

# Multi-focused laboratory experiments based on Quorum Sensing and Quorum Quenching for acquiring Microbial Physiology concepts

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**Abstract:** After a time away from the classrooms and laboratories due to the global pandemic, the return to the teaching activities during the semester represented a challenge to both teachers and students. Our particular situation in a Microbial Physiology course was the necessity of imparting in a shorter time, laboratory practices that usually take longer. This article describes a two-week long laboratory exercise that covers several concepts in an interrelated way: conjugation as a gene transfer mechanism, regulation of microbial physiology, production of secondary metabolites, degradation of macromolecules and biofilm formation. Utilizing a Quorum Quenching (QQ) strategy, the Quorum Sensing (QS) system of *Pseudomonas aeruginosa* is first attenuated. Then, phenotypes regulated by QS are evidenced. QS is a regulatory mechanism of the microbial physiology that relays on signal molecules. QS is related in *P. aeruginosa* to several virulence factors, some of which are exploited in the laboratory practices presented in this work. QQ is phenomenon by which QS is interrupted or attenuated. We utilized a QQ approach based on the enzymatic degradation of the *P. aeruginosa* QS signals in order to put in evidence QS-regulated traits that are relevant for our Microbial Physiology course.

**Keywords:** Microbial physiology - Quorum Sensing - Quorum Quenching - *Pseudomonas aeruginosa*

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## INTRODUCTION

In our Faculty, Microbial Physiology is a mandatory course for undergraduate students for the Bachelor's degree in Biotechnology. This course involves 6 weeks of practices in the lab, during a two-month period with one lab practice per week, meaning that they take, at most, six practice classes during the course. An extensive list of concepts must be acquired by the students throughout the Microbial Physiology course, including regulation of microbial physiology, synthesis and degradation of macromolecules, metabolic pathways, among others. Other concepts (e.g., microbial growth, nutrition, and antibiotics) and skills (e.g., aseptic techniques, preparation of culture media, seeding and incubation) are acquired in General Microbiology course in the previous semester.

The global pandemic situation that we have gone through in the last 2 and a half years, has led us to rethink our traditional laboratory practices. In our Faculty, the coming back to the in-person classes occurred in the middle of the semester. With less weeks for face-to-face teaching, teachers needed to achieve the study goals without lowering the education quality. For students, they had to acquire, in a short time, the corresponding skills related to their degree.

Employing *Pseudomonas aeruginosa* strain PAO1, we propose a series of lab practices in which students, in a short period of time, learn different concepts of microbial physiology in an interrelated way:

- Bacterial conjugation as a gene transfer mechanism
  1. Bioactive molecules
  2. Signaling and regulation of microbial physiology through cell-to-cell communication
  3. Degradation of macromolecules

*Pseudomonas (P.) aeruginosa* is a well know Gram-negative strain; an opportunistic pathogen widely spread in soil and aquatic environments (Qin et al. 2022). In *P. aeruginosa* PAO1, several virulence factors, including extracellular enzymes, production of biofilm, toxins, secondary metabolites, and siderophores, are regulated by its Quorum Sensing (QS) system (Chadha, Harjai, and Chhibber 2022).

QS is a well know cell density-dependent intercellular communication system that allows individual cells to act as a community. QS mechanisms are based on the production, release, and detection of signal molecules called autoinducers (Fuqua and Greenberg 2002). Similar to several other Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, in *P. aeruginosa*, these bioactive molecules are *N*-acyl homoserine lactones (AHLs) (Chadha, Harjai, and Chhibber 2022). To date, three QS systems intimately linked among them and arranged in a hierarchical regulatory cascade are known in *P. aeruginosa*. The autoinducer synthase LasI and RhII produces AHL molecules as autoinducers: *N*-oxododecanoyl-L-homoserine lactone (3OC12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL), respectively. When these autoinducers reach a critical threshold level by accumulation due to bacterial population growth, they interact with their receptors LasR and RhIR, respectively, which are also transcriptional activators (Chadha, Harjai, and Chhibber 2022). LasR and RhIR stimulates the *lasI* and *rhII* expression, creating autoinduction loops. A third QS system that relies on the production of 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal or PQS) is interconnected with the LasI-LasR and RhII-RhIR systems.

In nature, microorganisms interfere with the QS systems of competitors mainly through the enzymatic inactivation of the QS signals, or the production of metabolites that impede the normal functioning of the signal receptors, a phenomenon known as Quorum Quenching (QQ) (Grandclément et al. 2016). In the lab, the cell-to-cell communication can be blocked with easy and fast experiments to understand the functioning of the QS system. In the approach chosen for the present work, a *aiiA* gene encoding the AiiA lactonase from *Bacillus* sp. A24, an AHL-degrading

enzyme, is expressed under a constitutive *plac* promoter in *P. aeruginosa* PAO1, reducing the accumulation of autoinducers and the expression of several traits (Reimann et al. 2002). The easiest way to expose the QQ phenomenon is evidencing with biosensor strains the decrease in the AHL levels. These strains have a genetic deficiency on signal production, but detect and respond to exogenous signals by means of a functional receptor coupled to a reporter gene that produces an observable phenotype when specific QS signals are present (Miller and Gilmore 2020; Steindler and Venturi 2007). *Chromobacterium* (*C.*) *subtsugae* CV026 and *P. putida* F117 (pKR-C12) are the biosensors used in these practical lab works. *C. subtsugae* CV026 produce violacein, a purple pigment, in presence of AHLs with acyl side chains from C4 to C8 in length (McClellan et al. 1997). *P. putida* F117 (pKR-C12) produce green fluorescent protein in presence of AHLs with acyl side chains from C10 to C14 in length (Riedel et al. 2001).

The experimental workflow presented in this work is detailed in Figure 1. Each individual assay is thought of as a piece of a puzzle that fits together giving a single, complete, overall, and multi-focused approach for the lab classes.

## MATERIALS AND METHODS

### Equipment

The experimental procedures require the use of laminar flow chambers, micropipettes, centrifuges, vortex, transilluminator, spectrophotometers, optical microscope, a rotary shaker, a rotary evaporator and a 37 °C lab stove.

### Biosafety

Even when these lab practice are not particularly risky, all students must wear appropriate lab clothes and protective equipment including gloves and glasses for handling *Ps. aeruginosa* PAO1, considering that this strain is a Biosafety Level 2 organism.

### Strains and culture conditions

Bacterial strains used in this work are listed in Table 1. The culture media used for the assays are Luria Bertani (LB) broth composed of 10 g L<sup>-1</sup> tryptone, 5 g L<sup>-1</sup> yeast extract, 10 g L<sup>-1</sup> NaCl; and nutrient yeast broth (NYB) composed of peptone 10 g L<sup>-1</sup>; NaCl 5 g L<sup>-1</sup>; meat extract 10 g L<sup>-1</sup>; yeast extract 5 g L<sup>-1</sup>. The composition of Cetrimide agar utilized for selective isolation of transconjugants is gelatin peptone 20 g L<sup>-1</sup>, MgCl 1.4 g L<sup>-1</sup>, K<sub>2</sub>SO<sub>4</sub> 10 g L<sup>-1</sup>, centrimide 0.3 g L<sup>-1</sup>, glycerol 10 mL L<sup>-1</sup>. When required, culture media are solidified with agar 15 g L<sup>-1</sup> and/or tetracycline (Tc) 125 µg ml<sup>-1</sup> sterilized by filtration through sterile 0,22 µ membrane filters. *P. aeruginosa* PAO1 and the transconjugants are grown at 37 °C and 180 rpm in an orbital shaker. Biosensor strains, *Chromobacterium subtsugae* CV026 and *P. putida* F117 (pKR-C12) are grown at 30 °C and 180 rpm in an orbital shaker.

### Attenuation of Quorum Sensing activity

A Quorum Quenching (QQ) strategy is utilized to achieve the attenuation of *P. aeruginosa* PAO1 QS activity. Plasmid pME6863 contains the *Bacillus* sp. A24 *aiiA* AHL-lactonase gene cloned in the pME6000 control vector (Reimmann et al. 2002). AiiA lactonase degrades the AHLs leading to the attenuation of the QS activity. Both pME6863 and pME6000 are conjugated from *E. coli* S17-1 into *P. aeruginosa* PAO1 by biparental mating, in two independent assays, as follows. Overnight cultures of *P. aeruginosa* PAO1, *E. coli* S17-1 (pME6000) and *E. coli* S17-1 (pME6863) on LB agar are utilized for the conjugation. For each conjugation, an inoculation loop of *P. aeruginosa* PAO1 is mixed with two loops of *E. coli* S17-1 (pME6000) or *E. coli* S17-1 (pME6863). The mixtures are prepared as small spots of 0.5-1 cm diameter on top of a LB agar plate. Conjugations are performed at 30 °C for 24 h. The cell pellets are aseptically withdrawn and suspended in physiological solution. Transconjugants are selected after these dilutions on cetrimide agar plates supplemented with Tc. Cetrimide is a quaternary ammonium compound that inhibits *E. coli* strains; tetracycline allows the growth of only *P. aeruginosa* transconjugants. Isolated colonies are finally streaked on fresh cetrimide agar plates with Tc.

#### AHLs production and extraction

*P. aeruginosa* PAO1, *P. aeruginosa* PAO1 (pME6000) and *P. aeruginosa* PAO1 (pME6863) are independently pre-cultured for 18 h in NYB broth. These precultures are utilized to inoculate 125 mL flasks containing 20 mL of fresh culture media without antibiotics at an initial  $OD_{600nm}=0,1$ . After 4h and 8h, samples of 5 mL are withdrawn for AHLs extraction following the protocol described elsewhere (Shaw et al. 1997). Briefly, one volume of ethyl acetate acidified with 0.1 % acetic acid is mixed with one volume of cell-free supernatants. The samples are then vigorously extracted in a vortex for 5 min. After resting, the organic phase is removed, and a second extraction is performed from the aqueous phase. Both organic extractions are combined, and the solvent is evaporated to dryness in a rotary evaporator at 35-40 °C. Finally, the extracts are solubilized in 10  $\mu$ L acetonitrile.

#### Bioassays

Biosensor strains *C. subtsugae* CV026 and *P. putida* F117 (pKR-C12) lack their own AHL production, but respond to exogenous AHLs through the production of violacein and green fluorescent protein, respectively. 10 mL of melted LB soft (0.6 %) agar are independently seeded with overnight cultures of each biosensor strain, and then poured on top of a layer of LB 1.5 % agar. 2  $\mu$ L of the organic extracts are spotted on top of the soft layer, and the solvent is left to dry spontaneously. Plates are incubated at 30 °C for 18-24 h. A positive result with *C. subtsugae* CV026 is evidenced by the presence of violet spots, which can be observed at the naked eye without the requirement of specific equipment. A positive result with *P. putida* F117 (pKR-C12) is evidenced by green fluorescent spots that observed with a UV transilluminator or a blue light transilluminator.

## Evaluation of Quorum Sensing-regulated phenotypes

In all assays, *P. aeruginosa* PAO1, *P. aeruginosa* (pME6000) and *P. aeruginosa* (pME6863) are grown overnight in NYB broth and then 5  $\mu$ L are used to inoculate the corresponding plates. All incubations are carried out at 37 °C for 24 h. Skim milk agar medium is used for the evaluation of protease production (Ohman, Cryz, and Iglewski 1980). A positive proteolytic activity is observed as clear halos around colonies due to degradation casein protein. Interestingly, although King B medium is recommended for the evaluation of siderophore production, this phenotype can also be observed in skim milk agar (see below). Hemolytic activity is visualized on blood agar plates. A positive hemolytic activity is observed as clear halos around the colonies. In this medium, differences in the morphology of the colonies between the strains also can be observed.

## Implementation

The practice is proposed in three days during two weeks (Table 2). While the first week is planned for *P. aeruginosa* conjugations, the second week is used for the analysis of AHL production and the characterization of the phenotypes regulated by QS in the transconjugants. In addition, Table 2 shows the material required for each assay and the theoretical concepts to be learned that open the discussion and encourage critical thinking in the students. The class design is based on a typical lab seance in our Faculty that is organized in 4 hours. At the beginning, an appropriate introductory explanation is provided by the teacher before starting the lab practice. Then, students carry out the experimental part. Finally, the analysis and discussion of the results obtained is carried out with the aim that students reinforce the concepts learned during the class.

## RESULTS AND DISCUSSION

### First week

First day, Petri dishes containing sterile LB agar medium and overnight cultures of bacterial strains are available for conjugations assays (see Table 2). After a review about how to work under aseptic techniques, students are organized in working groups, as required, and conjugations with *E. coli* S17-1 strains carrying pME6000 or pME6863 are performed as described before. To note, functions for transfer helper are integrated in the chromosome of *E. coli* S17-1, which allows a biparental mating. Students also prepare cetrimide agar supplemented with Tc solution required for the selection of transconjugants. Next day, students aseptically remove the bacterial growth from the plates where the conjugations were performed, and serial dilutions in sterile physiological solution are seeded on cetrimide agar plates supplemented with Tc. The plates are incubated at 37 °C for 24 hours. As negative controls, *P. aeruginosa* PAO1, *E. coli* S17-1 (pME6000) and *E. coli* S17-1 (pME6863) should also be plated on cetrimide agar supplemented with Tc. The next day, isolated colonies from biparental mating can be obtained. No colonies should be observed in the negative controls. In this point, a proper

explanation about the importance of the composition of the culture medium and of the complementation with Tc in the selection of transconjugants is critical. The teacher can also exemplify a hypothetical situation where no antibiotic or a non-selective medium (e.g., LB medium) is used in the selection of transconjugants. To note, cetrimide is the selective component that allows the growth of only *P. aeruginosa*, due to its surfactant activity. Finally, students select a plate with isolated colonies to continue the assays the following week.

### Second week

Previous to the lab session, liquid cultures of each strain are prepared as described above and, after 4h and 8h of incubation, samples are withdrawn, which can be stored frozen until the organic extractions. First day, AHLs are extracted from supernatants with acidified ethyl acetate, and the extracts are concentrated to dryness. The teacher supports this experience explaining the higher affinity of AHLs molecules for the organic phase recovered during the extraction process. To be utilized the following day, students also prepare cultures of *P. aeruginosa* PAO1, *P. aeruginosa* PAO1 (pME6000) and *P. aeruginosa* PAO1 (pME6863) for protease and hemolytic activity assays, as well as cultures of *C. subtsugae* CV026 and *P. putida* F117 (pKR-C12) for bioassays. On the second day, students analyze the concentrated extracts using the two biosensor strains. Plates for protease and hemolytic activities are also seeded using overnight cultures of *P. aeruginosa* PAO1, *P. aeruginosa* PAO1 (pME6000) and *P. aeruginosa* PAO1 (pME6863). Ten  $\mu$ L of each culture are seeded as equidistant spots on skim milk agar plates and blood agar plates. Finally, on the third day, students examine and discuss the results found in the bioassays (Figure 2 and 3) and for the phenotypic properties (Figure 4 and 5).

In the bioassays, the production of short- and long-chain AHLs can be distinguished, using *C. subtsugae* CV026 and *P. putida* F117 (pKR-C12) biosensor strains, respectively (see more details in Table 1). Students can also observe the effect of the QQ strategy in *P. aeruginosa* PAO1 (pME6863). The lactonase enzyme, coded in the pME6863 vector, degrades the synthesized AHLs; then, a smaller or barely detectable spots in the bioassays is expected. It should be noted that the synthesis of AHLs is not blocked, but their accumulation is prevented by the enzymatic degradation. The violet spots at the top of the plates in Figures 2A (samples obtained after 4 h of incubation), corresponding to *P. aeruginosa* PAO1 (left) and *P. aeruginosa* PAO1 (pME6000) (right), can be attributed to the induction of violacein production in *C. subtsugae* CV026 by C4-HSL. In the lower part of the plate, where the extract obtained from the *P. aeruginosa* PAO1 (pME6863) culture was seeded, the pigment production is not detected due to degradation of the QS signal molecules. Figure 2B shows the results with samples obtained after 8 h of incubation. In this case, no differences are observed with respect to the extracts obtained after 4 h of incubation, which contrasts when *P. putida* F117 (pKR-C12) biosensor strains is used. In this biosensor, 3OC12-HSL induce the production of green fluorescent protein (Figure 3). The

analysis of the extracts of both *P. aeruginosa* PAO1 (left) and *P. aeruginosa* PAO1 (pME6000) (right) exhibits similar size of fluorescence spots. The small spot, corresponding to *P. aeruginosa* PAO1 (pME6863), is due to the lower levels of 3OC12-HSL related to the AHL degradation after 4 h of incubation (Figure 3A), and that is not detectable after 8 h of incubation (Figure 3B). The differences observed between the biosensor strains and the incubation time are related not only to the different levels of the AHLs but also to the different sensitivity to the QS signals.

Figure 4 shows the results obtained for protease activity. As expected from the bioassays, similar behaviors with *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 (pME6000) are observed. Both strains show positive protease activity evidenced by the presence of a clear halo around the colonies due to the casein degradation. Among other, several genes coding for extracellular proteases are activated by QS in *P. aeruginosa* PAO1. LasA protease and LasB elastase are the best characterized and the ones that give the name to the LasI-LasR QS system (Simanek and Paczkowski 2022; Schuster et al. 2003). Though both Las and Rhl QS systems upregulate the expression of the corresponding *lasA* and *lasB* coding genes, the former is more prevalent than the latter. In addition, the green color of the colonies can be attributed to the siderophores production. Siderophores are organic iron chelators, which also gives the typical green colour to the *Pseudomonas* culture. *P. aeruginosa* produces two siderophores in iron-limited media: pyoverdine and pyochelin. Its production can also be evidenced in skim milk agar (Figure 4). *pvd* and *pch* genes, which are involved in pyoverdine and pyochelin, are upregulated by LasI-LasR (Stintzi et al. 1998). In *P. aeruginosa* PAO1 (pME6863), with an attenuated QS system, both protease activity and siderophore production are affected.

A similar situation is observed on blood agar plates (Figure 5A) where positive hemolytic activities are detected for *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 (pME6000), but not for *P. aeruginosa* PAO1 (pME6863). Bacterial phospholipases aid in the hemolysis, though this activity is mainly related to the production of the surfactant rhamnolipids. Expression of *rhlA* and *rhlB*, which code for enzymes involved in the rhamnolipid synthesis, is positively regulated by the RhlI-RhlR QS system (Ochsner and Reiser 1995).

Differences in the morphology of the colonies can also be observed on blood agar plates (Figure 5 B, C and D). Bacterial colonies are static biofilms formed in the interphase between the culture medium and the air (Merritt, Kadouri, and O'Toole 2005). A large set of genes are involved in biofilm formation in *P. aeruginosa* PAO1 (Waite et al. 2005). Among them, *alg* genes involved in the production of the exopolysaccharide alginate, and the aforementioned *rhlA* and *rhlB* are upregulated by the RhlI-RhlR QS system (Ochsner and Reiser 1995).

Finally, the teacher may introduce students to the biotechnological applications derived from the use of QQ strategies for blocking the cell-to-cell communication systems. Reference materials including scientific articles can support this discussion (Chen et al. 2018; Romero, Acuna, and Otero 2012).

Students are assessed through a final report covering the entire practical, which also contributes to the scientific training of how to formulate the hypothesis and objectives of a work.

### Final considerations

In the present work, *P. aeruginosa* PAO1 is used as a model strain because of its QS system involved in the regulation of several readily demonstrable traits, including proteases and surfactants. These properties can be directly observed by students in a simple and practical laboratory class. It has to be noted that the general concepts explored in these experiments are not specific to PAO1. Other bacteria with well-known QS systems can also be used, considering that the methodology should be adapted accordingly. However, in our opinion *P. aeruginosa* PAO1 is an excellent choice due to the availability of protocols and the facility for the observation and interpretation of the results. These laboratory experiences allow, in a short time, to familiarize the students with several concepts and techniques, ranging from the regulation of genetic expression to the evidence of several physiological traits.

Acknowledgments: This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 2015-0946, PIP 2021-2436, PUE 22920160100012CO), Agencia Nacional de Promoción Científica y Tecnológica (PICT 2016 N° 0532; PICT 2019 N° 03336), and Secretaría de Ciencia, Arte e Innovación Tecnológica from the Universidad Nacional de Tucumán (PIUNT D609). We thank Patricia Peralta (PROIMI-CONICET) for her valuable support in the preparation of photos and images.

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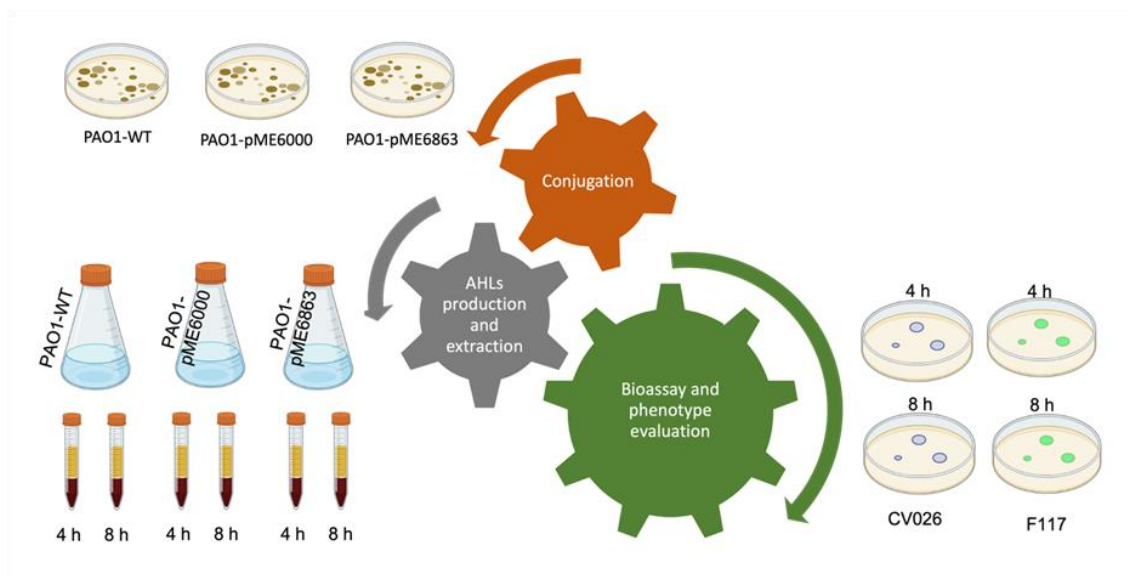
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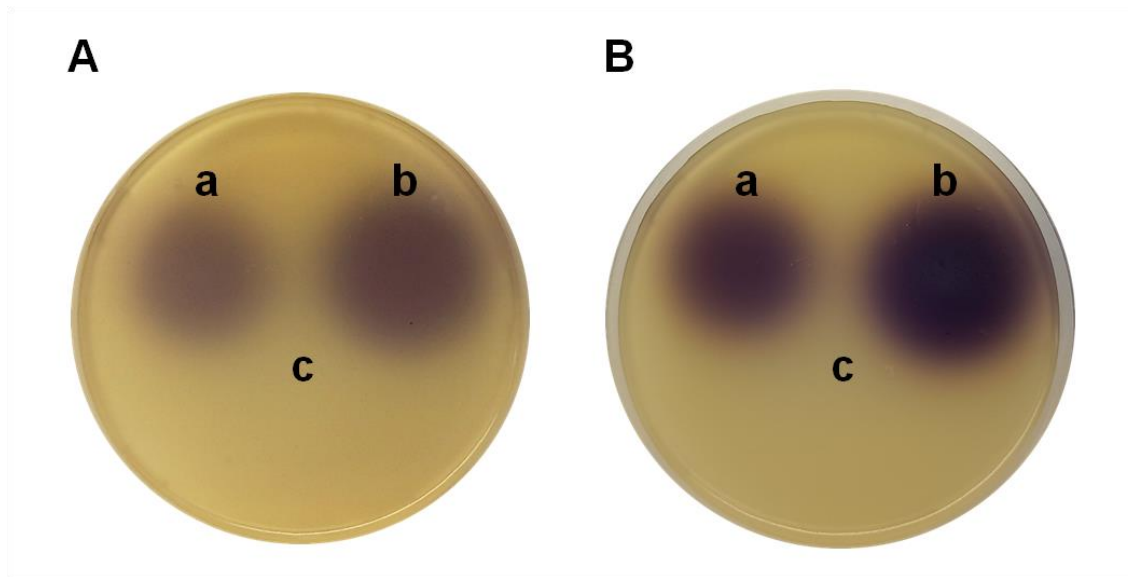
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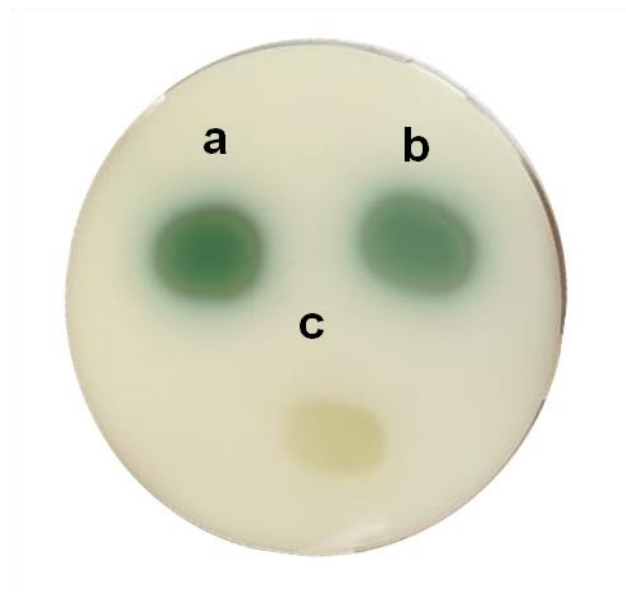


**Figure 1.** Workflow for the proposed laboratory class. Each gear represents the individual tests to be performed to get a complete, overall, and multi-focused approach.

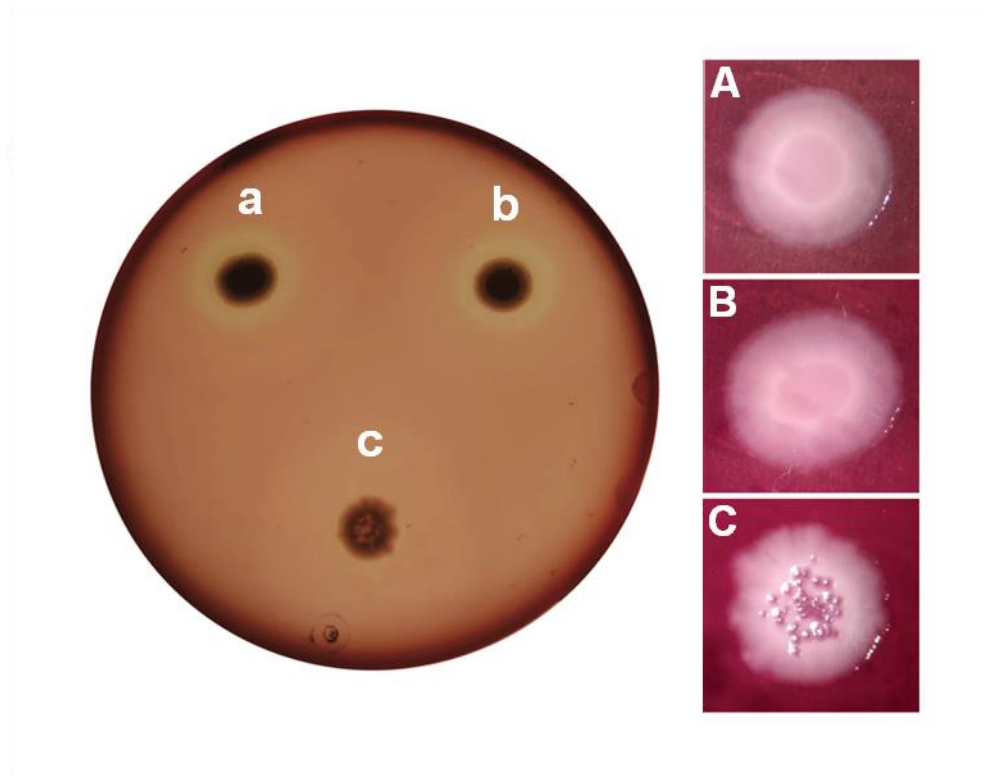


**Figure 2.** Analysis of short-chain AHLs in extracts obtained from samples withdrawn after 4 h (A) and 8 h (B) of culture. *P. aeruginosa* PAO1 (a), *P. aeruginosa* PAO1 (pME6000) (b) and *P. aeruginosa* PAO1 (pME6863) (c). Bioassay was developed using *C. subtsugae* CV026.

**Figure 3.** Analysis of long-chain AHLs in extracts obtained from samples withdrawn after 4 h (A) and 8 h (B) of culture. *P. aeruginosa* PAO1 (a), *P. aeruginosa* PAO1 (pME6000) (b) and *P. aeruginosa* PAO1 (pME6863) (c). Bioassay was developed using *P. putida* F177 (pKR-C12). Plates were observed in a blue light transilluminator.



**Figure 4.** Influence of QS on protease activity on skim milk agar. After 24 h of incubation, proteolytic activities are observed with *P. aeruginosa* PAO1 (a) and *P. aeruginosa* PAO1 (pME6000) (b). Lower or no activity is observed with *P. aeruginosa* PAO1 (pME6863) (c). Production of siderophores (see the characteristic green color) is also observed in a and b.



**Figure 5.** Influence of QS on hemolytic activity on blood agar. Hemolysis is observed as clear halos around the colonies (A). Wider halos of hemolysis are observed with *P. aeruginosa* PAO1 (a, A) and *P. aeruginosa* PAO1 (pME6000) (b, B), than with *P. aeruginosa* PAO1 (pME6863) (c, C). A direct observation of the colonies (right) allows also to distinguish differences in the morphologies of the colonies.

**Table 1.** Bacterial strains required for this laboratory work

Microorganism	Characteristics
<i>P. aeruginosa</i> PAO1	Wild type strain

<i>P. aeruginosa</i> (pME6000)	PAO1 harboring pME6000
<i>P. aeruginosa</i> (pME6863)	PAO1 harboring pME6863
<i>E.coli</i> S17.1 (pME6000)	Donor of control vector
<i>E.coli</i> S17.1 (pME6863)	Donor of QQ vector
<i>C. subtsugae</i> CV026	Biosensor of short-chain AHLs
<i>P. putida</i> F117 (pKR-C12)	Biosensor of long-chain AHLs

**Table 2.** Class design and organization of activities.

1 <sup>st</sup> week			
Day	Activities	Material provided	Theoretical concepts
1	-Conjugation	-Fresh plates of <i>P. aeruginosa</i> PAO1, <i>E. coli</i> S17-1 (pME6863), <i>E. coli</i> S17-1 (pME6000) - LB plates	-Bacterial conjugation as a tool for transferring genetic information
2	-Serial dilutions and planting	-Cetrimide agar plates supplemented with Tc -Sterile microcentrifuge tubes with physiological solution	-Relevance of antibiotics and cetrimide for the selection of transconjugants
3	-Isolation of transconjugants	-Cetrimide agar plates supplemented with Tc	
2 <sup>nd</sup> Week			
1	-AHLs extraction -Cultures of <i>C. subtsugae</i> CV026 and <i>P. putida</i> F117 -Cultures of <i>P. aeruginosa</i> PAO1, <i>P. aeruginosa</i> (pME6000) and <i>P. aeruginosa</i> (pME6863)	-Samples previously withdrawn of <i>P. aeruginosa</i> PAO1, <i>P. aeruginosa</i> PAO1 (pME6000) and <i>P. aeruginosa</i> PAO1 (pME6863) -LB broth. -NYB broth -Tc solution	-Relevance of ethyl acetate for the AHL extraction -Molecular mechanisms involved in the functions of biosensor strains
2	-Bioassays with <i>C. subtsugae</i> CV026 and <i>P. putida</i> F117 -Protease and hemolytic activity tests	-LB agar plates and melted LB soft agar -Skim milk agar blood agar plates -Sterile Petri dishes	-Bioassay as a strategy to detect the production of AHLs -Quorum Quenching as a strategy for disrupting Quorum Sensing regulation -Relevance of genetic regulation for the expression of different

		traits
3	-See results	-Global interpretation and final discussion