

ARTICLE / INVESTIGACIÓN

Microorganisms isolated from seabirds feathers for mercury bioremediation

Lorena Monserrate-Maggi¹, Lizette Serrano-Mena¹, Louise Delahaye², Paola Calle³, Omar Alvarado-Cadena³, Omar Ruiz-Barzola^{3,4}, Juan Manuel Cevallos-Cevallos^{1,3*}

DOI. 10.21931/RB/2023.08.01.19

¹ Escuela Superior Politécnica del Litoral, ESPOL, Centro de Investigaciones Biotecnológicas del Ecuador, CIBE, Guayaquil, Ecuador.² VIVES University College Post-graduate International Cooperation North-South, Kortrijk, Belgium.³ Escuela Superior Politécnica del Litoral, ESPOL, Facultad de Ciencias de la Vida, FCV, Guayaquil, Ecuador.⁴ Universidad de Salamanca, Departamento de Estadística; Campus Miguel de Unamuno, Salamanca, España.Corresponding author: pccalle@espol.edu.ec

Abstract: Environmental pollution caused by mercury has received increasing attention in recent years. Several studies have warned of the high rates of biomagnification in superior levels of marine food networks affecting seabirds. Although seabird feathers are reported as bioindicators of mercury, the possibility of using the microbiota associated with them for the bioremediation of this metal has not been considered. Despite the potential of the seabird feather microbiota, the cultivable microorganisms from this sample matrix have not been identified. In this study, we isolated and identified the organisms in the feathers from three types of seabirds, two species of penguins (*Pygoscelis antarctica* and *Pygoscelis papua*) and the brown skua bird (*Catharacta lonnbergi*) through poisoned media a final concentration of 10 mg / L Hg²⁺ in the culture medium for the microbial consortia. Yeast isolates belonged to the genus *Debaryomyces*, *Meyerozyma*, *Papiliotrema*, and *Rhodotorula*, and fungi genera *Leiotrametes*, *Penicillium*, *Pseudogymnoascus*, and *Cladosporium* were identified. Adult bird feathers with high mercury concentrations can serve as a matrix to isolate microorganisms capable of removing mercury.

Key words: Antarctica, bioremediation, feathers, mercury, microorganisms.

Introduction

Mercury (Hg) is among the most severe pollutants due to its accumulation in food chains, resulting in risks to human, animal, and environmental health¹⁻⁴. The atmospheric transport of this metal affects the most remote and cleanest areas of the planet, such as the polar zones, reaching even higher levels of deposition than in other parts of the world⁵⁻⁸, thus affecting the aquatic ecosystems of Antarctica^{9,10}.

The elemental mercury (Hg⁰) and ionic mercury (Hg²⁺) that reach Antarctica fall on sediment and water bodies, while some elemental mercury remains dissolved in the water column. Another part of mercury is transformed by microorganisms, through the biomethylation process, to a more toxic organometallic compound, the methylmercury (CH₃Hg⁺), which will be bioaccumulated and biomagnified along the marine trophic chain¹¹. With a trophic magnification factor (TMF) of 4 to 8 for each step of the trophic level^{12,13}, the amount of CH₃Hg⁺ in predatory species can be up to 100 times higher than their primary food source so that birds, among other species, are the most exposed in the marine ecosystems of Antarctica⁹. In addition to its high position in the food chain, the bioaccumulation of Hg in birds is favored by factors such as its wide distribution, population variety, long life cycles, and its type of diet^{14,15}.

Although in Antarctica there is no industrial development that contributes to mercury emissions^{4,6,16}, its proximity to the southern hemispheres, the tourism, the pollution from logistics activities of scientific stations¹⁷⁻²⁰ as well as contamination of natural origin from volcanic activity^{10,21} contribute to the increase of Hg in predatory seabirds from different

locations in this continent^{14,22}.

Complex communities of microorganisms are found in birds' feathers whose composition can be influenced by exposure to heavy metals²³. It is well known that organisms living in contaminated or toxic conditions have developed different mechanisms to adapt to high levels of various forms of mercury present in the environment and can be used for bioremediation or mitigation of the contaminant²⁴⁻²⁶. Bioremediation is an option that uses those strategies that microorganisms have developed to deal with Hg, with exceptional advantages that include high efficiency, low cost and environmentally friendly²⁷. Therefore, it is essential to identify the microorganisms living in high-mercury environments²⁸⁻³⁰.

Current research primarily focuses on the ability of Antarctic seabird feathers to act as bioindicators of Hg³¹⁻³³ or in the isolation of microorganisms from soils and water contaminated with Hg^{24,25,27-30}. However, the microbiota associated with bird feathers having high mercury levels has not been fully described.

Therefore, the objective of this study was to identify the culturable microorganisms from the feathers of three Antarctic seabirds known to biomagnify mercury, including the geentoo penguins *Pygoscelis papua* and chinstrap *Pygoscelis antarctica* and the skuas brown *Catharacta lonnbergi*, which inhabit the surroundings of the Pedro Vicente Maldonado Scientific Station in Antarctica, as a first step that can aid further mercury bioremediation studies.

Citation: Monserrate-Maggi, L.; Serrano-Mena, L.; Delahaye, L.; Calle, P.; Alvarado-Cadena, O.; Ruiz, O.; Cevallos-Cevallos, J., Microorganisms isolated from seabirds feathers for mercury bioremediation. *Revis Bionatura* 2023;8 (1)19. <http://dx.doi.org/10.21931/RB/2023.08.01.19>

Received: 26 September 2022 / **Accepted:** 15 October 2022 / **Published:** 15 March 2023

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Materials and methods

Sample collection

The present investigation corresponds to an exploratory study using a purposive (judgmental) sampling method. The seabird feather samples were collected during the scientific expeditions to Antarctica carried out by the Instituto Antártico Ecuatoriano (INAE) during the summer of 2013 and 2014. The sampling was carried out in the surrounding areas of the Ecuadorian Scientific Station Pedro Vicente Maldonado (PEVIMA), located in the South Shetland archipelago of the Antarctic Peninsula. The islands evaluated were Barrientos (n = 2 sites), Dee (n = 1 site) and Greenwich (n = 2 sites). Figure 1 shows the sampling sites.

The molting feathers were collected using a non-invasive method and following the guidelines of the Antarctic Treaty (1959), in which animal welfare is preserved and the capture of living individuals is avoided (34). Therefore, fallen feathers were randomly collected in nests and colonies of three bird species: *P. antarctica* and *P. papua*, corresponding to chicks and adults with feathers lengths measured between 3-6 cm, while *C. lonnbergi*, compared to juveniles and adults with feathers lengths measured between 15-30 cm. The description of the samples is shown in table 1.

Year	Bird feathers isolation	Island	Sample
2013	<i>Pygocelys antarctica</i>	Barrientos	1
	<i>Pygocelys papua</i>	Barrientos	2
	<i>Chataracta lonnbergi</i>	Greenwich	3
	<i>Chataracta lonnbergi</i>	Barrientos	4
2014	<i>Chataracta lonnbergi</i> data	Dee	5
	<i>Chataracta lonnbergi</i> data	Greenwich	6
	<i>Pygocelys antarctica</i>	Barrientos	7
	<i>Pygocelys papua</i>	Barrientos	8

Table 1. Origin of the collection of feathers by years.

The samples were collected in Ziploc bags and delivered to the PEVIMA station laboratory, where they were rinsed with deionized water, dried at room temperature, wrapped in aluminum foil, and kept in the freezer (-20°C) until they were analyzed at the Centro de Investigaciones Biotecnológicas del Ecuador (CIBE-ESPOL) in Guayaquil, Ecuador.

Isolation of microorganisms from feathers

The feathers were subjected to an individual cleaning process in which the barbs exposed to external conditions and/or feather age, which could influence the interpretation of the results, were removed^{35,36}. The rachis was cleaned with hypochlorite solution (30%) for 30 seconds and immersed in 99% and 70% ethanol for 30 seconds each. Finally, they were rinsed with plenty of ultrapure water. The rachis of the feathers was then ground under liquid nitrogen (LN2) in a porcelain mortar and collected in 15-ml falcon tubes to enrich microorganisms in the feathers.

For the enrichment process of the samples, 1 gr of the crushed sample was weighed and placed in a 15ml falcon tube with 9ml of liquid culture medium. The culture media used were Peptone water (AP, Oxoid, Thermo Scientific, USA), Luria Bertani (LB, Oxoid, Thermo Scientific, USA), and Potato Dextrose Broth (PDB, Oxoid, Thermo Scientific, USA) that were previously autoclaved at 121 °C for 25 minutes. Then, the samples were incubated at 10 °C for seven days with constant shaking at 110 rpm (Innova 44R, New Brunswick, USA).

Then, to determine the tolerance of microorganisms to Hg, the reference standard of inorganic mercury, Hg2+ (HACH, Germany), was added, using aseptic techniques, at a concentration 10 times higher than that reported in certain feathers of Antarctic birds³¹ and taking into account the toxicity threshold for adverse effects in seabird feathers of between 5-30 mg / L as reported by some authors^{14,37}. For this reason, a final concentration of 10 mg / L Hg2+ in the culture



Figure 1. The geographical location of the sampling sites.

medium was used for the microbial consortia and 5 mg / L Hg²⁺ for the isolates cultured from the microbial consortia. The three poisoned media without samples were used as a blank, and each treatment was performed in duplicate.

Isolation and molecular identification of microorganisms present in the consortia feathers

Seven days after adding Hg to the microbial cultures of bird feather consortia, 100 µl of the culture were taken and dispersed in Petri dishes previously prepared with 57.5 g / L of Potato Dextrose Agar medium (PDA, Oxoid, Thermo Scientific, USA) plus 10 mg / L Hg²⁺, and incubated at 10 °C for isolation of cultivable microorganisms. After this, the obtained strains were separated into yeasts and fungi according to their macro and micromorphology.

The DNA extraction from isolates was carried out using a rapid fungal DNA extraction protocol according to Cenis (1992)³⁸. Molecular identification was performed by PCR amplification and sequencing of the internal transcribed spacer regions (ITS1, 5.8S, and ITS2) using ITS1 (TCCG-TAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers, the samples that did not amplify, a PCR of nesting using primers ITS3 (GCTTCGATGAAGAACGCA-GC) and ITS4. The master mix for both PCRs was: 1X PCR buffer, 0.2 mM dNTP, 1.5 mM MgCl₂, 5 U / µL Taq polymerase (ThermoFisher, USA), 0.4 µM of each primer, and the DNA concentration comprised 15-20 ng / µl. The program in the thermal cycler (Eppendorf, Mastercycler Nexus GSX1-6345, Germany) for the first set of primers consisted of initial heating of 1 minute at 94 °C, followed by 30 cycles of 1 minute at 94 °C, 1 min at 55 °C, 1 min at 68 °C, and a final extension for 3 min at 68 °C. For the second set of primers, the PCR conditions were: 6 min at 95 °C, 30 cycles from 0:30 min to 95 °C, 0:30 min to 55 °C, 0:30 min to 70 °C, followed by a final extension of 0:30 min at 72 °C. Amplification was verified by electrophoresis of a 1.5% agarose gel in 1X TAE solution (Tris base, boric acid, and 0.5M EDTA, pH 8.0), loading 5 µL of PCR product with 1 µL of loading dye (Loading dye, Promega, USA) at 100 volts for 30 minutes. The size of each DNA fragment was estimated using a 100 bp DNA marker (cat. 15628050, Invitrogen™). Gel images were analyzed using the Gel Doc XR Imager program (Bio-Rad, Philadelphia, PA).

The obtained PCR products were sequenced by Sanger at Macrogen, Inc., an external laboratory in South Korea, according to Genetic Resource Access Contract No MAE-DNB-CM-2017-0059, material transfer agreement MAE-DNB-CM-2017-0059-000-ATM-0001, and sample export authorization No 074-17-EXP-IC-FAU-DNB/MA granted by the Ministry of the Environment of Ecuador.

The chromatograms of the DNA sequences of the different isolates were visualized and edited manually using the program Finch TV version 1.4.0 (Geospiza Inc.) and then compared with the database of the National Center for Biotechnological Information (NCBI) using BLAST. The sequences were deposited in the GenBank public database (<http://www.ncbi.nlm.nih.gov/GenBank>), and the isolates collection resides in the Microorganism Culture Collection of CIBE at Escuela Superior Politécnica del Litoral (http://www.wfcc.info/ccinfo/index.php/collection/by_id/1151/).

Results and discussion

Seabirds' bioaccumulation mercury has been repor-

ted from different locations in the Antarctic and including in petrel feathers *Pagodroma nivea* 0.54 ± 0.18 µg g⁻¹ dry wt¹⁸, antarctic petrel *Thalassoica antarctica* 2.71 ± 0.25 mg g⁻¹ dw³⁹; Gentoo penguins *Pygoscelis papua* 1.83 ± 0.80 µg g⁻¹ dw^{31,40-42}; Chinstrap penguins *Pygoscelis antarctica* 1.53 ± 0.08 µg g⁻¹ dw^{31,40,41}, *Pygoscelis adeliae* 0.82 to 1.40 ± 0.13 µg g⁻¹ dw^{18,40,41}, *Aptenodytes forsten* 0.98 ± 0.2 µg g⁻¹ dw; skuas *Catharacta maccormicki* 2.91 ± 1.93 µg g⁻¹ dw¹⁸, *Catharacta lonnbergi* 2.86 ± 2.60 µg g⁻¹ dw³¹ and gull *Larus dominicanus* 426.6 ng g⁻¹.

In this study, isolated species of seabirds used in this study corresponding to the following yeast genera were identified: *Debaryomyces*, *Meyerozyma*, *Papiliotrema*, and *Rhodotorula* also fungi genera: *Leiotrametes*, *Penicillium*, *Pseudogymnoascus*, and *Cladosporium*. The most abundant species in the bird consortiums correspond to yeasts of the genus *Debaryomyces* followed by the fungi genus *Pseudogymnoascus* and *Penicillium*. Table 2 and table S1. Some of the yeasts belonging to the *Rhodotorula* and family *Saccharomycetaceae* and fungi between these *Cladosporium* and *Penicillium* have been reported with efficient accumulation strategies and biovolatilization of mercury regardless of their origin. They are considered suitable for application in remedial technology⁴³⁻⁴⁵.

Regarding the efficiency of the culture media for metal removal, they were AP and PDB, and the fact that only yeasts and fungi have been isolated may be due to these culture media being a broad spectrum range favoring the growth of these groups⁴⁶.

On the other hand, the differences in the level of resistance to metals among genera and strains depend on different growth requirements (such as temperature, pH, and nutrients), biological function⁴⁷, or pressure origin⁴³.

One of the mechanisms responsible for removing mercury in the medium from cultivable isolated may be due to the biosorption capacity of the fungal cell wall, which contains polysaccharides with reactive functional groups, amino, carboxyl, and phosphate. Of these, it is known that the carboxyl and phosphate groups carry negative charges that allow fungal cell wall components to be highly metal ion-retaining⁴⁸. The peptide links of nitrogen and oxygen could be accompanied by the displacement of protons, depending on the pH, which also favors the removal of the metal^{49,50}. The number of available binding sites determines metal biosorption⁵¹. On the other hand, fungal mycelium secretes many extracellular enzymes and acids that decompose metals and has a huge potential for degrading contaminants^{45,52,53}.

Urik *et al.* (2014)⁴³ indicated fungal mercury uptake increases linearly with increased initial media mercury concentration until a threshold concentration near 8.2 mg L⁻¹. When the amount of mercury remaining in media with higher than threshold concentration decreased by 75 % or more, sorption via mercury immobilization on the fungal cell wall and bioaccumulation in the intracellular compartments play insignificant roles in mercury resistance strategy; hence the fungal necessity to trigger other detoxification mechanisms confirm that biovolatilization is the main mechanism of detoxification of mercury by fungal strains. Also, some authors confirm that biovolatilization is the primary mechanism of the detoxification of mercury by fungal strains⁴³.

Other fungi strains isolated from soil samples like *Aspergillus niger* removed more than 90% and proved an excellent mercury absorber. *Aspergillus flavus* strain and *Cladosporium* can eliminate more than 90% of 10 mg L⁻¹ of initial mercury concentration in static culture for 7 days

Samples	Closest related taxon (blast search)	Similarity (%)	Source
1AP3, 1PDB3 2LB3, 2PDB3 3LB3, 3PDB3 4AP3 5PDB4 6PDB4 7PDB4 8AP4, 8LB4	<i>Debaryomyces hansenii</i> (MK394104.1)	100	1: P.antartica- I. Barrientos 2013 2: P.papua- I. Barrientos 2013 3: C.lonnbergi-I.Barrientos 2013 4: C.lonnbergi-I.Greenwich 2013 5: C.lonnbergi-I.Deer 2014 6: C.lonnbergi-I. Greenwich 2014 7: P.antartica- I. Barrientos 2014 8: P.papua- I. Barrientos 2014
2AP3	<i>Meyerozyma guilliermondii</i> (MH986817.1)	100	2: P.papua- I. Barrientos 2013
5RAP4	<i>Papiliotrema flavescens</i> (FN428902.1)	100	5: C.lonnbergi-I.Deer 2014
6PDB4			6: C.lonnbergi-I. Greenwich 2014
6PDBP4	<i>Papiliotrema terrestris</i> (NG_062961.1)	100	6: C.lonnbergi-I. Greenwich 2014
4APR4	<i>Rhodotorula mucilaginoso</i> (KY104874.1)	100	4: C.lonnbergi-I.Greenwich 2013
5RLB4	<i>Cladosporium cycadicola</i> (NR_156279.1)	100	5: C.lonnbergi-I.Deer 2014
2LB3 4LB3, 4PDB4 5PDB4 7RAP4 8AP4, 8PDB4, 8RPDB4	<i>Pseudogymnoascus pannorum</i> (MH864459.1 y MH864756.1)	100	2: P.papua- I. Barrientos 2013 4: C.lonnbergi-I.Greenwich 2013 5: C.lonnbergi-I.Deer 2014 7: P.antartica- I. Barrientos 2014 8: P.papua- I. Barrientos 2014
1RPDB3	<i>Leiotrametes flavida</i> (KC589130.1)	100	1: P.antartica- I. Barrientos 2013
1RPDB3 6AP, 6PDB4 8PDB4	<i>Penicillium adametzioides</i> (LT558904.1, KT279815.1, NR_103660.1 y AF033403.1)	100	1: P.antartica- I. Barrientos 2013
			6: C.lonnbergi-I. Greenwich 2014
			8: P.papua- I. Barrientos 2014
1PDB3	<i>Penicillium bialowiezense</i> (MH854996.1)	100	1: P.antartica- I. Barrientos 2013
2LB3 3PDB3 5RPDB4	<i>Penicillium brevicompactum</i> (KF465776.1)	100	2: P.papua- I. Barrientos 2013
			3: C.lonnbergi-I.Barrientos 2013
			5: C.lonnbergi-I.Deer 2014
4AP_75 5RAP_55	<i>Penicillium sp.</i> (MW018928.1)	100	4: C.lonnbergi-I.Greenwich 2013
			5: C.lonnbergi-I.Deer 2014

Table 2. Molecular identification according to ITS region of the cultivable isolates.

and have been reported with biovolatilization efficiency rendering them the most suitable for application in remedial technology^{43,54,55}.

The precise fungal mercury volatilization mechanism is not currently elucidated, but it most likely involves some intra or extracellular reducing factor and/or methylation agent when considering mercury volatilization in dimethyl form^{56,57}. However, it should not be ruled out that another mechanism of action of the cultivable isolates proposed by Kelly *et al.* (2006) where mercury deposition as HgS in microfungi dominates at low mercury concentrations⁵⁸.

Findings focused on bioremediation, comparing the use of consortia (multiple or heterogeneous systems) with pure isolates (homogeneous systems), describe the advantages of living in the community. Many factors can influence passive and active mechanisms in the removal of metals, as well as considering the relationship with the use of car-

bon sources and biodegradation processes⁵⁹, and they can withstand higher concentrations of heavy metals. The use of these represents a closer approximation to what occurs in nature. This also allows the development of experimental model systems, which can explain the lag between the bioadsorption of pure cultures *in situ*⁶⁰. While pure isolates can resist lower concentrations of mercury compared to consortia, and few mechanisms of action on metal could be focused on, like bioaccumulation, biosorption, bioprecipitating, and/ or biovolatilization^{45,52,59}.

Conclusions

Microorganisms isolated from bird feathers that bio-magnify Hg in Antarctica are yeast genera identified: *Debaryomyces*, *Meyerozyma*, *Papiliotrema*, and *Rhodotorula*

also, fungi genus: *Leiotrametes*, *Penicillium*, *Pseudogym-noascus*, and *Cladosporium*. Of these, yeasts belonging to the genus *Rhodotorula* and family *Saccharomycetaceae* and fungi between these *Cladosporium* and *Penicillium* have been reported with efficient strategies of accumulation and biovolatilization of mercury and are considered suitable for application in remedial technology according to the re-ported bibliography.

This study opens the opportunity for bioprospecting microorganisms isolated from other matrices, not mer-cury-contaminated water and soil, but bird feathers that bio-magnified this metal. However, is a need to evaluate in time and elucidate the mechanisms used for these microorga-nisms in mercury removal and include other factors such as growth requirements between these pH and temperature, tolerance indices to major concentrations, and evaluation of their potential as adsorbents a low-cost and environmen-tally friendly. Therefore, the bioremediation of mercury from microorganisms isolated from bird feathers is still a develo-ping technology.

Supplementary Materials

Table S1. Cultivable isolates with source and Genbank accession number.

Author Contributions

Conceptualization, Cevallos-Cevallos.J., Calle.P. and Monserrate-Maggi.L.; methodology, Cevallos-Cevallos.J., Monserrate-Maggi.L., Delahaye.L., Alvarado-Cadena. O. and Ruiz-Barzola.O.; formal analysis, Ruiz-Barzola.O. and Serrano-Mena. L.; investigation, Cevallos-Cevallos.J., Calle.P., Monserrate-Maggi.L., Delahaye.L.; resources, Calle.P., Alvarado-Cadena. O., Ruiz-Barzola.O. and Serra-no-Mena. L.; data curation, Serrano-Mena. L.; writing—ori-ginal draft preparation, Cevallos-Cellos. J., Monserrate-Ma-ggi.L. and Serrano-Mena. L.; writing—review and editing, Monserrate-Maggi.L., Cevallos-Cevallos.J. and Calle.P.; vi-sualization, Monserrate-Maggi.L., Cevallos-Cevallos.J. and Calle.P.; supervision, Calle.P. and Cevallos-Cevallos.J.; project administration, Cevallos-Cevallos.J. All authors have read and agreed to the published version of the ma-nuscript.

Funding

This research was funded by Instituto Antártico Ecuatoriano (INAE), Secretaría de Educación Superior, Ciencia, Tecnología e Innovación (SENESCYT) and Escuela Supe-rior Politécnica del Litoral financed.

Data Availability Statement

The partial DNA sequences obtained in this study were deposited in the GenBank database. Accession numbers for each of the isolates are given in Table S1.

Conflicts of Interest

The authors declare no conflict of interest.

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