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Prospecting Microbial Strains for Bioremediation and Probiotics Development for Metaorganism Research and Preservation

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Federal University of Rio de Janeiro
Department of Environmental Microbiology
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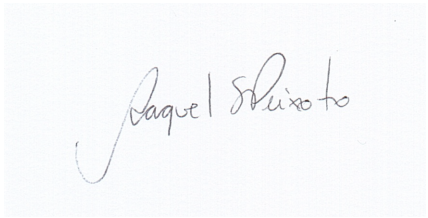
May 08, 2019

Dear Dr. Ronald Myers,

Marine ecosystems have been affected by different impacts. Global and local impacts, including global change and seawater pollution, are pointed out as some of the causes. There are several possible solutions to remediate marine pollutants and local contamination, and bioremediation techniques present several advantages, such as sustainability, relative low cost, and the fact they can be applied in different ecosystems, causing minimal or no impacts to the environment. As an extra advantage, the manipulation of endogenous microbiomes, including putative beneficial microorganisms for corals (pBMCs) – may have probiotic effects for marine animals. In this context, we suggest the use of the two approaches – bioremediation and pBMC inoculation – combined. This strategy would promote the degradation of specific pollutants, that can be harmful to corals and other marine organisms, while it may increase host resistance and resilience to deal with pollution and other threats. But a few groups have manipulated bacteria and managed to properly use conventional microbiology methods for the selection of marine probiotics. Hereby we submit the manuscript entitled "Prospecting Contaminant-degrading Microbial Strains Presenting Putative Beneficial Characteristics for Corals" where we focus on the selection of pBMCs to degrade two contaminants: the synthetic estrogen 17a-ethinylestradiol (EE2) and crude oil. Both have been reported to cause negative impacts on marine animals (including corals), and humans. First, we show how to isolate and test bacteria capable of degrading the specific contaminants. Additionally, we describe how to detect some putative beneficial characteristics of these associated microbes to their coral host. The methodologies described here are relatively cheap, easy to perform, and highly adaptable – almost any kind of soluble target compound can be used instead of EE2 and oil.

We believe the protocols will be very useful for people from diverse fields such as petroleum science, organic pollutants, environmental remediation, coral reefs' ecotoxicology, marine microbial ecology, technological mapping, etc. The authors also declare no conflict of interests.

Sincerely,



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TITLE:

Prospecting Microbial Strains for Bioremediation and Probiotics Development for Metaorganism Research and Preservation

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KEYWORDS:

microbial biodegradation, bioremediation, oil-degrading bacteria, ethinylstradiol-degrading bacteria, coral reefs, beneficial microorganisms for corals (BMCs), environmental probiotics, bioprospecting, ocean pollution, ocean conservation, oil pollution, endocrine disruptor

SUMMARY:

Pollution affects all biomes. Marine environments have been particularly impacted, especially coral reefs, one of the most sensitive ecosystems on Earth. Bioremediation is the capacity of organisms to degrade contaminants. Here, we describe methodologies to isolate and test microbes presenting bioremediation ability and potential probiotic characteristics for corals.

ABSTRACT:

Pollution affects all biomes. Marine environments have been particularly impacted, especially coral reefs, one of the most sensitive ecosystems on Earth. Globally, 4.5 billion people are economically dependent on the sea, where most of their livelihood is provided by coral reefs. Corals are of great importance and therefore their extinction leads to catastrophic consequences. There are several possible solutions to remediate marine pollutants and local contamination, including bioremediation. Bioremediation is the capacity of organisms to degrade contaminants. The approach presents several advantages, such as sustainability, relatively low cost, and the fact

that it can be applied in different ecosystems, causing minimal impacts to the environment. As an extra advantage, the manipulation of endogenous microbiomes, including putative beneficial microorganisms for corals (pBMCs), may have probiotic effects for marine animals. In this context, the use of the two approaches, bioremediation and pBMC inoculation combined, could be promising. This strategy would promote the degradation of specific pollutants that can be harmful to corals and other metaorganisms while also increasing host resistance and resilience to deal with pollution and other threats. This method focuses on the selection of pBMCs to degrade two contaminants: the synthetic estrogen 17a-ethinylestradiol (EE2) and crude oil. Both have been reported to negatively impact marine animals, including corals, and humans. The protocol describes how to isolate and test bacteria capable of degrading the specific contaminants, followed by a description of how to detect some putative beneficial characteristics of these associated microbes to their coral host. The methodologies described here are relatively cheap, easy to perform, and highly adaptable. Almost any kind of soluble target compound can be used instead of EE2 and oil.

INTRODUCTION:

Pollution is a major issue affecting human, animal, and plant health worldwide. Although pollution can be natural, such as volcanic ashes[1], human activities are the primary cause of most pollution. Anthropogenic activities are contaminating soil, water, and air, which directly or indirectly lead to almost 20 million premature human deaths[2] and decimate billions of other forms of life annually. Pollutants are present even in the most remote areas of the planet. For instance, heavy metals and persistent organic compounds have been detected in deep sea invertebrates and polar mammals, respectively[3,4].

Marine environments have been especially impacted by pollution. For a long time, it was assumed that the ocean would remain unaffected and supply an endless source of goods because of its massive volume of water[5]. For this reason, all types of industry and institutions freely released waste in water bodies for centuries[6,7]. Several contaminants of all types, such as plastic[8], synthetic hormones[9], pesticides[10], oil[11], nutrients[12], heavy metals[3], and radioactive waste[13] have been reported as impacting ocean ecosystems. In this context, coral reefs are among the most important and sensitive ecosystems in marine environments[14]. Reefs are coastal protectors, crucial to the development of thousands of marine species by playing essential roles in nutrients cycling and climate control. Reefs also contribute to the economy by providing fish, goods, and tourism, among others[15]. For instance, 4.5 billion people depend on ocean fish as their main food source[16], which are greatly supported by coral reefs.

Regardless of their ecological, social, and economic importance, coral reefs are being decimated[17,18]. Anthropogenic activities are primarily responsible for contributing to the three main causes of corals' death: climate change, overfishing, and water pollution[19]. Even though it is important to work on the mitigation of global warming, it is also important to work on minimizing local contamination, including water pollution, that can critically contribute to coral decline[20]. Thus, there is an urgent need for the development of strategies to increase corals' lifetime, which could provide them with extra time to adapt and survive.

In this regard, it is extremely important to find solutions to minimize contamination and to develop strategies to increase the fitness of corals. Strategies to remediate marine pollutants are highly diverse and can be grouped into physical, chemical, and biological approaches. Physical approaches are helpful. However, they are not always efficient. For instance, plastic waste can be minimized by physical removal, while water-soluble compounds need other methodologies to be eliminated. Examples of such compounds are crude oil, released by oil industry activities and spills, as well as other micropollutants, such as synthetic hormones, normally used as the estrogenic component in oral contraceptives and present in sewage[21,22]. The use of chemical substances to decrease contamination can solve a specific problem, but it may also represent an extra source of pollution. This is the case with chemical dispersants to mitigate oil contamination, which have been described as even more toxic to marine ecosystems than the oil contamination itself[23]. For these reasons, biological approaches present several advantages when compared to the other methods. Bioremediation is the capacity of living organisms, or their metabolic products, to transform contaminants into less toxic or non-toxic forms[24]. The main advantages of using biological methods are sustainability, relative low cost, the fact they are ecologically friendly, and that they can be applied in different ecosystems, causing minimal or fewer impacts to the environment[21,25–27].

Additionally, the manipulation of the microbial community present in an environment allows an extra potential advantage. There are microbiomes that are associated with hosts and are essential to their health. It is well known that these associated symbiotic microbiomes are necessary to maintain host homeostasis[19]. The manipulation of these associated microorganisms has been well explored for hosts such as plants and mammals[28,29], but the use of coral probiotics is still novel[15]. Corals also host, interact with, and depend on large and specific populations of microorganisms to survive[19]. The role of these microbial communities in the health and dysbiosis of corals is under active study, but it is still far from being fully understood[30]. One of the most popular hypotheses is called the coral probiotic hypothesis. It suggests the existence of a dynamic relationship between symbiotic microorganisms and environmental conditions which brings about the selection of the most advantageous coral metaorganisms[31]. Based on this information, key potential probiotic mechanisms, as well as the strategies for isolation, manipulation, and delivery of beneficial microorganisms for corals (BMCs) for several purposes, were proposed[32] and tested[33]. These potential beneficial characteristics include resistance to temperature increase, protection from reactive oxygen species (ROS), nitrogen fixation, resistance to contaminants, and biological control against pathogens, among others[32].

This study focuses on the selection of BMCs and free-living microorganisms presenting the ability to degrade two contaminants commonly found in marine environments: the synthetic estrogen 17a-ethinylestradiol (EE2) and crude oil. Pollutants containing hormone active agents are often present in water bodies[34–42]. Among them, synthetic estrogenic endocrine-disruptor compounds (EDCs) mimic the action of estrogens on target cells, causing several impacts on animals, including breast cancer, infertility, and hermaphroditism[9]. EE2 is excreted by humans because of the use of oral contraceptives. It is not removed from sewage by traditional wastewater-treatment plants and has negative effects even at very low concentrations (e.g., ng/L or µg/L)[43–45]. Little is known about the effects of estrogens on coral physiology[46,47]. However, on other marine invertebrates,

such as sponges, crustaceans, and mollusks, estrogens were reported to cause several negative effects mainly related to reproduction, such as development and/or stimulation of gametes, alteration in enzymatic and protein actions, problems in embryonic processes, and others[48–52]. The negative consequences caused by EE2 contamination highlight the necessity to develop sustainable approaches to remove this compound from the environment without impacting marine life.

In parallel, with oil currently accounting for almost 40% of the world's consumed energy sources[53], chronic contamination and oil spills often occur near reef areas[11]. Oil contamination was reported to cause negative effects in several species of marine animals, birds, plants, and humans[54–57]. On corals, it causes bleaching, reduces the resistance of larvae to thermal stress[58], disrupts the microbial associated communities[21], and causes tissue necrosis. In addition, chemical dispersants, an oil remediation technique commonly used by oil companies to remediate spills, are even more toxic to corals than the oil itself[23]. Beneficial microorganisms isolated from corals, in contrast, are known for playing crucial roles on host health. However, the manipulation of these potential probiotics must be better explored in order to investigate possible negative side effects and the metabolic capacities that can be screened to improve the fitness of the metaorganism. In this context, characteristics such as the antimicrobial activity against coral pathogens, the production of catalase to fight oxidative stress, the ability to degrade urea (which may have important roles in the calcification process), and the presence of genes that confer potential beneficial characteristics, among others, must be the focus of investigation. Here, we show how bioremediation and probiotics can be used to concomitantly mitigate the impacts of pollution and enhance coral health. The development of innovative approaches that can be used as interventions to increase marine species persistence represent a step towards a more sustainable and healthier planet.

PROTOCOL:

1. Water and coral collection and storage for microbial isolation

NOTE: It is essential to take the coordinates and temperature of the sampling sites. If possible, metadata such as salinity, pH, depth, and light intensity can also help in finding fine-tuned cultivation approaches and future interpretation of data. For reliable results, keep the samples stored for the minimum length of time possible. The water/coral microbiomes may change considerably if the samples are not kept at the right temperature and/or are stored for long periods. If the isolation step is not performed instantly after collection, it is crucial to maintain samples at 4 °C until processing. The longer the samples are stored, even at 4 °C, the more the microbial community will change.

1.1. Sample and store seawater.

1.1.1. Collect 500 mL samples of water in at least triplicate from each targeted sampling site. Preferably use sterile bottles with screw caps.

1.1.2. If processing the water instantly after collection, keep the bottles at RT for a short interval. If sample processing is happening later, keep the bottles at 4 °C.

1.2. Sample and store the coral.

1.2.1. Use a sterile pair of pliers to cut coral fragments from the same sampling site of the water samples. To avoid contamination, touch corals only with sterile gloves.

1.2.2. Rinse the sampled coral fragment using 20 mL sterile saline solution (3% NaCl in distilled water) or artificial seawater to get rid of the loosely attached free-living bacteria of the seawater.

1.2.3. Using forceps, place each coral fragment into a sterile 250−500 mL container with a screw cap containing sterile saline solution.

1.2.4. In the laboratory, using sterile forceps and pliers, weigh 5 g of coral fragments using sterile 100 mm x 20 mm Petri dishes on a weighing scale.

1.2.5. Transfer the 5 g of coral sample to a sterile mortar and macerate it using a sterile pestle.

1.2.6. Using a sterile spatula, transfer the macerated sample to a sterile culture flask containing 45 mL of 3% NaCl sterile solution and 10−15 glass beads of 5 mm. Use some of the 45 mL sterile saline solution to wash the mortar and recover the maximum amount of the macerate.

1.2.7. Keep the flasks under constant agitation (150 x *g*) for 16 h at the water temperature of the sampling site.

NOTE: For shallow-water corals the optimum temperature will range from 24−28 °C. This step will detach microorganisms from different coral compartments, such as the ones attached to the host cells, or the ones living inside the tissue and the skeleton. After this step, the coral macerates should not be stored, and the isolation step must be instantly performed.

2. Isolation of EE2-degrading bacteria from seawater and/or corals

2.1. Select bacteria.

NOTE: After steps 1.1.2 and 1.2.7, the concentration of microorganisms in seawater that have detached from the different coral macerates will be unknown and variable. In order to guarantee the isolation of individual microbial colonies in Petri dishes containing agar media, serial dilutions are needed.

2.1.1. Perform serial dilutions up to 10^{-9} in sterile saline solution for coral samples and up to 10^{-6} for water samples. Pipette dilutions up and down 5x before discarding the tip. Vortex samples for 5 s every time before performing the next serial dilution.

2.1.2. Pipette 100 µL of each dilution on Petri dishes containing 3% NaCl lysogeny broth (LB) agar medium, and plate them.

NOTE: Use marine agar (MA) as an alternative medium. Triplicates of each dilution are required for reliable results.

2.1.3. Incubate the plates for 1−3 days at the target temperature (e.g., 26 °C). Check the plates once a day.

2.1.4. Select and isolate the colonies presenting distinct growth morphologies on new plates using the streak plate technique. Repeat this step as many times as needed to have pure colonies growing on the plates.

2.1.5. If the procedure is not instantly continued to step 2.3.1, store isolates at 4 °C or in glycerol as described in section 2.2.

2.2. Prepare glycerol stocks.

NOTE: This step is optional and can be used for long-term bacterial stocks storage.

2.2.1. Pick single colonies from the fresh plate or from the plates stored at 4 °C and independently inoculate them in 2 mL of sterile LB medium.

2.2.2. Place tubes under constant agitation (150 x *g*) at 24−28 °C overnight (ON).

2.2.3. Add 1 mL of the bacterial cultures from step 2.2.2 and sterile glycerol to a final concentration of 20% to the 2 mL cryovials.

2.2.4. Leave the cryovials ON at 4 °C.

2.2.5. Place the glycerol bacterial stocks at -80 °C until needed.

2.3. Perform the EE2-degradation ability test.

2.3.1. Activate the isolates in LB broth or alternative media. For this, pick a single colony from the fresh plates or the plate stored at 4 °C, and inoculate 2 mL of sterile LB medium. In case it is a glycerol stock, first streak it on LB agar plates and incubate at 24−28 °C ON to have single colonies growing. Place the tube containing LB medium inclined under constant agitation (150 x *g*) at 24−28 °C ON.

2.3.2. After bacterial growth, pellet the cells by centrifuging them at 8,000 x *g* for 8 min at room temperature (RT). Discard the supernatant and, gently pipetting up and down, resuspend the cells in an equal volume (2 mL) of saline water to wash the remaining LB broth.

2.3.3. Repeat step 2.3.2 twice to guarantee that there are no traces of carbon source, resuspending the cells in an equal volume of saline solution. For instance, if it was started with a 2 mL culture, resuspend the cells in a final volume of 2 mL saline solution.

2.3.4. Inoculate the washed and resuspended cells in minimum Bushnell Haas culture medium (BH Broth) containing EE2 as the only carbon source[59].

NOTE: EE2 is dissolved in ethanol at a final concentration of 5 mg/L in the culture medium. Make changes in the pollutant type and/or concentration if needed.

2.3.5. Assess bacterial growth by optical density at 600 nm and/or colonies forming units (CFU) on LB agar medium[33], for 16–72 h of incubation.

NOTE: Alternatively, the microorganisms can be directly isolated on minimum media containing EE2, or other compounds, as the only carbon source. This step would direct the selection and avoid undesirable growth.

3. Isolation of oil-degrading bacteria from seawater and/or corals

3.1. Prepare minimum media containing an oil water-soluble fraction (oWSF) and oil water-insoluble fraction (oWIF) as the only carbon source[21].

3.1.1. Add 1–2% crude oil to 500 mL of sterile distilled water. Use a filter flask opened on the bottom to take the soluble fraction out without disturbing the upper layer of the insoluble fraction.

3.1.2. Keep the mixture under constant agitation at 24–28 °C at 150 x *g* for 48 h.

3.1.3. Place the filter flask containing the crude oil fractions on a stable surface and wait 10–20 min to allow soluble and insoluble fraction separation.

3.1.4. Transfer the oWSF to a new sterile flask, saving the oWIF by opening the bottom filter flask and taking out the soluble fraction.

3.1.5. Using all the oWSF recovered in the previous step (~400 mL), prepare 1 L of BH agar minimum medium containing oWSF as the only carbon source.

3.1.6. Using the oWIF remaining in the flask from step 3.1.4, prepare 1 L of BH agar minimum medium containing oWIF as the only carbon source.

3.2. Isolate oWSF- and oWIF-degrading bacteria.

3.2.1. Using water and coral macerate from steps 1.1.2 and 1.2.7, respectively, dilute the samples up to 10^{-6} in sterile saline solution as described in step 2.1.1.

3.2.2. Pipette 100 µL of each dilution on Petri dishes containing BH-oWSF and BH-oWIF agar media and plate them.

3.2.3. Repeat the procedures described from step 2.1.3 to step 2.1.5.

4. Consortium member selection

4.1. Extract and sequence the DNA for taxonomic identification.

4.1.1. Activate the isolates stocked in glycerol as described in step 2.3.1.

4.1.2. Extract the DNA using a DNA extraction kit (see **Table of Materials**).

4.1.3. Use primers 27f (5'-AGA GTT TGA TCA TGG CTC AG-3') and 1492r (5'-GTT TAC CTT GTT ACG ACT T-3') for amplification of the 16S rRNA gene.

4.1.4. Perform 50 µL PCR reactions according to the following protocol: 5 µL of 10x polymerase buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 5 mM of each primer, 10 ng of genomic DNA, and 2.5 U of Taq DNA polymerase. Add negative controls (i.e., blank DNA extractions and PCR reactions without template DNA) to ensure that there is no contamination.

4.1.5. Set up the following thermal cycling steps: a first denaturation cycle at 94 °C for 4 min; 35 cycles at 94 °C for 1 min, followed by 50 °C for 1 min, and 72 °C for 2.5 min; a final extension cycle for 10 min at 72 °C.

4.1.6. Check amplicon integrity in 1.2% agarose gel using 80 V.

4.1.7. Gel purify the samples using a gel purification kit (see **Table of Materials**).

4.1.8. Quantify PCR products using a fluorometer.

4.1.9. Send product for sequencing.

NOTE: For better taxonomical classifications, the Sanger method is recommended[60], because it provides long sequences. The universal primers 27f and 1492r can be used to amplify nearly the entire length of the 16S rRNA gene[61]. If contigs cannot be assembled using one pair of primers, an extra pair in the middle of the sequence should be considered.

4.2. Determine the growth curve.

4.2.1. Activate the isolates in glycerol stocks from step 2.2.5 as described in step 2.3.1 but using 5 mL instead of 2 mL.

4.2.2. Add 1% (v/v) of the grown 5 mL culture from step 4.2.1 in triplicates, to a 250 mL flask containing 100 mL of 3% LB media. Make sure to prepare triplicates of a negative control (no inoculum) as well.

4.2.3. Place the flasks in an incubator under constant agitation (150 x *g*) at 24−28 °C.

4.2.4. Take 1 mL aliquots every 4 h for 48 h. If the strains present a high growth rate, decrease the 4 h interval.

4.2.5. Measure the optical density (OD) estimation at 600 nm wavelength and colony forming units (CFU) counted from serial dilutions plated on rich media plates (100 µL should be inoculated in each plate and normalized to the volume of 1 mL).

4.2.6. Plot the OD and CFU curves and analyze the correlation of OD/CFU of each individual strain. From now on, calculate the number of cells based on the OD values.

4.3. Perform antagonism test.

4.3.1. Activate the isolates as described in step 2.3.1.

4.3.2. Inoculate one strain at a time along the middle of plates containing agar media and the other ones perpendicular to the central one. Repeat until every selected microbial strain is tested against all others.

4.3.3. Incubate the plates at 24−28 °C and monitor them daily in order to observe potential halos indicating antagonistic activity. If halos are observed indicating antagonistic activity between two strains, one of them should be excluded.

4.4. Perform consortium assembly.

4.4.1. Activate the isolates as described in step 2.3.1.

4.4.2. Pellet the cells at 3,500 x *g* and gently wash them 2x in an equal volume of saline solution. Resuspend the cells in an equal volume (i.e., if the final volume was 2 mL of cells, wash them using 2 mL of saline solution and resuspend them in a final volume of 2 mL).

4.4.3. Inoculate 1 mL culture in 100 mL 3% NaCl LB medium and incubate ON at 150 x *g*.

4.4.4. Repeat step 4.5.2, inoculate the 100 mL grown, washed, and resuspended cultures in 10 L cultures. Incubate ON in sterile air-lift bioreactors, receiving filtered air from a pump. If more culture is needed, always inoculate 1% of grown culture in fresh media.

4.4.5. Centrifuge grown cultures at 8,000 x *g* for 8 min at 4 °C. Discard supernatant after centrifugation.

4.4.6. Wash the pellet, gently resuspending the cells in 500 mL of sterile saline solution.

4.4.7. Repeat steps 4.5.5 and 4.5.6.

4.4.8. Resuspend each individual culture in 100 mL of saline solution and measure the OD to estimate the number of cells.

4.4.9. Calculate the volume of each culture necessary to reach a final concentration of 10^7 cells mL^{-1}.

4.4.10. Perform CFU counts on rich media for a final confirmation of cell viability and concentration.

4.4.11. Mix an equal volume of each individual culture in sterile flasks and aliquot the consortium into 50 mL sterile tubes.

4.4.12. Keep at 4 °C until inoculation.

NOTE: Prepare the consortium assembly as fresh as possible to guarantee cells will still be viable. Alternatively, CFU counts can be performed before inoculation. To assemble an ideal consortium, it is necessary to use isolates presenting different and complementary metabolic capacities. Usually, 6−10 isolates are used to form consortia, but this number will vary depending on the members' characteristics and purposes.

5. Detection of putative beneficial characteristics for corals

5.1. Activate the isolates from the glycerol stocks by streaking them on LB agar plates and incubating them at 24−28 °C ON to have single colonies growing. Pick a single colony from the plates and inoculate it in 2 mL of sterile LB medium. Place tube containing LB medium inclined under constant agitation (150 x *g*) at 24−28 °C ON.

5.2. Perform DNA extraction as described in section 4.1 for detection of potentially beneficial genes by PCR.

NOTE: PCR reaction conditions and primers will depend on the targeted gene. Methodologies to test BMC characteristics of individual strains and PCR detection for potentially beneficial genes are described in **Table 1**.

REPRESENTATIVE RESULTS:

Based on the methods described here, it was possible to isolate microorganisms from different water sources and coral nubbins presenting putative BMC characteristics and capable to degrade different classes of contaminants (**Figure 1**). Using water samples collected at a sewage treatment plant, obtained from CESA-UFRJ (Experimental Center of Environmental Sanitation of

the Federal University of Rio de Janeiro), and based on the procedure presented here, 33 bacterial strains able to degrade EE2 at a final concentration of 5mg/L were isolated (**Figure 2A**). Additionally, using the technique for selecting oil-degrading bacteria, 20 strains able to degrade both oWSF (**Figure 2B**) and oWIF (**Figure 2C**) were isolated.

Putative BMC characteristics were screened in microorganisms isolated from different coral species under diverse conditions. Among them, a strain presenting strong antagonistic activity against the coral pathogen *Vibrio coralliilyticus* (**Figure 3A**), strains able to degrade urea (**Figure 3B**), a good catalase producer (**Figure 3C**), and microorganisms presenting potentially beneficial genes (**Figure 3D**) were found.

Employing the two approaches combined (i.e., bioremediation and BMC inoculation), it was possible to protect corals from oil exposure impacts. For this, an oil bioremediator pBMC consortium, isolated from the coral *Mussismilia harttii*, was inoculated on coral nubbins exposed to 1% oil in triplicates[21]. The treatments exposed to oil presented a progressive decrease in Fv/Fm from the fourth day onwards, reaching values close to zero by the tenth day. Variable fluorescence/maximum fluorescence (Fv/Fm) provided a measure of maximal photosystem II (PSII) photochemical efficiency of the zooxanthellae, representing an indirect measurement of coral health. On the other hand, coral nubbins present in the aquariums inoculated with the consortium showed a better-preserved photochemical ability (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Summary of the main steps of a bioremediator-pBMC consortium selection and assembly. Scheme of pollutant-degrading microorganisms selection steps (in gray) and final steps used for the consortium microbial selection (DNA sequencing, growth curve, antagonism test, and consortium assembly in red).

Figure 2: Selection of pollutant-degrading bacteria. (**A**) Bacterial isolates growing on minimum media plates containing EE2 as the only carbon source. (**B**) Bacteria colonies growing on minimum media plates containing oWSF as the only carbon source. (**C**) Bacteria colonies growing on minimum media plates containing oWIF as the only carbon source.

Figure 3: Detection of pBMC characteristics. (**A**) Spots in triplicates of strain presenting antagonistic activity against the coral pathogen *Vibrio coralliilyticus* (in black) and a control strain (in green). (**B**) Strains growing on media containing urea as the only carbon source. (**C**) Strain producing catalase (+) and a bad catalase producer strain (-). (**D**) Example of PCR detection of the nirK gene (lane 1 = 1kb ladder; lane 2 = blank DNA extraction negative control; lane 3 = nirK detection; lane 4 = PCR reactions without template DNA).

Figure 4: Fv/Fm measurements of *M. harttii* nubbins dark-adapted at 5 PM, using a diving-PAM chlorophyll fluorometer. Fv/Fm measurements of the treatments control consortium, oil, and oil with consortium were performed in triplicates every day for 10 days. Standard deviation is shown. Features of the graph were modified with permission from previous results[21], available

Table 1: Detection of putative BMC characteristics, mechanism of action (MOA), reported microorganisms presenting the potential and technique used to detect the characteristic.

DISCUSSION:

Bioremediation approaches have been massively explored over the past 50 years. For instance, over 200 microbes among bacteria, cyanobacteria, microalgae, and fungi in several different habitats, have been designated as able to indicate the presence and/or degrade oil hydrocarbons[62–64]. Additionally, other classes of compounds that cause impacts to the environment and to humans, such as plastic, bisphenol A, endocrine disruptors, and heavy metals, are targets for bioremediation technique development[65–67]. On the other hand, marine probiotic development has been limited to the fields that have an obvious impact on the economy, such as fish probiotics in aquaculture[68,69]. However, isolation and characterization of beneficial microorganisms to protect coral reefs, marine ecosystems that support fishery, tourism, and other profitable activities, are starting to be valued[15]. Here, a cheap, easy, and accessible protocol to select pollutant-degrading microorganisms that can also present potentially beneficial characteristics to local marine ecosystems, especially putative beneficial microorganisms to corals (pBMCs), is described.

Additionally, the method demonstrated here is highly adaptable to several compounds and diverse types of microbial sources. It is possible to target different pollutants by replacing the only carbon source added to the minimum media. For this, instead of oil or EE2, other compounds should be added at the desired concentration. This would be the selective pressure to isolate degraders for the targeted pollutants. For instance, microorganisms capable of degrading other classes of endocrine disruptors have been already selected and tested using the same methodology[70]. Moreover, other marine and terrestrial organisms, such as sponges and plants[71,72], as well as distinct types of environmental samples, such as soil, fuel, and rocks can be used as the degrading-microbial sources[25,73,74]. For instance, it was possible to detect and isolate hydrocarbon-degrading bacteria from different soil and sediment samples[25,54,63,64,75]. Finally, performing slight modifications in the media, microorganisms other than bacteria can be easily selected as the degrading-microbes. For instance, a microalgae strain with the ability to efficiently degrade estrogen compounds has been reported[76].

Ideally, bioremediation-probiotic consortia must be assembled for each specific compound or area. Microbes that grow in a specific environment may not grow as well in new sites compared to their native conditions. Because researchers have not found a product that can be efficiently applied under all different environmental conditions, new consortia assembly should be performed for each specific situation. This would be akin to personalized medicine for environment-tailored recovery. For this reason, the creation of a central bank of microbial strains with potential probiotic characteristics and degradation capacity is a crucial step for the progress of this field. This initiative would save time and work, contributing to the assembly of new specific consortia worldwide.

Microorganisms associated with corals (i.e., microalgae, bacteria, archaea, fungi, and viruses) have a complex and intricate role in maintaining host homeostasis[19]. Environmental stressors, such as pollution, can also destabilize the coral microbiome, resulting in dysbiosis, which may cause disease and mortality[30]. The mechanisms by which the coral microbiome may support coral health are starting to be revealed. These mechanisms are the key to understanding coral resistance and resilience to environmental stressors and, consequently, to promote reef persistence and preservation. Additionally, findings in the field will help to understand general host-microbiome interactions, which may contribute to the development of better probiotics and health-promoting strategies in other areas. It is also important to better investigate how these probiotics inoculations can interfere on the metaorganism's health during stress events. For instance, work showing that the augmentation in coral performance is due to the probiotics and not simply the coral using bacteria as a food source are still needed.

In parallel, the development of new consortia delivery approaches and the improvement of the existing ones are of great importance. Alternative methods for consortium immobilization as well as innovative approaches, such as inoculating coral food (i.e., artemia and rotifers) and using them as vectors, are promising. These delivery systems can also be modified to target other marine organisms and will be essential to the success of the marine probiotics field.

Pollution mitigation and coral reef persistence are currently two of the main topics highlighted in environmental conferences regularly. The Agenda 2030, a document published by the United Nations that describes the global goals society should reach to allow a sustainable future, dedicates specific goals for each issue. While Goal 6 highlights the importance of water quality improvement by reducing pollution, Goal 14 reinforces the relevance of conservation and sustainable use of the oceans, seas, and marine resources[77]. In this context, coral reef conservation depends on changes that should be achieved in the near future, including pollution mitigation. This is of great importance, because most massive coral losses occurred when other factors were added to climate events, such as local habitat destruction and contamination[78,79]. This paper demonstrated that it is possible to combine bioremediation and pBMC inoculation to degrade specific pollutants, while it may increase coral's resistance and resilience to deal with pollutants and other issues. The optimization of existent protocols and/or the development of innovative methods, combined or independently applied, will be crucial to determine the future of marine ecosystems.

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The authors have nothing to disclose.

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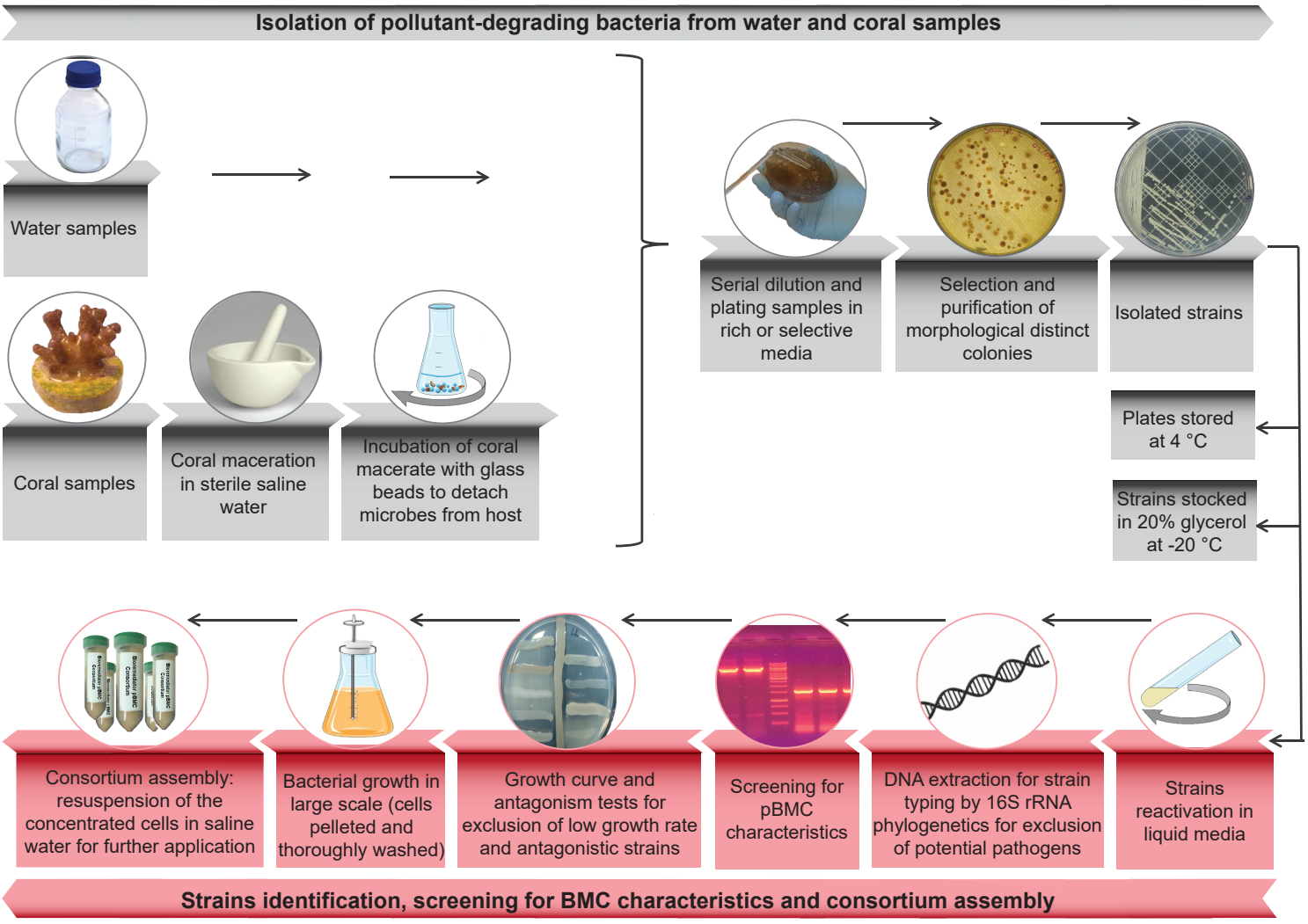
Figure



pBMC characteristic	MOA
Degradation of dimethylsulfoniopropionate (DMSP)	Climate regulation; surfur cycling; antimicrobial compounds; increase antioxidant protection of cells.
Antimicrobial activity	Biological regulation of pathogens.
Urea uptake (urease production)	Benefit to the calcification process; source of nitrogen for scleractinian corals.
Nitrogen fixation (*NifH* genes)	Nitrogen cycle; increase nitrogen fixation.
Denitrification (*NirK* genes)	Nitrogen cycle; decrease of the ammonium concentration.
Production of antioxidant compounds (catalase, superoxide dismutase, etc.)	Holobiont protection against reactive oxygen species (ROS).

Microorganism	Detection technique
Aspergillus sydowii	PCR for *dddP* gene
Pseudovibrio sp. P12	Culture medium with DMSP
Pseudoalteromonas sp.	PCR for *dmdA* gene
Microbiome from *Acropora palmata* mucus	Clear zone of inhibition
Extracts from corals	Growth inhibition assay
Marinobacter sp.	Swarming assays
Pseudoalteromonas sp.	Agar plate cross-streaking
Pseudoalteromonas sp.	Agar-diffusion method
Symbiodinium spp.	Colorimetric method
Microbiome from *Stylophora pistillata* mucus	___
Microbiome from *Acropora alciminata*	Method by Bolland et al.[80]
Microbial community	qPCR
Microbial community	PCR
Microbial community	qPCR
Microbial community	Adapted acetylene (C_2H_2) reduction technique
Pseudoalteromonas sp. and *Halomonas taeanensis*	PCR
Pseudoalteromonas sp.	PCR
Microbiome from Tubastraea coccinea	PCR
Microbiome from Xestospongia testudinaria	Predictive metagenome analysis
Pseudoalteromonas sp., *Cobetia marina* and *Halomonas taeanensis*	Catalase test
Symbiodinium spp.	Amplex red
Vibrio pelagius and *Sync- chococcus* sp.	Horseradish peroxidase-scopoletin method
Vibrio fischeri	Multiple methods

References
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Name of Material/ Equipment	Company
500 mL PYREX Media Storage Bottle	thomas scientific/Corning
500 mL Aspirator Bottles	thomas scientific/Corning
6-inch wire cutter plier	thomas scientific/Restek
17a-Ethinylestradiol	LGC Standards
Agar	Himedia
Bushnell Haas Broth	Himedia
Erlenmeyer Flask	thomas scientific/DWK Life Sciences (K
GFX PCR DNA and Gel Band Purificatio	GE Healthcare
Glass Beads	MP Biomedicals
Laminar Flow Hood	
Luria Bertani Broth, Miller (Miller Luri	Himedia
Marine Agar 2216 (Zobell Marine Agar)	Himedia
Orbital-Shaker Incubator	
Plates Incubator	
Porcelain Mortar and Pestle	Thomas scientific/United Scientific Sup
Qubit 2.0 Fluorometer	Invitrogen
Refrigerated Centrifuge	
Spectrophotometer	
Wizard Genomic DNA Purification kit	Promega

Catalog Number

1743E20/1395-500

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DRE-C13245100

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M1245-1KG

M384-500G

1201U69/JMD150

A1120

Comments/Description
Used to sample water.
Used to separate the oil fractions.
Used to cut coral fragments.
Used as the only carbon source to make the selective media.
Used to make solid media.
Used as minimum media to be supplemented with carbon sources.
Used to incubate coral macerate with glass beads.
Used to purify PCR products before sending them for sequencing.
Used to detach the microorganisms from coral structures.
Needed to work at sterile conditions.
Used as rich media to grow bacteria.
Used as rich media to grow bacteria.
Used to incubate liquid media and oil.
Used to incubate plates.
Used to macerate coral fragments.
Used for nucleic acids quantification of DNA and PCR products.
Used to centrifuge bacterial cultures.
Used to measure optical density of bacterial cultures.
Used for microbial strains DNA extraction.

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Kind regards,

Paloma Hammond
Rights Assistant

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Author(s): Villela H DM; Vilela C; Vasena N; Burke C; Coil D; Eren AM; Peixoto RS

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Editorial Comments:

1. Table of Materials: Please list information of all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol (e.g., DNA extraction kit, gel purification kit, fluorometer, etc.). Please remove any ™/®/© symbols and sort the materials alphabetically by material name.

A: Changes were made in JoVE Materials Table according to the recommendations.

2. Figure 1: Please fix typo (e.g., glycerol instead of glicerol).

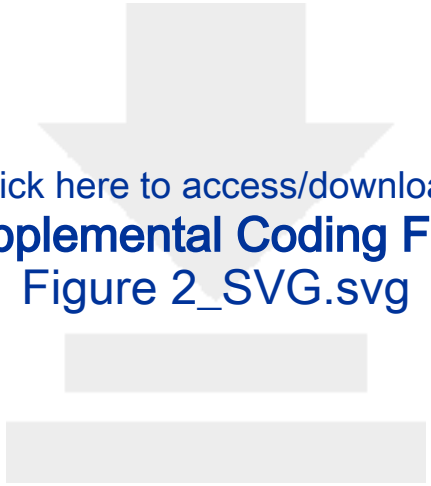
A: Figure 1 was fixed according to the recommendation.

3. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). Please save any .ai files as a .pdf for submission but maintain .ai files for production purposes. If submitting as a .tiff or .jpg (not preferred), please ensure that the image is 1,440 pixels x 480 pixels or 300 dpi.

A: All figures were saved in .svg format.

Figure 1_svg

Figure 2_svg



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