AHL production was more frequently observed for the *Proteobacteria* associated with *M. laxissima* than those with *I. strobolina* [Mohamed N.M. et al. 2008]. In the case of specific sponge-bacteria association, it is possible that specifically associated bacteria may thrive or at least survive with sponge. In fact a ribosomal RNA study of axenic cell cultures of *Suberites domuncula* showed a 16S rRNA band specific for bacteria [Thakur N.L. et al. 2003].

The finding of two different bacteria, associated with the same sponge, (*Dysidea avara*) with contrasting response in bioassay for AHL synthesis, could add an interesting dimension to the study of signalling interactions in sponges microcosm. It is well known

of the beneficial coexistence of microorganisms and sponges, and that the microbial communities are species-specific and represent a stable population [Friedrich A.B. et al. 2001; Webster N.S. et al. 2001], and that multispecies bacterial communities regulate their behaviour by intraspecies and interspecies cell-cell communication [Bassler B.L. 2002], so it is likely that microorganisms living in association with marine sponge had opposing response in QS mechanism. The reason of this matter could be the mutual control on the growth of different microorganisms in the same host (sponge).

In a previous paper [De Rosa S. et al. 2003] it was described the isolation and chemical characterization of compounds belonging to a class of diketopiperazine from cell-free supernatant of *Bacillus* sp. associated with marine sponge *Ircinia variabilis*. Now it is demonstrated that one of these compounds, cyclo-(cis-4-hydroxy-D-prolyl-L-leucine), activated QS mechanism. Moreover others cyclic dipeptides, cyclo-(L-prolyl-L-leucine) and cyclo-(L-prolyl-L-phenylalanine), isolated from *Vibrio* sp. associated with marine sponges *D. avara* showed the ability to activate quorum sensing mechanism. It is known that QS signals are either small organic molecules or peptides, Gram-negative bacteria employ AHLs, while cyclic peptides (diketopiperazines) are employed by Gram-positive bacteria [Williams P. 2007]. Moreover, other low molecular mass diffusible factors are also described as modulators of communication in bacterial community [Williams P. 2007]. It is not unexpected that from cell-free supernatant of marine sponges-associated bacteria cyclic dipeptides were isolated as factors involved in QS mechanism, perhaps this finding could be interesting within of sponge-bacteria interaction, and it could be of interest to understand the cross-kingdom chemical communication mechanism.

Detection of signal molecules in *Archaea* has never been rigorously investigated and defined. These microorganisms can live in prohibitive environmental conditions for the other forms of life, thus it is possible to suppose that QS is involved in their survival mechanism.

Although *Haloterrigena hispanica* supernatant activated the AHL bioreporters pSB401 and NTL4 (pCF218; pCF372), no AHL signal molecules were present. The active compound was a diketopiperazine compound (DKP), cyclo-L-prolyl-L-valine. This compound also activated QS system in *Vibrio anguillarum* DM27 strain by producing *gfp*.

V. anguillarum DM27 strain present a null mutation of *vanM*-gene that suppressed the production of AHLs. Moreover it contains an insertion (*luxR*-P_{luxI}-RBSII::*gfp*mut3*-T_0) that makes bacteria able to detect exogenous AHL or other signal molecules (in this case DKP) source by producing *gfp* [Sewald X. et al. 2007]. The active DKP activated *gfp* expression in the *V. anguillarum* bioreporter at a much higher concentration than AHL standard (2,5 mM of DKP respect to 50 nM of C6-HSL). The amount of active compound produced by *H. hispanica* was in a range of mM (12 mg/10L), thus it was likely that the cyclo-L-prolyl-L-valine was capable to activate or antagonize *lux*-based AHL biosensor in halophilic mixed communities. Until now there are no report describing the isolation of DKPs from *Archaea*. DKPs are a wide class of natural compounds with increased interest by several research groups [Huanga R. et al. 2010]. Microorganisms of marine and terrestrial origins are a rich source of these compounds [Mitova M. et al. 2004, De Rosa S. et al. 2003]. They show different bioactivities including cytotoxic, antimicrobial, antioxidant, plant-growth regulatory activities. In fact a class of disulfides cyclic dipeptides (rostratins) isolated from a marine-derived fungus *Exserohilum rostratum* showed *in vitro* a potent cytotoxicity

against human colon carcinoma (HCT-116). Also, DKPs isolated from a marine-derived fungus *Aspergillus* sp. and *Chromocleista* sp. showed interesting biological activities, in particular a moderate antimicrobial, antioxidant and ultraviolet-A protecting activities. [Park Y.C. et al. 2006, Schultz A.W. et al. 2008, Li Y. et al. 2004, Tan R.X. et al. 2004]. These compounds were also previously demonstrated to activate AHL bioreporters [Holden M.T.G. et al. 1999]. Supernatants of *Pseudomonas aeruginosa* contained the DKPs cyclo-(L-Ala-L-Val) and cyclo-(L-Pro-L-Tyr). Both compounds activated an AHL bioreporter in a concentration-dependent manner, presumably competing for the same LuxR binding site.

A previous paper reported the detection of QS molecules belonging to AHLs produced by haloalkaliphilic archaeon *Natronococcus occultus* [Paggi R.A. et al. 2003]. However, the chemical structures of the activating compounds were not confirmed using MS and NMR techniques. It may be that the compounds produced by *N. occultus* were DKPs, or indeed other AHL mimic compounds, and not AHLs. Although Paggi et al. demonstrated correlation between the production of the signal molecule and an extracellular protease , this may also be due to DKP activity [Paggi R.A. et al. 2003]. DKPs have been shown to influence the transcription of specific stationary phase-regulated genes in *Escherichia coli* [de Nys R. et al. 2000].

The physiological role of these compounds in the metabolic pathway of *H. hispanica* is not clear. It is possible to suppose that they are involved in the strategy evolved by *Archaea* to adapt to their hypersaline habitat. Whether DKPs function as QS signal molecules for *Archaea* remains to be established. However, under certain conditions, these compounds may function to communicate with AHL-signaling bacteria within mixed communities.

Growing number of reports investigate the role of signal molecules in inter-kingdom communication mechanism.

The co-evolution between bacteria and host, thus the mutual exposure to the respective communication system for billions of years, could suggest a potential ability of prokaryotic and eukaryotic cells to intercept and translate signal molecules released by each other.

The mechanism by which AHLs produce a response in eukaryotic cells remains unknown. Recently, researchers showed that oxo-C12-HLS could modulate the intense inflammatory response due to *P. aeruginosa* infection inside the lungs of cystic fibrosis (CF) patients.

It was also described the ability of mammalian cells to disrupt QS signaling, probably throw the production paraoxonases (PONs). These enzymes have a calcium dependent esterase activity, therefore they can hydrolyze AHLs, as a defense against bacterial infection.

Catecholamines (epinephrine and norepinephrine) can stimulate bacterial growth. Exposure to norepinephrine produces increased intestinal colonization of *Salmonella enterica* in mouse models [Pacheco A.R. and Sperandio V. 2009].

The evaluation of QS activity of the four models, as well as reports considered in this extensive analysis, revealed a complex diversification, but also the abundance in interconnections, of communication mechanisms.

It has been detected the presence of bacteria with contrasting response in bioassay for AHL synthesis (activation/disruption of QS in CV026 bioreporter) in the same host, the marine sponge *D. avara*.

Concerning the analysis of the consortium of bacteria diesel degrading strains, it has been assumed a common communication mechanism due to short chain AHLs.

The diesel-degrading consortium also showed multiple heavy-metal and antibiotic resistance characteristics. It is possible to suppose that QS mechanism is involved in the strategy evolved by these strains to adapt themselves to polluted environments.

The aim of the study of the 4th model, bacteria-plant association, was to detect differences in signal molecules production, due to the interaction between bacteria and plants, choosing poplars as model.

All samples, excluding Hoagland medium extract (sample 7, Hoagland 0), activated NTL4 AHLs bioreporter. In particular the highest activity was revealed in the extract of "Bacteria + plant with roots culture (in Hoagland's nutrient solution)" (sample n. 1, Cuttings 10^8).

This activity was also revealed by means Lux screen assay, in which sample n.1 activated *E. coli* pSB536 (production of bioluminescence).

These results could explain that both bacteria and plant synthesize molecules able to activate QS mechanism.

It's also interesting the increase of signal molecules synthesis (activation of NTL4 bioreporter) in Cuttings 10^8,"Bacteria + plant with roots culture (in Hoagland's nutrient solution)", compared to Cuttings 0, uninoculated cuttings culture, which suggests a possible role of QS, in particular of signalling molecules produced by the bacterium *Pseudomonas putida* W619, in root development process of the poplar cultivar *Grimminge* [*Populus deltoides* x (*Populus trichocarpa* x *Populus detoides*)].

Moreover, it has also been detected induction of QS of NTL4 in an halophilic archaeon, *Haloterrigena hispanica*.

Finally, it was analyzed the potential ability of prokaryotes to intercept and translate signals released by eukaryotes (i.e. norepinephrine in *Salmonella enterica*) and vice versa (AHLs in *P. aeuruginosa*).

The analysis revealed the existence of a possible universal mechanism of molecular communication among prokaryotes, but there are also evidences that it could be intercepted by eukaryotes. This molecular language is largely unknown yet, therefore new chapters need to be opened in inter-species and inter-kingdom research.

SUPPLEMENTARY DATA

- **Cyclo-(cis -4-hydroxy-D-prolyl-L-leucine)**; ^{1}H-NMR: δ 4.56 (1H, m, H-8), 4.46 (1H, dd, J= 8.3 and 8.1 Hz, H-6), 4.00 (1H, m, H-3), 3.65 (1H, dd, J= 12.3 and 3.5 Hz, H-9a), 3.49 (1H, dd, J= 12.3 and 5.6 Hz, H-9b), 2.53 (1H, m, H-7a), 2.17 (1H, m, H-7b), 1.70 (2H, m, H-10), 1.54 (1H, m, H-11), 0.94 (3H, d, J= 6.1 Hz, H-12), 0.91 (3H, d, J= 6.1 Hz, H-13); EIMS m/z (%) $[M]^{+}$ 226(6), 183(12), 170(100).

- **Cyclo-(trans-4-hydroxy-L-prolyl-L-leucine)**; ^{1}H-NMR: δ 4.44 (2H, m, H-6 and H-8), 4.15 (1H, m, H-3), 3.59 (1H, dd, J= 13.1 and 4.2 Hz, H-9a), 3.32 (1H, d, J= 13.1 Hz, H-9b), 2.17 (1H, dd, J= 13.4 and 6.3 Hz, H-7a), 2.00 (1H, ddd, J= 13.4, 13.2 and 4.2 Hz, H-7b), 1.66 (2H, m, H-10), 1.46 (1H, m, H-11), 0.76 (6H, d, J= 6.1 Hz, H-12 and H-13); EIMS m/z (%) $[M]^{+}$ 226(10), 183(15), 170(100).

- **Cyclo-(trans-4-hydroxy-L-prolyl-L-phenylalanine)**; ^{1}H-NMR: δ 7.43 (3H, m, H-13-15), 7.26 (2H, m, H-12 and H-16), 4.66 (1H, t, J= 4.4 Hz, H-3), 4.41 (2H, m, H-6 and H-8), 3.80 (1H, dd, J= 12.8 and 5.4 Hz, H-9a), 3.34 (1H, dd, J= 12.8 and 1.2 Hz, H-9b), 3.30 (1H, dd, J= 14.1 and 4.5 Hz, H-10a), 3.18 (1H, dd, J= 14.1 and 4.5 Hz, H-10b), 2.04 (1H, dd, J= 13.1 and 5.9 Hz, H-7a), 1.12 (1H, ddd, J= 13.1, 12.8 and 5.0 Hz, H-7b); EIMS m/z (%) $[M]^{+}$ 260(80), 169(86), 141(100).

- **Cyclo-(glycyl-L-leucine)**; ^{1}H-NMR: δ 4.03 (1H, d, J= 17.8 Hz, H-3a), 3.91 (1H, dd, J= 6.8 and 6.5 Hz, H-9a), 3.85 (1H, d, J= 17.8 Hz, H-3b), 1.84 (1H, m, H-6), 1.71 (1H, m, H-7); 1.01 (3H, d, J= 7.1 Hz, H-8), 0.99 (3H, d, J= 7.1 Hz, H-9); EIMS m/z (%) $[M]^{+}$ 170(38), 127(55), 114(100), 113(80).

- **Cyclo-(L-prolyl-L-tyrosine)**; ^{1}H-NMR: δ 7.02 (2H, d, J= 8.5 Hz, H-12 and H-16), 6.81 (2H, d, J= 8.5 Hz, H-13 and H-15), 4.48 (1H, m, H-3), 4.01 (1H, dd, J= 6.1 and 2.1 Hz, H-6), 3.46 (1H, m, H-9a), 3.28 (1H, m, H-9b), 3.17 (1H, dd, J= 14.2 and 4.0 Hz, H-10a), 2.95 (1H, dd, J= 14.2 and 4.4 Hz, H-10b), 1.97 (1H, m, H-8a), 1.71 (2H, m, H-8b and H-7a), 0.70 (1H, ddd, J= 12.0, 11.6 and 9.6 Hz, H-7b); EIMS m/z (%) $[M]^{+}$ 260(6), 154(100), 107(96).

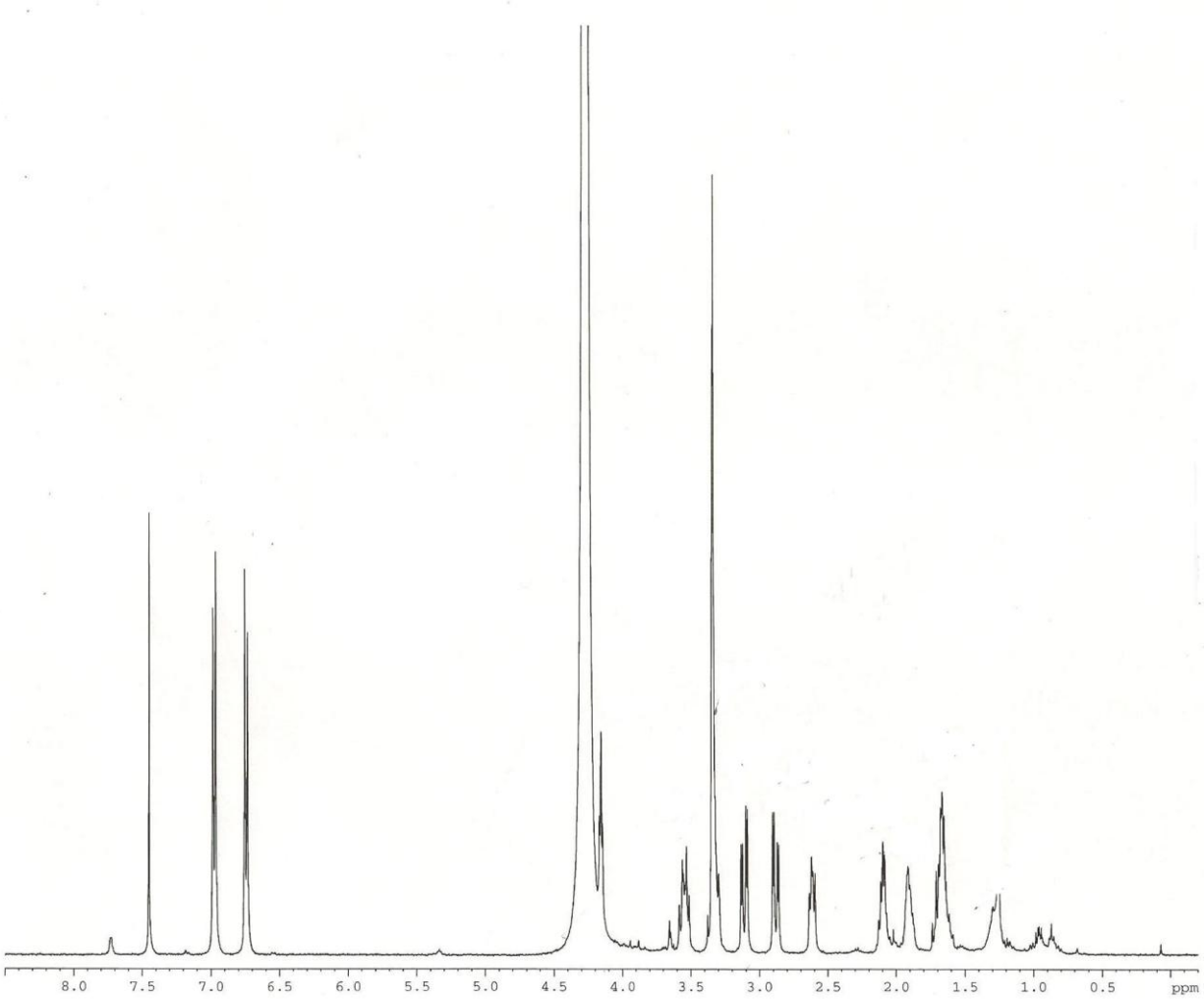
- **Cyclo-(D-prolyl-L-tyrosine)**; ^{1}H-NMR: δ 7.02 (2H, d, J= 8.5 Hz, H-12 and H-16), 6.81 (2H, d, J= 8.5 Hz, H-13 and H-15), 4.17 (1H, m, H-3), 3.57 (1H, m, H-9a), 3.16 (1H, dd, J= 14.2 and 4.0 Hz, H-10a), 3.13 (1H, m, H-9b), 2.90 (1H, dd, J= 14.2 and 4.4 Hz, H-10b), 2.65 (1H, dd, J= 6.1 and 2.1 Hz, H-6), 2.10 (1H, m, H-8a), 1.92 (1H, m, H-7a), 1.68 (2H, m, H-7b and H-8b); EIMS m/z (%) $[M]^{+}$ 260(8), 154(100), 153(36), 125(68), 107(96).

- **Cyclo-(cis-4-hydroxy-D-prolyl-L-phenylalanine)**; ^{1}H-NMR: δ 7.34 (3H, m, H-13-15), 7.21 (2H, m, H-12 and H-16), 4.41 (1H, t, J= 4.4 Hz, H-3), 4.33 (1H, m, H-8), 3.58 (1H, dd, J= 12.4 and 3.8 Hz, H-9a), 3.34 (1H, dd, J= 12.4 and 5.7 Hz, H-9b), 3.22 (1H, dd, J= 13.6 and 4.2 Hz, H-10a), 3.06 (1H, dd, J= 13.6 and 5.0 Hz, H-10b), 2.62 (1H, dd, J= 8.5 and 8.4 Hz, H-6), 2.26 (1H, m, H-7a), 1.86 (1H, m, H-7b); EIMS m/z (%) $[M]^{+}$ 260(15), 169(86), 154(33), 141(100).

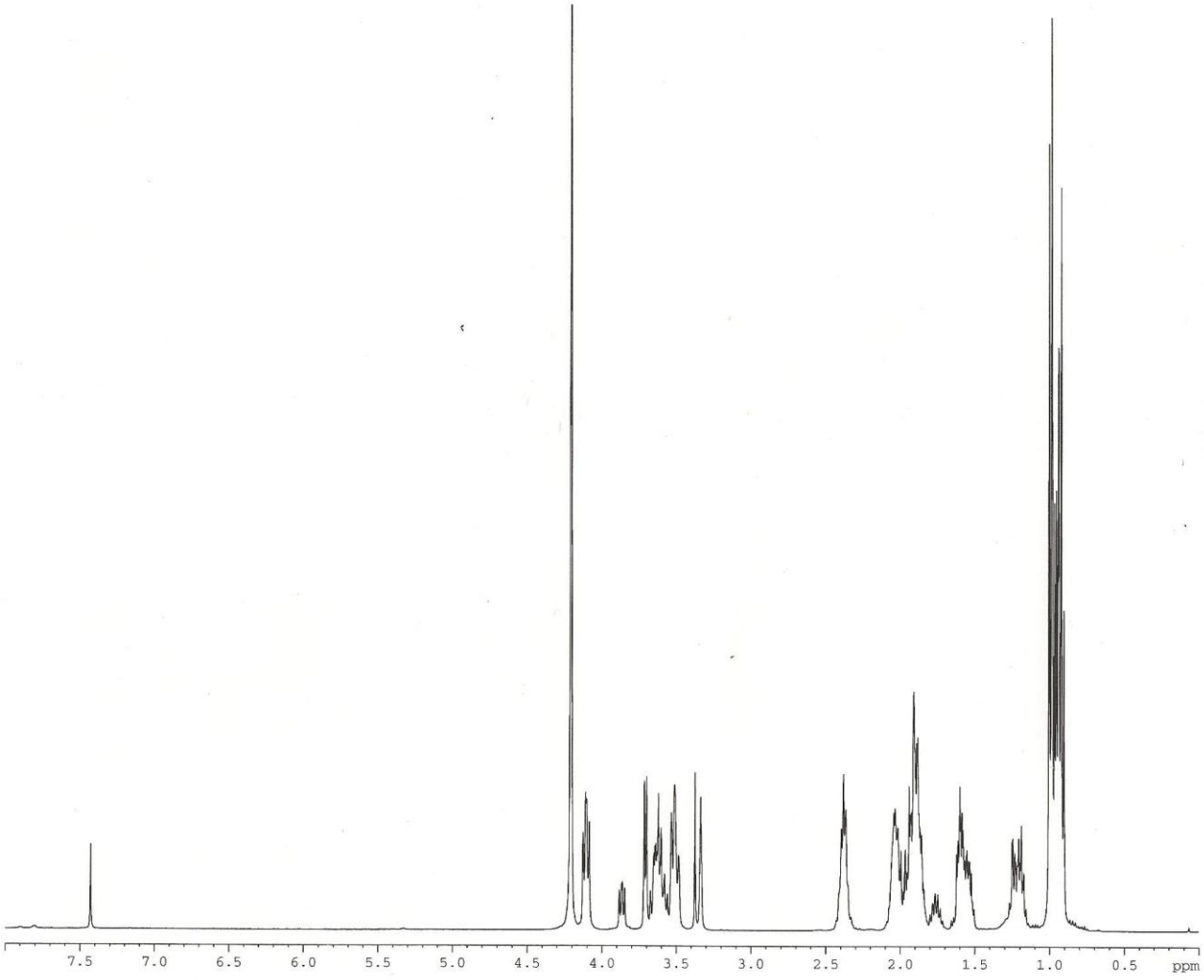
- **Cyclo-(L-prolyl-L-phenylalanine)**; ^{1}H-NMR: δ 1.8-2.15 (2H, m, Pro 4-Ha,b); 2.16 (1H, m, Pro 3-Ha); 2.35 (1H, m, Pro 3Hb); 2.79 (1H, dd, J= 10.6 Hz/-14.6 Hz Pha 3-Ha); 3.50-3.75 (2H, m, Pro 5-Ha,b); 3.63 (1H, dd J= -14.6 Hz/3.7 Hz

Phe 3Hb); 4.09 (1H, dt J= 1.0 Hz/≈8.5 Hz Pro α-H); 4.29 (1H, m J= 10.6 Hz/3.7 Hz/≈1 Hz Phe -H); 5.62 (1H, bs, NH); 7.15-7.50 (5H, m, Phe aromatic H).

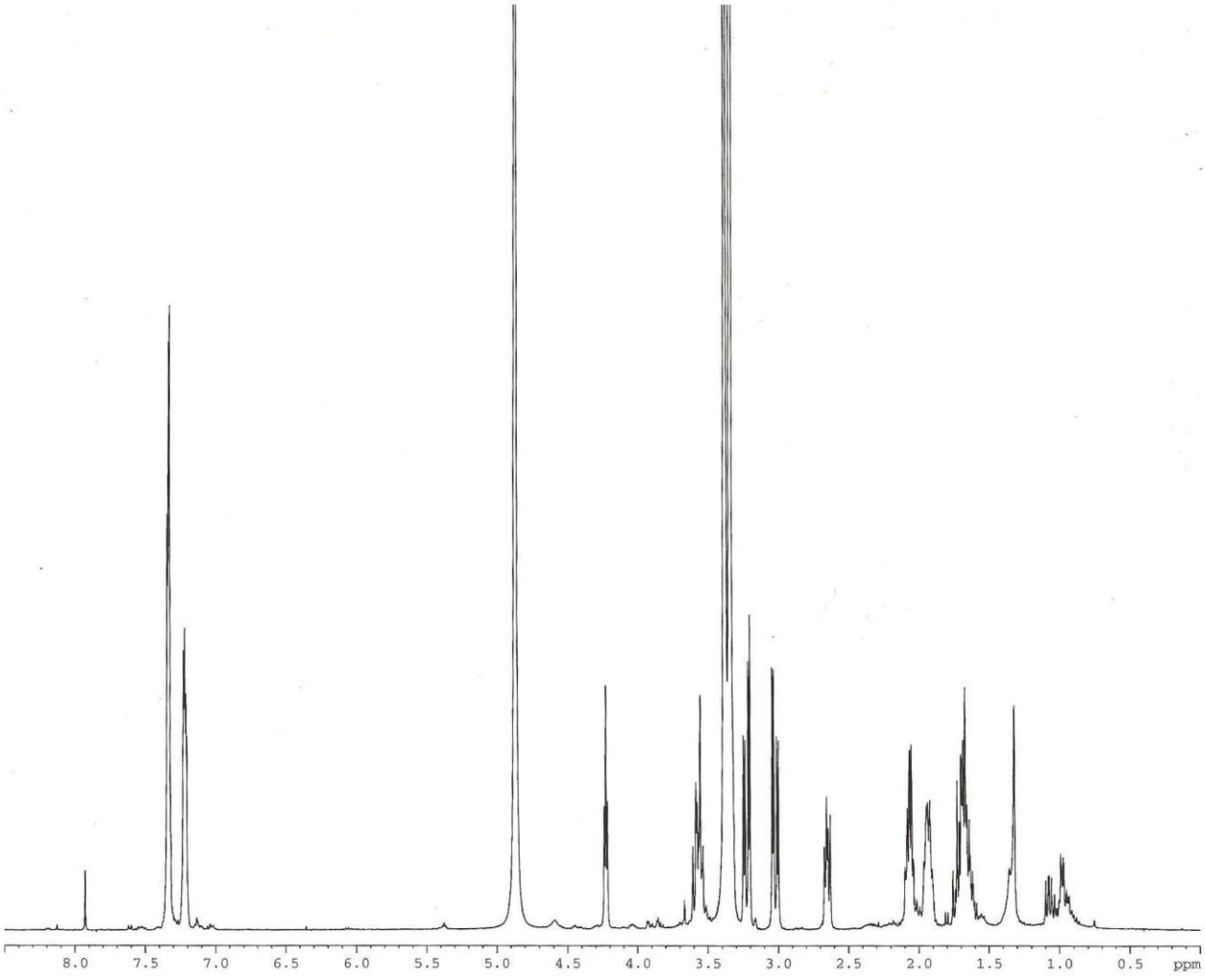
- **Cyclo(L- prolyl -L- leucine)**; ^{1}H-NMR: δ 0.96 (3H, d J= 6.5 Hz Leu CH_3); 1.01 (3H, d J= 6.4 Hz Leu CH_3); 1.55 (1H, m J= 4.9 Hz/ 9.3 Hz/ -14.2 Hz Leu 3-Ha); 1.75 (1H, m, Leu 4-H CH); 1.92 (1H, m, Pro 4-Ha); 2.05 (1H, m, Pro 4-Hb); 2.07 (1H, m, Leu 3-Hb); 2.15 (1H, m, Pro 3-Ha); 2.37 (1H, m, Pro 3-Hb); 3.5-3.7 (2H, m, Pro 5-Ha,b); 4.02 (1H, dd J= 9.2 Hz/ 4.4 Hz Leu α-H); 4.12 (1H, dt J ≈8 Hz/ ≈1 Hz, Pro α-H); 5.80 (1H, bs, NH).



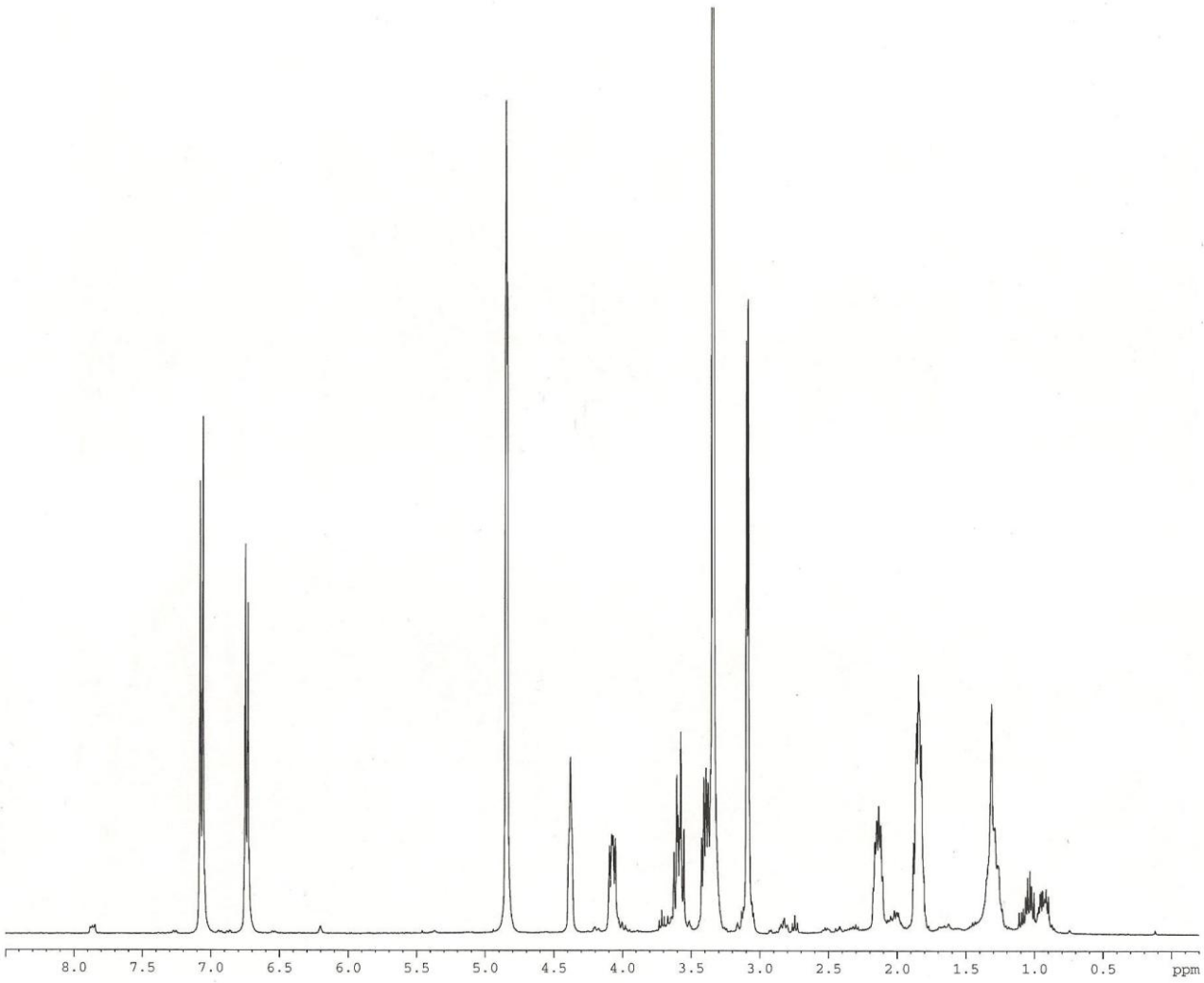
^{1}H-NMR spectrum of cyclo-(D-prolyl-L-tyrosine),
recorded on a Bruker AMX-400 spectrometer in CD_3OD



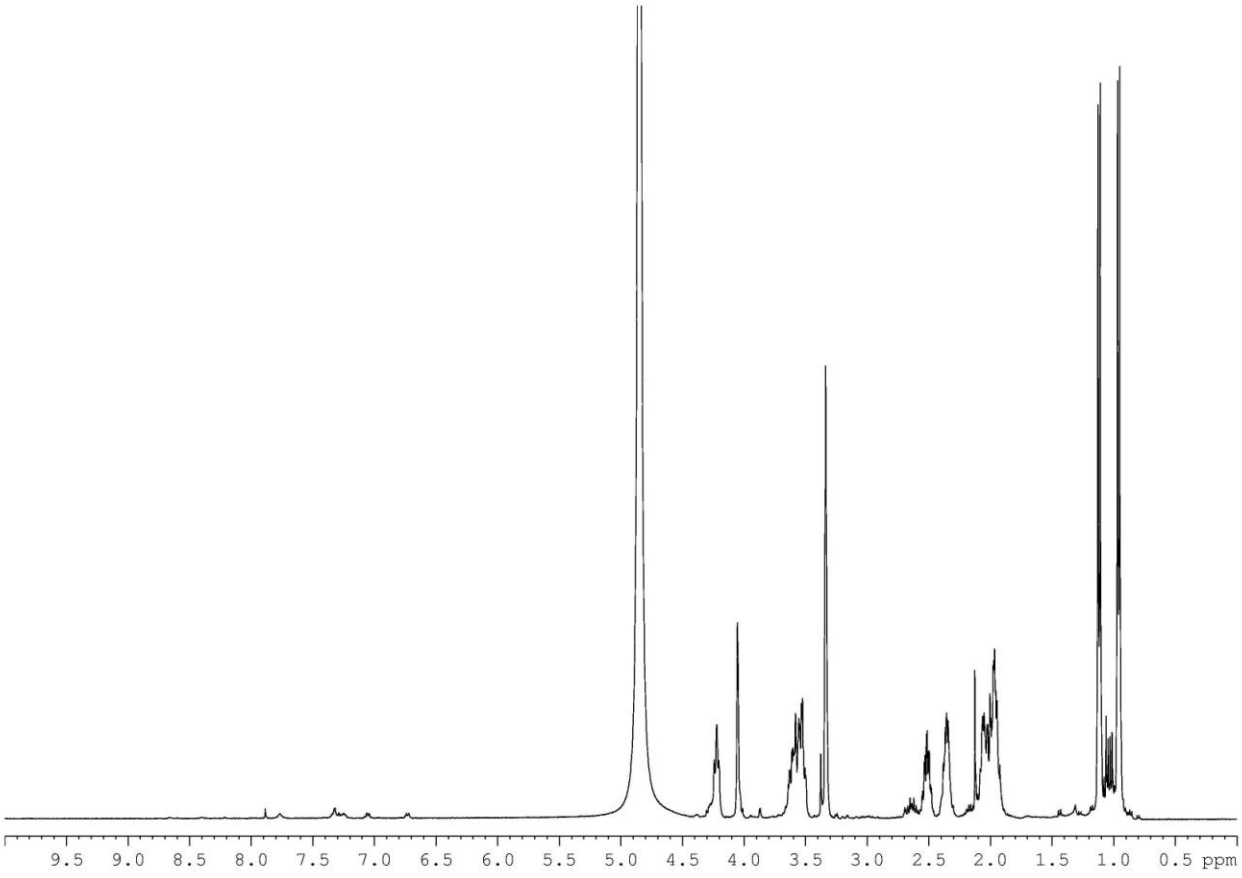
^{1}H-NMR spectrum of cyclo-(L-prolyl-L-isoleucine),
recorded on a Bruker AMX-400 spectrometer in CD_3OD



^{1}H-NMR spectrum of cyclo-(L-prolyl- L-phenylalanine),
recorded on a Bruker AMX-400 spectrometer in CD_3OD



^{1}H-NMR spectrum of cyclo-(L-prolyl-L-tyrosine),
recorded on a Bruker AMX-400 spectrometer in CD_3OD



^{1}H-NMR spectrum of cyclo-(L-prolyl–L-valine),
recorded on a Bruker AMX-400 spectrometer in CD_3OD

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