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IMPLEMENTACIÓN DE UN SISTEMA DE TRATAMIENTO BIOLOGICO PARA LA DETOXIFICACIÓN DE ARSENICO UTILIZANDO BACTERIAS UREOLITICAS-CALCIFICANTES ARSENITO-OXIDANTES, CAPACES DE BIOMINERALIZAR AS(V) Y AS (III).

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RESUMEN

El Arsénico es un compuesto tóxico con propiedades mutagénicas y cancerígenas, que posee la capacidad de formar variados compuestos orgánicos e inorgánicos como, arsenito (As(III)) y arseniato (As(V)). La presencia del arsénico en fuentes de agua natural, tanto, superficiales como subterráneas, constituyen un permanente riesgo para la población expuesta, quienes deben utilizar dichos recursos como fuente de agua potable y de riego. En Chile, la principal fuente de arsénico en aguas tiene origen natural. Sin embargo, se ha descrito que este compuesto es también producto de algunas actividades industrial, presentándose en altas concentraciones en ciertos ríos y en el suelo del Norte de nuestro país, donde se encuentran niveles de concentración 100 veces mayores que el límite recomendado por la Organización Mundial de la Salud, la Comunidad Europea y la EPA para agua de consumo humano.

Para cumplir con las normativas establecidas y para proteger la salud humana, se hace necesario contar con sistemas de tratamiento efectivos que puedan remover el arsénico de las aguas contaminadas. Los tratamientos químicos son altamente eficientes, sin embargo presentan altos costos operaciones y en algunos casos se utilizan químicos que pueden generar efectos negativos mayores en los cuerpos de agua.

Una alternativa para la remoción e inmovilización de arsénico, es la biomineralización basada en la precipitación de calcita inducida microbiológicamente (MICP). Los microorganismos secretan productos metabólicos que reaccionan con iones en el ambiente, resultando en la precipitación de minerales. Estos estudios de bio-precipitación han sido realizados utilizando bacterias ureolíticas principalmente. Calcita, un producto de biomineralización, puede adsorber en su superficie iones de arsénico. Esta habilidad de biomineralizar iones tóxicos ha sido propuesta como una metodología de remediación *in situ* para descontaminar ambientes. En este contexto, el objetivo de esta tesis fue desarrollar un sistema de tratamiento biológico para la remoción de arsenito y arseniato, desde aguas contaminadas con arsénico, mediante la bio-precipitación microbiológica de

calcita, utilizando bacterias ureolíticas, resistentes a As(III) y As(V), aisladas desde ambientes naturales contaminados con arsénico.

Las poblaciones bacterianas As-tolerantes, fueron aisladas y caracterizadas desde muestras de sedimentos, obtenidas desde entornos naturales altamente contaminados con arsénico (rio Loa y rio Camarones, Norte de Chile). Las muestras fueron caracterizadas taxonómica y funcionalmente mediante la secuenciación masiva de ADN con MiSeq de Illumina. Además desde las muestras de sedimentos se seleccionaron y caracterizaron cepas bacterianas ureolíticas-calcificantes. La capacidad de transformar As(III) a As(V) fue monitoreada utilizando HPLC. Se seleccionó una cepa ureolíticas-calcificantes (EM-6), que se identificó como *Pseudomonas marginalis* y se estudiaron sus mecanismos genéticos de resistencia a arsénico. Ademas, se evaluó la capacidad detoxificante de arsénico de la cepa seleccionada con vistas a su aplicación biotecnológica, mediante un sistema biológico de tratamiento.

Los análisis moleculares de las comunidades bacterianas asociadas a sedimentos de los ríos estudiados, demostraron que las secuencias de los grupos taxonómicos dominantes (abundancia ≥1%) en las muestras de sedimento del rio Camarones afiliaron con los Phylum: *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* y *Nitrospirae*. Sin embargo, una afiliación diferente se detecto para las secuencias provenientes de las muestras del rio Loa, las cuales fueron clasificadas dentro de los Phylum, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, *Lentisphaerae*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia y Chlorobi*, principalmente.

La caracterización de cepas bacterianas ureolíticas-calcificantes aisladas desde los sedimentos demostró, que *Pseudomonas marginalis* EM-6, aislada desde el rio Loa, fue la cepa bacteriana que presento mayor capacidad ureolítica y de tolerancia a arsénico. Además, análisis de microscopía electrónica asociadas a difracción de rayos x, demostraron que la cepa fue capaz de biomineralizar calcita. Por lo anterior, esta cepa bacteriana fue utilizada para implementar un sistema de tratamiento biológico (en Batch), para eliminar el arsénico desde sistemas acuáticos. Se estudio, la capacidad de la cepa

Pseudomonas marginalis EM-6 de eliminar la toxicidad del compuesto en el medio, mediante cromatografía liquida de alta resolución acoplada a un sistema de absorción atómica y mediante el estudio de la viabilidad celular en células de cordón umbilical (HUVECs). Los resultados demostraron que a medida que aumentaba la precipitación de calcita, la concentración de As(III) disminuía hasta en un 100%, al igual que la toxicidad. Estos resultados demuestran que existe una relación entre el aumento de la producción de biocalcita y la disminución del As en el sistema con la disminución de la toxicidad, donde a medida que la concentración de arsénico disminuye en el sistema la viabilidad celular de las células HUVECs aumenta. Por otra parte, los análisis demostraron que el sistema de tratamiento biológico, basado en MICP, es capaz de biomineralizar el 100 % de arsénico presente en efluente real (1124 ug L^{-1}), obtenido desde el rio Loa, eliminado su toxicidad.

El aporte de este trabajo, fue evidenciar la potencialidad biotecnológica de la bio-precipitación microbiológica de calcita, para la implementación de sistemas de tratamiento de aguas contaminada con arsénico, utilizando bacterias ureolíticas-calcificante, aisladas desde ambientes naturales, capaces de oxidar el arsenito y resistir arseniato, como una alternativa biotecnológica, económicamente viable y ambientalmente amigable.

ABSTRACT

Arsenic is a toxic compound with mutagenic and carcinogenic properties that has the ability to form various organic and inorganic compounds such as arsenite (As(III)) and arsenate (As(V)). The presence of arsenic in natural water sources, both superficial and underground, constitute a permanent risk for the exposed population, who must use these resources as a source of drinking water and irrigation. In Chile, the main source of arsenic in waters has natural origin. However, it has been described that this compound is also the product of some industrial activities, occurring in high concentrations in some rivers and sediments of the north of Chile. In addition, several authors reported levels of As, 100 times higher than the concentration limit recommended by the Organization World Health Organization, the European Community and the EPA for water for human consumption, in rivers of Atacama Desert.

To accomplish with the regulations established to protect human health, it is necessary to have an effective treatment systems that can remove arsenic from contaminated waters. The chemical treatments are very efficient. However, they present high operations costs and in some cases, used chemicals product that can generate highest negative effects in water systems.

An alternative for the removal and immobilization of arsenic is, biomineralization based on microbially induced calcite precipitation (MICP). The microorganisms secrete metabolic products that react with ions in the environment, resulting in the precipitation of minerals.
Bio-precipitation studies have been carried out using mainly ureolytic bacteria. Calcite, a bio-mineralization product, can adsorb arsenic ions on its surface. This ability to bio-mineralize toxic ions has been proposed as an *in situ* remediation methodology to decontaminate environments. In this context, the objective of these thesis was to develop a biological treatment system for the removal of arsenite and arsenate, from water contaminated with arsenic by MICP, using ureolytic bacteria resistant to As (III) and As (V), isolated from natural environments contaminated with arsenic.

The As-tolerant bacterial populations, were isolated and characterized from sediment samples obtained from natural environments, highly contaminated with arsenic (Loa River and Camarones River, Northern Chile). Samples were taxonomically and functionally characterized by massive DNA sequencing Illumina MiSeq. In addition, from the sediment samples, ureolytic-calcifying bacterial strains were selected and characterized. The ability to transform As (III) to As (V) was monitored using HPLC. We selected an ureolytic-calcifying strain and the genetic mechanisms of arsenic resistance were studied. The ability to detoxify arsenic of the selected strains with a view to their biotechnological applications was evaluated through a biological treatment system.

Molecular analyzes of the bacterial communities associated with sediments of the studied rivers, showed that the sequences of the dominant taxonomic groups (abundance ≥1%) in Camarones river, sediment samples, affiliated with the Phylum: *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* and *Nitrospirae*. However, a different affiliation was detected for the sequences from Loa river samples, which were mainly classified as: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, *Lentisphaerae*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia* and *Chlorobi*.

The characterization of ureolytic-calcifying bacterial strains isolated from the sediments showed that, *Pseudomonas marginalis* EM-6 isolated from the Loa river was the bacterial strain with the highest ureolytic capacity and tolerance to arsenic. In addition, electron microscopy analysis associated with x-ray diffraction showed, that the strain was able to biomineralize calcite. Due to the above, this bacterial strain was used to implement a biological treatment system (in Batch), to eliminate arsenic from aquatic systems. The ability of the *Pseudomonas marginalis* EM-6 strain to eliminate the toxicity of the compound in the medium, was assessed by high-performance liquid chromatography coupled to an atomic absorption system and through viability of HUVECs cells. The results showed that as the calcite precipitation increased, the arsenite concentration decreased in a 100%, as did the toxicity. These results demonstrate that there is a relation between increased biocalcite production and decreased arsenic in the system with decreased toxicity, where as the arsenic concentration decreases in the system the cellular viability of the HUVEC cells

increases. On the other hand, the analyzes showed that the biological treatment system, based on MICP, is able to biomineralize 100% of the arsenic present in the effluent, obtained from the river Loa (1124 ug uL^{-1}), eliminating its toxicity.

The contribution of this work was to highlight the biotechnological potential of microbiological bio-precipitation of calcite, for the implementation of water treatment systems contaminated with arsenic, using ureolytic-calcifying bacteria isolated from natural environments, able to oxidize the arsenite and resist arsenate, as a biotechnological alternative, economically viable and environmentally friendly.

CAPÍTULO I: INTRODUCCIÓN

El arsénico (As) es un elemento tóxico, fácilmente asociable a enfermedades en el hombre por su exposición reiterada. Las principales manifestaciones del arsenicismo son melanosis, queratosis, y diferentes formas de cáncer (cáncer de piel, vejiga, pulmón y próstata entre otros) (Hsu y col., 2013). La forma de exposición más común es a través de su ingesta desde aguas contaminadas. Bangladesh, India, Mongolia, China, Taiwán, México, Argentina y Chile son países donde el envenenamiento con As aparece como un problema de salud pública, producto principalmente del consumo y utilización de aguas contaminadas con el metaloide (Mandal y Suzuki, 2002).

El norte de Chile, especialmente el desierto de Atacama, ha sido descrito como un ambiente rico en As. Los minerales que contienen sulfuros metálicos se disuelven en las montañas de los Andes, contaminando tanto aguas superficiales como subterráneas que cruzan el desierto, las cuales son utilizadas luego por los habitantes de Atacama para su consumo general. Desde 1970, el agua es tratada para remover estas cantidades de As. Esto implica altos costos asociados a estos tratamientos para abastecer a grandes ciudades de la región (e.g. Antofagasta) (Mandal y Suzuki, 2002). A pesar de la existencia del tratamiento del agua para las ciudades principales, los habitantes de poblados rurales se mantienen expuestos constantemente a aguas contaminadas con As. El arsenicismo crónico es un problema que afecta a 50.000 personas, principalmente en poblaciones rurales del desierto de Atacama. La población afectada bebe agua desde pequeñas cascadas y ríos con contenidos de As sobre los 1000 μg L^{-1} (Yañez y col., 2005; Valenzuela y col., 2009). Esta situación supera ampliamente la concentración de As recomendada tanto por la organización mundial de la salud, así como por la Agencia de Protección Ambiental de EE.UU (máximo 10 μg L^{-1}) (Smith y col., 2002; U.S EPA, 2001; OMS, 2001).

En general, el arseniato (As(V)) es la forma predominante de arsénico en ambientes acuosos oxigenados, mientras que el arsenito (As(III)) es la forma predominante bajo condiciones ambientales anóxicas o reductoras, siendo esta forma 100 veces más tóxica que su forma oxidada (arseniato) y presenta mayor dificultad para ser removido por métodos físico-químicos (Al-Abed y col., 2007; Taerakul y col., 2007).

Los procesos de tratamiento convencional para la remoción de As incluyen coagulación, filtración, suavizado con soda, adsorción con alúmina activada, intercambio iónico, osmosis reversa, electrodiálisis reversa y nano filtración (Campos y col., 2009). Si bien todos estos métodos son efectivos y pueden remover cerca de 80-95% del As de una solución, los costos operacionales son elevados. De hecho la oxidación de As(III) a As(V) es un pre-requisito para todas las técnicas convencionales (Khoe y col., 1997). Sin embargo, la oxidación del As con oxígeno es extremadamente lenta y se deben utilizar oxidantes más fuertes y costosos, como el cloro, el peróxido de hidrogeno o el ozono (Kim y Nriagu, 1999). Por lo anterior, opciones económicas y amigables con el ambiente, como la bio-transformación, ofrecen una alternativa interesante a las rutas químicas tradicionales. Respecto a esta visión, un factor importante en la especiación del As son los microorganismos presentes en suelos y ambientes acuáticos, produciendo diferencias en la solubilidad, biodisponibilidad y toxicidad del metaloide (Simeonova y Luster, 2004). Muchas bacterias involucradas en estos procesos de transformación que comprenden reacciones de reducción, oxidación y metilación de especies de arsénico (bacterias arsénico resistentes), han sido propuestas para la implementación de sistemas de tratamiento biológico (Heinrich-Salmerón y col., 2011; Kruger y col., 2013; Osborne y Santini, 2012). En este contexto, los métodos de transformación de As siguen siendo un punto de interés para nada despreciables. Sin embargo, los sistemas descritos anteriormente no son capaces de inmovilizar los compuestos tóxicos y generalmente solo son capaces de remover una sola forma de As. Es por esto que una forma efectiva para el tratamiento de As debe permitir la remoción de ambas formas: As(III) y As(V). Una alternativa es la biomineralización basada en la precipitación de calcita inducida microbiológicamente (MICP) que se muestra como una técnica con proyección para la remediación de As desde ambientes contaminados, haciendo uso de las ventajas de la biorremediación (Pan, 2009). Este proceso es activo en casi todos los ambientes de la tierra. Los microorganismos pueden secretar uno o más productos metabólicos que pueden reaccionar con iones o compuestos en el ambiente, resultando en la consiguiente precipitación de partículas minerales. Calcita, un producto de biomineralización, puede adsorber en su superficie o incluso incorporar los iones metaloides de As en su estructura cristalina (Di Benedetto y col., 2006). Una parte importante de los estudios de precipitación

de bio-calcita han sido realizados utilizando bacterias ureolíticas (Muynck y col., 2010; Achal y col., 2009). Estas bacterias ureolíticas son capaces de influenciar la precipitación de carbonato de calcio por la acción de una enzima, la ureasa. Por lo que, la precipitación del carbonato de calcio ocurre como consecuencia de la actividad metabólica bacteriana.

La ubiquidad de la MICP y su habilidad de inmovilizar (biomineralizar) elementos tóxicos ha sido propuesta como una potencial metodología de remediación in situ para descontaminar sistemas naturales. La mayor parte de estos estudios han sido elaborados para remediar metales pesados como, 90Sr, Pb, Cd y Cr, en muestras de suelo, arena, desechos de la industria del cromo y aguas subterráneas (Fujita y col.,2004; Achal y col., 2012a; Achal y col., 2012b; Achal y col., 2012c; Achal y col., 2013).

Basados en el hecho de que la mayoría de las bacterias son capaces de precipitar $CaCO_3$, producto de sus procesos metabólicos como la fotosíntesis, hidrólisis de la urea y reducción de sulfato, la biorremediación de aguas contaminadas con As con MICP puede ser una alternativa viable (Martin y col., 2012; Hammes y col., 2003). Ademas, cabe señalar que la interacción o secuestro de arsénico por calcita ha sido objeto de numerosos estudios sintéticos, motivados por el potencial que presenta este mineral en la inmovilización del As. Román-Ross y col. (2006) demostraron que los oxianiones de As(III) pueden reemplazar grupos CO_3^{2-} en la red de calcita quedando incorporados en su estructura. Sin embargo, Sø y col. (2008) reportaron que esa sustitución solo podía ocurrir a valores relativamente altos de pH y concentración de As(III). Alexandratos y col. (2007) fueron los primeros que mostraron que también el arseniato As(V) puede remplazar al carbonato en la red de calcita mediante experimentos de adsorción realizados en batch a pH 8.3, sin cambiar la geometría tetraédrica o estado de oxidación de As(V). Recientemente, Yokoyama y col. (2012) confirmaron este resultado en un amplio rango de pH (7-12) y además mostraron que la incorporación de As(V) también puede ocurrir a partir de As(III) en solución, después de una etapa de oxidación y la formación de complejos de arseniato de calcio.

Reforzando esta propuesta existen muchos reportes de bacterias capaces de sintetizar arsenito oxidasas, capaces de oxidar el As(III) a As(V), como por ejemplo; *Alcaligenes faecali*, *Agrobacterium tumefaciens*, *Pseudomonas arsenicoxydans*, *Pseudomonas* sp. y

Rhizobium sp. (Anderson y col., 1992; Inskeep y col., 2007; Campos y col., 2010). La oxidación bacteriana permite a las bacterias obtener una fuente de electrones requerida para su metabolismo y a su vez le confiere un mecanismo de detoxificación, ya que el arseniato es menos tóxico que el arsenito. Finalmente, este arsenito resultante podría ser adsorbido por la bio-calcita producto de un proceso MICP. Achal y col. (2012a) describen una bacteria ambiental tolerante a As(III), capaz de biorremediar mediante MICP suelos contaminados con arsenito. No obstante, ellos no describen el mecanismo bacteriano involucrado en tal proceso, ni el estado de oxidación del As resultante.

Lamentablemente no han sido reportados en Chile estudios similares ni han sido evaluadas las capacidades depurativas, con el fin de obtener parámetros de diseño para un biorreactor especializado para la remoción de As(III) y As(V) bajo un sistema biológico MICP, utilizando bacterias ureolíticas tolerantes a As. En este contexto, el reto principal de esta propuesta es de caracterizar poblaciones bacterianas tolerantes a compuestos de As capaces de mineralizar carbonato de calcio presentes en sistemas naturales contaminados con el metaloide, con el propósito de aislar e identificar cepas bacterianas capaces de oxidar As(III) y tolerar As(V).

El objetivo general de este proyecto será evaluar la capacidad de biomineralizar As(V) y As(III) de bacterias ureoliticas-calcificantes arsenito-oxidantes en sistema de tratamiento biológico del metaloide, basado en la precipitación de calcita inducida microbiológicamente.
Para alcanzar este objetivo, las poblaciones de bacterias tolerantes a As deben ser aisladas desde muestras de suelo obtenidas desde entornos altamente contaminados (región de Arica y Parinacota), y desde estas seleccionar y caracterizar aquellas que sean capaces de calcificar. La capacidad de transformar As(V) a As(III) será monitoreada utilizando HPLC. Se estudiaran los mecanismos genéticos en la resistencia a arsénico de la cepa seleccionada. Se evaluará la capacidad depurativa de las cepas seleccionadas con vistas a sus aplicaciones biotecnológicas.

CAPÍTULO II: ANTECEDENTES GENERALES

II.I Importancia del arsénico

El arsénico (As) es un compuesto químico que puede ser encontrado en suelos, sedimentos, agua y en la atmósfera. Las diferentes especies de As (orgánicas e inorgánicas) tienden a permanecer en solución e incluso a altas concentraciones a pH casi neutro (Smedley y Kinniburgh, 2002). Por lo que millones de personas en todo el mundo están expuestas al As en su agua potable, incluyendo un estimado de 50 millones en Bangladesh, 30 millones en la India, 15 millones en China y millones más en Estados Unidos, Europa y América Central y Sudamerica (Steinmaus y col., 2013). Como resultado, la exposición al As a través del agua potable se considera la mayor causa de envenenamiento de personas en todo el mundo. Y existen fuertes evidencias que vinculan el As con una amplia variedad de problemas de salud, que van desde una toxicidad aguda hasta enfermedades crónicas que pueden tardar años en desarrollarse. Las enfermedades relacionadas con el As (Arsenicismo) incluyen lesiones cutáneas (Hiperpigmentación y Hiperqueratosis), hipertensión, isquemia, algunos trastornos vasculares periféricos (e.g., “enfermedad del pie negro"), diabetes, arteriosclerosis severa, neuropatías (Gong y col., 2001; Hall y col., 2017). Por otra parte, el cáncer a la piel, vejiga, riñón, hígado, próstata y pulmón, también han sido asociados a la ingesta de arsénico en humanos (Ferreccio y col, 2000; Liaw y col., 2008, Dauphiné y col., 2010; Rahman y col., 2013). Por lo cual, el compuesto ha sido clasificado por la Agencia Internacional para la Investigación del Cáncer (IARC, siglas en inglés), como uno carcinógeno humano de clase I (IARC, 2004; Filella y col., 2009; Fu y col., 2016) y como un contaminante tóxico prioritario por la Agencia de Protección Ambiental de Estados Unidos (US EPA) y la Unión europea (Fu y col., 2016).

En el norte de chile la principal causa de contaminación por As inorgánico proviene de la minería, de las actividades agrícolas y asociadas al suelo en condiciones naturales (Ferreccio y Sancha, 2006; Liaw y col., 2008; Steinmaus y col., 2013). El problema derivado del alto contenido de As del ambiente es delicado, principalmente en comunidades rurales. Considerando que el agua de ciertos ríos y arroyos contienen altas concentraciones del compuesto, y que esta agua es utilizada para el riego de vegetales y hortalizas que luego

son consumidas por las personas, el riesgo de intoxicación crónica es evidente. Además el agua es muchas veces obtenida sin los tratamientos correspondientes, y bebida directamente por la comunidad (Figueroa y col., 2001; Yáñez y col., 2005).

La Región de Antofagasta del norte de Chile es extremadamente seca, sin pozos privados o fuentes alternativas de agua, por lo que toda la población utiliza agua municipal. Antes de 1958, el agua de la principal ciudad de Antofagasta contenía aproximadamente 90 μg L^{-1} de As. En 1958, cambió su fuente de agua a un sistema de tuberías procedentes de 2 ríos contaminados con As y las concentraciones del metaloide en el agua aumentaron inmediatamente de aproximadamente 90 μg L^{-1} a una concentración promedio de 870 μg L^{-1} (Marshall y col., 2007). En 1970, una planta de remoción de As fue instalada y los niveles bajaron drásticamente a alrededor de 110 μg L^{-1}. La remoción de As ha mejorado y el agua de la ciudad ahora contiene menos de 10 μg L^{-1} (Ferreccio y Sancha, 2006).

La organización mundial de la salud, la unión europea y la EPA, basadas en estudios epidemiológicos han determinado como aceptable aquellas concentraciones de As no superiores a 10 μg L^{-1} en agua para consumo humano (Gurian y col., 2001). En chile, la norma chilena para agua potable establece un máximo de 50 μg L^{-1} de As. Sin embargo, el As en el agua de ríos utilizados por las comunidades, como el Río Camarones (Provincia de Arica, Comuna de Camarones) supera ampliamente el estándar, alcanzando niveles de 1000 μg L^{-1} (Figueroa y col., 2001). O el río Loa cuyos principales afluentes proporcionan agua para uso doméstico, minero (e.g. mina de cobre de Chuquicamata), industrial (industria química del salitre) y de riego en esta árida región y que presenta tres secciones con concentraciones diferentes de As (Loa Alto, Medio y Bajo). Loa Alto comprende la zona entre el origen del río (al pie del volcán Miño) y la confluencia con el río Salado, cerca del pueblo de Chiu-Chiu, con una concentración de As de 0,2 mg L^{-1}. Loa Medio comprende la zona entre la confluencia Loa-Río Salado y la confluencia Loa-Río San Salvador en el pueblo de Chacance y posee una concentración de As de 1,4 mg L^{-1}. Por último, Loa Bajo comprende la zona entre el pueblo Chacance y la descarga del río hasta el Océano Pacífico con una concentración de As de 2,2 mg L^{-1} (Romero y col., 2003). La concentración de As en las aguas a lo largo de la cuenca del Río Loa es alta debido a varios factores: el clima desértico extremo favorece altas tasas de evaporación, alta salinidad, la falta de afluentes

superficiales y contribuciones de agua dulce subterránea impide la dilución, mientras que el pH neutro a alcalino del agua dificulta la adsorción de As en fases sólidas.

II.II Arsénico y ambiente

La principal característica química del As, explicando su comportamiento en el ambiente así como en sistemas biológicos activos, se deben a la fácil conversión entre sus formas reducidas y oxidadas. Un ambiente acido favorece la reducción inorgánica desde arseniato (As(V)) a arsenito (As(III)) (E^o(potencial de reducción estándar)=0,56 volts); es por esto que, a pH bajo 7 la especie predominante en solución es el As(III) en la forma H_3AsO_3. La reacción inversa, es decir la oxidación, esta favorecida en ambientes básicos (E^o=-0,67 volt); donde se encuentra como $H_2AsO_4^{-1}$ y $HAsO_4^{-2}$ a pH entre 7 y 10 (National Research Council (NRC), 1999). Este comportamiento del As y especialmente su comportamiento con enzimas involucradas en el complejo de fosforilación oxidativa, son algunas de los factores de riesgo que lo caracterizan como un poderoso tóxico natural (Hu y col., 2013).

Respecto a las formas orgánicas de este elemento, pueden ser encontradas en organismos vivos como metabolitos resultantes de sucesivas oxido-reducciones de las especies inorgánicas. De esta manera es posible encontrar estas formas orgánicas en peces, mariscos y algas, con pequeñas cantidades de ácido metilarsónico (MMA), ácido dimetil arsónico (DMA) en adición a la trimetilarsina (TMA) o el óxido de trimetilarsina (TMAO) (formas mono, di y trimetiladas de arsénico respectivamente), arsenobetamina y arsenocolina además de algunas arsenoazucares (Bergés-Tiznado y col., 2013; Le y col., 2004).

La forma predominante del As inorgánico en entornos acuosos es el As(V), mientras que el As(III) es la forma más habitual en ambientes anóxicos. El As(V) se encuentra fuertemente adsorbido a la superficie de minerales comunes como la ferrohidrita y la alúmina. El As(III) en cambio muestra una menor adsorbancia y comportándose más como un oxianión móvil (Smedley y kinniburgh, 2002). Numerosas formas metiladas de As orgánico (ácidos metilarsónico, metilarsenioso y dimetilarsénico) son encontrados en aguas como resultado

de actividades metabólicas de la biota acuática o como resultado de la excreción urinaria de animales, incluyendo al hombre (Sharma y Sohn, 2009).

II.III Toxicidad del arsénico

La toxicidad del As está bien documentada y depende no solo de su estado de oxidación, sino también de su especiación, siendo las formas inorgánicas las más tóxicas. Los arsenitos son entre 20 y 100 veces más tóxicos que los arseniatos en ambientes marinos (Neff, 1997) y 60 veces más tóxicos para humanos (Ferguson y Gavis, 1972). El As(V) es un reconocido carcinogénico y su máxima concentración permitida por USEPA, es 10 μg L^{-1} (U.S. EPA, 2001) en la unión europea los niveles de arsénico no debe sobrepasar los 10 μg L^{-1}. Estos requerimientos hacen necesario desarrollar nuevos tratamientos para su eliminación. El arseniato, análogo al fosfato, entra a la célula usando la vía de transporte de fosfato, inhibiendo la fosforilación oxidativa. El arsenito se une a los grupos sulfuro (-SH), alterando la función de muchas proteínas (National Research Council (NCS), 1999). Este también afecta la función respitatoria, uniéndose a los grupos tiol de la piruvato deshidrogenasa y las enzimas 2-oxoglutarato deshidrogenasa. Recientemente ha sido reportado que interacciona con receptores glucocorticoides (Kaltreider y col., 2001).

II.IV Bacterias y arsénico.

Los microorganismos presentes en ambientes acuáticos y de suelo juegan un rol fundamental en el ciclo biogeoquímico del As (Ciclo rédox). Esto influye de manera importante en el ambiente, pues las distintas especies muestran diferencias en su solubilidad, biodisponibilidad y toxicidad (Nriagu, 1994). Los estudios en poblaciones microbianas oxidantes y reductoras de As asociadas a ambientes enriquecidos de este metaloide han sido realizados mediante la amplificación de ADNr 16S utilizando partidores para el dominio universal bacteriano, análisis de electroforesis denaturante en gel (DDGE) y secuenciación directa (Butcher y col., 2000; Macur y col., 2004; Valenzuela y col., 2009; Campos y col., 2011).

II.V Mecanismos de resistencia bacteriana al arsénico.

II.V.I Disminución de la entrada de arsénico a la célula.

El mecanismo ha sido estudiado en *E. coli*, donde 2 diferentes mecanismos de transporte de fosfato han sido descritos: el sistema Pit, con baja afinidad por el fosfato, que también transporta As(V); y el sistema Pst, con gran afinidad por el fosfato (Rosenberg y col., 1977). Aquellos organismos que carecen del sistema Pit tienen una mayor resistencia al As(V) (Cervantes y col., 1994). En ciertas especies de plantas, la captura y toxicidad del As(V) puede ser reducida incrementando la concentración de fosfato en el ambiente (Abedin y col., 2002). Resultados similares han sido observados en bacterias que son cultivadas en presencia de diferentes concentraciones de As(V) y fosfato.

II.V.II Reducción del arsénico y expulsión de la bacteria.

El sistema de resistencia a As más estudiado en microorganismos es el sistema de genes ars. Se ha demostrado a través de estudios que el origen de estos genes puede ser tanto plasmidial como cromosomal (Diorio y col., 1995) y que median la resistencia a As(III) y a As(V) en *E. coli* (Mobley y col., 1983), Staphylococcus aureus (Götz y col., 1983) y Pseudomonas aeruginosa (Cervantes y Chávez, 1992) entre otras especies. En términos generales, el sistema *ars* consiste en una serie de tres o más genes codificantes para un sistema de eflujo transmembrana y una arseniato reductasa. Específicamente, el operón incluye un regulador del gen (*arsR*), un gen codificante de una bomba de expulsión de arsenito transmembrana (*arsB*) y un gen codificante para una arseniato reductasa (*arsC*)(Ji y col., 1993). El gen *arsC* es particularmente interesante y codifica una proteína arseniato reductasa soluble y cataliza la transformación intracelular del As(V) en As(III). Los genes ars son inducibles tanto por As(III) como por As(V), resultando en una reducción intracelular de As(V) por *arsC*, una reductasa citoplasmática, seguido por la excreción del As(III) al exterior de la célula (*arsB* y *arsA*). La reducción de As(V), como vía de detoxificación bacteriana puede contribuir a las aparentes condiciones de desequilibrio, en el entorno, siendo el As(III) encontrado en suelos oxigenados y en aguas superficiales (Escalante y col., 2009; Macur y col., 2001; Cullen y Reimer, 1989).

II.V.III Oxidación de arsénico.

Bacterias asenito oxidantes son fisiológicamente diversas y se encuentran en varios grupos de bacterias y archaeas e incluyen tanto bacterias As(III)-oxidantes heterótrofas (HAOs), como bacterias As(III)-oxidantes quimiolitoautotróficas (CAOs). La oxidación heterotrófica del As(III) generalmente se considera un mecanismo de detoxificación que convierte el arsenito en menos tóxico As(V), aunque puede ser utilizado como una fuente de energía suplementaria (Hoven y Santini, 2004). En contraste, CAOs usa As(III) como un donador de electrones durante la fijación de CO2 acoplada con la reducción de oxígeno (Santini y col., 2000). Además, ha sido descrito que la oxidación quimioautotrófica puede ocurrir via oxidación aeróbica, respiración anaeróbica nitrato y seleniato dependiente (Rhine y col., 2006; Hoeft y col., 2007; Budinoff y Hollibaugh, 2008) o fototroficamente (Budinoff y Hollibaugh, 2008). Por otra parte, algunos investigadores han informado de HAOs anaerobias facultativas capaces tanto de oxidar As(III) de manera aeróbica o de reducir As(V) de manera anaeróbica (Gihring y Banfield, 2001; Handley y col., 2009). En estudios recientes, bacterias oxidantes As(III) fueron aisladas de entornos ricos en As (Campos y col., 2009; Hamamura y col., 2013, Valenzuela y col., 2015), así como de suelos con bajos niveles de As y tierra de jardín no contaminada (Bachate y col., 2012; Bahar y col., 2012).

As(III) oxidasa, la enzima que cataliza la oxidación de As(III), ha sido caracterizada en bacterias CAOs y HAOs (Vanden Hoven y Santini, 2004; Lieutaud y col., 2010). En ambos casos, la enzima contiene dos subunidades, una subunidad grande que contiene un centro molybdopterina y un grupo [3Fe-4S] y una subunidad pequeña que contiene un grupo Rieske [2Fe-2S] (Ellis y col., 2001; Santini y Vanden Hoven, 2004). Aunque los genes homólogos que codifican estas dos subunidades fueron anteriormente asignados con diferentes nombres (*aoxB-aoxA / aroA-aroB* / *asoA-asoB*), la nomenclatura de los genes implicados en la oxidación procariota aeróbica del As(III) fue recientemente unificada y el nombre *aio* fue recientemente asignado. Por lo tanto, la subunidad grande y pequeña se denotan *aioA* y *aioB*, respectivamente (Lett y col., 2012). La subunidad AioA es similar a las subunidades que contienen molibdeno en la familia reductasa DSMO y distantemente relacionada a la subunidad catalítica de la reductasa respiratoria de As(V) (*arrA*) (Oremland y Stolz, 2003; Silver y Phung, 2005; Stolz y col., 2010). Genes homólogos que codifican

AioA han sido encontrados en cepas filogeneticamente diversas incluyendo *Alfa*-, *Beta*- y *Gama Proteobacterias*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Aquificae*, *Deinococcus-Thermus*, *Chlorobi*, *Chloroflexi*, *Nitrospirae* y *Crenarchaeota* (Yamamura y Amachi, 2014).

II.VI Biorremediación usando bacterias ureolíticas (Bacterias calcificantes).

Los proceso de biorremediación de As convencionales implican tanto la adsorción directa de As por la biomasa microbiana, como la adsorción y coprecipitación con hierro o hidróxidos de manganeso biogénicos (Wang y Zhao, 2009).

Además de la bio-eliminación de As y la formación mineral biogeogénica, la oxidación bacteriana del As(III) a As(V) es un enfoque prometedor para el tratamiento de agua contaminada en lugar de utilizar oxidantes convencionales ($KMnO_4$, cloro, ozono, peróxido de hidrógeno o óxidos de manganeso). En los últimos años, se han realizado varios estudios para evaluar la eficiencia de oxidación de diferente bacterias As(III)-oxidantes, adheridas en materiales inmovilizados. Ito y col. (2012) desarrollaron un biorreactor con células de *Ensifer adhaerens* inmovilizadas en gotas de alcohol polivinilo, para estudiar la capacidad oxidativa de la cepa en agua subterránea sintética que contenía 1 mg L^{-1} de As(III) y los autores demostraron que el As(III) fue oxidado a As(V) con una eficiencia de un 90%. Por otra parte, Dastidar y Wang (2009) desarrollaron un análisis de modelos de oxidación autótrofica de As(III) en un reactor usando una biopelícula de la cepa *T. arsenivorans* b6 bajo diferentes concentraciones de As(III) (500-4000 mg L^{-1}) y los autores concluyeron que la tasa de oxidación de As(III) del reactores variaba de 48,2 a 99,3%.

Después de la oxidación biológica del As(III), es necesario remover el As(V) producido mediante el uso de sorbentes. Los procesos combinados de oxidación biológica y la posterior eliminación químicas dentro de sorbentes sintéticos han sido investigados por varios autores. En una de las primeras aplicaciones de esta técnica, Lièvremont y col. (2003) encontraron que dos fases minerales, kutnahorita y chabacita, mostraron diferentes capacidades de adsorber As después de su oxidación biológica. Sus resultados también demostraron, que la cepa bacteriana estudiada realizo una rápida oxidación de As(III) a

altas concentraciones de As en presencia de chabacita y que el As(V) fue eficientemente removido por kutnahorita. Por lo tanto, se sugirió un proceso de desintoxicación de dos fases. Las ventajas de estas técnicas incluyen un bajo costo operacional, una minimización del volumen de químicos y/o lodos biológicos residuales para disponer, sumados a una alta eficiencia en la detoxificación en efluentes diluidos. La remoción de iones por bioabsorción es rápida, toma solo unos pocos minutos y es llevado a cabo bajo condiciones normales de presión y temperatura. De hecho la bioabsorción y bioadsorción son formas de inmovilización pasiva de metales en biomasa (Gabr y col., 2008).

Por lo anterior, uno de los más prometedores tratamientos biogeoquímicos para el suelo y el agua, es la obtención y utilización de sorbentes producidos por el metabolismo bacteriano, como lo es la precipitación de calcita (CaCO3) inducida microbiológicamente (MICP) en el ambiente (DeJong y col., 2010; Harkes y col., 2010; Nemati and Voordouw, 2003; Whiffin y col., 2007). Las posibles aplicaciones de MICP son numerosas e incluye, ingeniería geotécnica (Burbank y col., 2011; DeJong y col., 2006), rehabilitación estructural (De Muynck y col., 2008), arquitectura (Tiano y col., 1999) y remediación ambiental (Dupraz y col., 2009; Fujita y col., 2008).

La mayoría de las bacterias son capaces de inducir la precipitación de $CaCO_3$ a través de una variedad de vías metabólicas (Boquet y col., 1973). La vía más eficiente implica la hidrólisis de urea, que es catalizada por la enzima ureasa microbiana (urea amidohidrolasa) (De Muynck y col., 2010; Lloyd y Sheaffe, 1973). La hidrólisis enzimática de la urea es de aproximadamente 1014 veces más rápida que la reacción espontánea (Jabri y col., 1995). La urea se hidroliza inicialmente en carbamato y amoníaco (Mobley y Hausinger, 1989). Carbamato se hidroliza espontáneamente para dar ácido carbónico y amoníaco, que a su vez se someten a hidrólisis con constantes de equilibrio de pK1 6.3 y pKa 9.3, respectivamente. De acuerdo con estos valores , es evidente que habrá un aumento neto en el pH del suelo. En presencia de calcio disuelto, este proceso podría resultar en la precipitación de $CaCO_3$, a condición de que el medio está sobresaturado con respecto a $CaCO_3$. MICP es un proceso intrincado que se balancea por cuatro parámetros:(1) carbono inorgánico disuelto (DIC), (2) pH, (3) la abundancia de sitios de nucleación y (4) la concentración de calcio (De Muynck y col., 2010). Los primeros tres parámetros son

afectados directamente por la actividad ureolítica microbiana y por la abundancia de células bacterianas (bacterias que proporcionan sitios de nucleación).

La interacción o secuestro de arsénico por calcita ha sido objeto de numerosos estudios sintéticos, motivados por el potencial que presenta este mineral común en la inmovilización del arsénico. Román-Ross y col. (2006) demostraron que los oxianiones de As(III) pueden remplazar grupos CO32- en la red de calcita quedando incorporados en su estructura. Por otra parte, Sø y col. (2008) reportaron que esa sustitución solo podia ocurrir a valores relativamente altos de pH y concentración de As(III). Alexandratos y col. (2007) fueron los primeros que mostraron que también el arseniato As(V) puede remplazar al carbonato en la red de calcita mediante experimento de adsorción realizados en bacth a pH 8.3, sin cambiar la geometría tetraédrica o estado de oxidación de As(V). Recientemente, Yokoyama y col. (2012) confirmaron este resultado en un amplio rango de pH (7-12) y además mostraron que la incorporación de As(V) también puede ocurrir a partir de As(III) en solución, después de una etapa de oxidación y la formación de complejos de arseniato de calcio.

Particularmente, algunos estudios han utilizado la urea para promover la precipitación biogénica de $CaCO_3$ como una estrategia de remediación. Fujita y col. (2004) reportaron la incorporación de estroncio radioactivo (90Sr) en calcita generada por la ureolisis de *Bacillus pasturii*, como estrategia de biorremediación de aguas subterráneas contaminadas con el compuesto. Achal y col. (2012b) estudiaron la capacidad biorremediadora de plomo en suelos, de la bacteria calcita-precipitante *Kocuria flava*. Los resultados indicaron que la incubación de suelo contaminado, en presencia de la bacteria podría reducir el Pb activo, aliviar el estrés por la presencia de Pb y estabilizar el suelo contaminado. Por otra parte, la MICP de una *Halomonas* sp resistente a estroncio productora de ureasa, se caracterizó por su potencial papel en la biorremediación de estroncio (Sr) en acuíferos por Achal y col. (2012c). La cepa bacteriana elimino el 80% de Sr de la fracción soluble en la arena. Y la difracción de rayos X detectó una solución sólida de calcita-estroncianita (SrCO(3)) en la muestra biorremediada con indicios de que Sr se incorporó a la calcita. Achal y col. (2013) demostraron la biorremediación de cromato (Cr(VI)) a partir de desechos de la industria de cromo, por una bacteria ureolítica calcificante *Bacillus* sp. CS8 y se reportó que la movilidad del Cr(VI), disminuyo significativamente en la fracción intercambiable del desecho y que

posteriormente, la concentración de Cr(VI) incrementó en la fracción carbonatada. Además, las desechos de Cr(VI) se caracterizaron por SEM-EDS confirmando la utilización del proceso MICP en la biorremediación. Los resultados de estos estudios demuestran que el secuestro a base de MICP de metales pesados solubles a través de coprecipitación con calcita puede ser útil para la biorremediación de metales pesados tóxicos. Por otra parte Achal y col. (2012a) describiendo una bacteria ambiental tolerante a As(III), capaz de biorremediar mediante MICP suelos contaminados con arsenito. No obstante, estos autores no describen los mecanismos bacterianos involucrados en tal proceso, ni el estado de oxidación del arsénico absorbido a la calcita. Hasta ahora no han sido publicados estudios similares en Chile, así como tampoco se han descritos los parámetros para el diseño de un biorreactor especializado en la remoción de As(III) y As(V) bajo un sistema MICP. Esta propuesta de trabajo propone la utilización de bacterias ureolíticas tolerantes tanto a As(III) como a AS(V), para la implementación de un sistema de tratamiento biológico.

CAPÍTULO III: HIPÓTESIS, OBJETIVO GENERAL Y OBJETIVOS ESPECÍFICOS

El problema

El arseniato (As(V)) es la forma predominante de arsénico en ambientes acuosos oxigenados, mientras que el arsenito (As(III)) es la forma predominante bajo condiciones ambientales anóxicas o reductoras, siendo esta forma 100 veces más tóxica que su forma oxidada (arseniato) y presenta mayor dificultad para ser removido por métodos fisicoquímicos. Los procesos de tratamiento convencional para la remoción de As incluyen coagulación, filtración, suavizado con soda, adsorción con alúmina activada, intercambio iónico, osmosis reversa, electrodiálisis reversa y nano filtración. Si bien todos estos métodos son efectivos y pueden remover cerca de 80-95% del As de una solución, los costos operacionales son elevados. De hecho la oxidación de As(III) a As(V) es un prerequisito para todas las técnicas convencionales. Sin embargo, la oxidación del As con oxígeno es extremadamente lenta y se deben utilizar oxidantes más fuertes y costosos, como el cloro, el peróxido de hidrogeno o el ozono. Por lo anterior, opciones económicas y amigables con el ambiente, como la biotransformación, ofrecen una alternativa interesante a las rutas químicas tradicionales. Respecto a esta visión, un factor importante en la especiación del As son los microorganismos presentes en suelos y ambientes acuáticos, produciendo diferencias en la solubilidad, biodisponibilidad y toxicidad del metaloide.

HIPÓTESIS

Las comunidades bacterianas asociadas a sedimentos ricos en arsénico, presentan bacterias ureolíticas arsenito-oxidantes capaces de detoxificar aguas con As(V) y/o As(III), mediante biomineralización del metaloide, basada en la precipitación de calcita inducida microbiológicamente.

OBJETIVO GENERAL

Evaluar la capacidad de biomineralizar As(V) y As(III) de bacterias ureoliticas-calcificantes arsenito-oxidantes asociadas a sedimentos con altas concentraciones de arsénico, en sistema de tratamiento biológico para remoción del metaloide en cultivo Batch, basado en la precipitación de calcita inducida microbiológicamente.

OBJETIVOS ESPECÍFICOS

1. Estudiar la estructura de la comunidad bacteriana asociada a sedimentos con altas concentraciones de arsénico, a través del análisis del gen ADNr 16S y de genes funcionales envueltos en las reacciones redox del arsénico.

2. Aislar y caracterizar bacterias calcificantes As(III)-oxidantes desde sedimentos con altas concentraciones de As, capaces de biomineralizar As(V), con vista a su potencial utilización en sistemas de tratamientos biológicos MICP.

3. Evaluar la capacidad de remoción de As(III) y/o As(V) del método basado en la precipitación de calcita inducida microbiológicamente (MICP), usando bacterias ureolíticas arsenito-oxidantes en un sistema biológico Batch.

CAPÍTULO IV: Bacterial communities in Arsenic-rich sediments of Camarones river, Atacama Desert, Chile.
(submitted to PLoS ONE)

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ABSTRACT

Arsenic is a highly biologically toxic metalloid naturally present in Camarones River (Atacama Desert, Chile), representing a great health concern for the local population.

In this study, the taxonomic and functional characterization of bacterial communities associated with metal-rich sediments, from three sites (M1, M2 and M3) along a gradient of arsenic (As) concentration in Camarones River, were evaluated using a combination of approaches. Diversity of bacterial communities was evaluated by Illumina sequencing.

Strains resistant to different arsenic concentrations (0.5 to 100 mM sodium arsenite or sodium arsenate) were isolated and investigated for the presence of genes coding for enzymes involved in the oxidation (*aio*) and reduction (*arsC*) of arsenic.

Bacterial communities possessed moderate diversity, which slightly increased across the river, from M1 to M3 sites, according to the concomitant decreasing of arsenic concentrations. Sequences of the dominant taxonomic groups (high abundance ≥1%) across all sediment samples were affiliated with *Proteobacteria* (mainly represented by *Deltaproteobacteria*), *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* and *Nitrospirae*. Changes in their relative abundances from high to low abundant phylotypes or *vice-versa* resulted in different bacterial assemblages along the river. A great diversity was found within *Deltaproteobacteria* and *Firmicutes*, mainly represented by anaerobic members, particularly active in the metal reduction.

Aio gene was detected more frequently in isolates from M1 (54%), whereas *arsC* gene was present in almost all of isolates from all sediments, suggesting that bacterial communities play an important role in the detoxification of the arsenic compounds.

Keywords: Arsenic-resistant bacteria; bacterial diversity; Bio-reduction Arsenic; Bio-oxidation arsenic, *aio* gen; *ars* gen

INTRODUCTION

Arsenic (As) is one of the most prevalent toxic metalloids on Earth, occurring primarily as the inorganic species oxyanion arsenate (H_3AsO_4) [As(V)] and arsenite (H_3AsO_3) [As(III)]. Arsenates are the predominant species in soil and oxygenated surface water, whereas arsenite predominates under anoxic or reduced conditions [1] and it is 100 times more toxic and 4 to 10 times more soluble than As(V) [2, 3]. Arsenic present in the environment originates from both natural and anthropogenic sources. There is also a growing concern of As contamination from agricultural and other anthropogenic sources, such as copper and sodium-based arsenicals from herbicides and pesticides [4].

Microorganisms in As-rich environments have evolved mechanisms to utilize arsenic for metabolic processes or to detoxify the cell. Therefore, they influence the biochemical cycle of As, bio-transforming As-species varying in solubility, mobility, bioavailability and toxicity [5-7]. While many heavy metals are essential elements at low concentrations, they can exert toxic effects at high concentrations, such as those present in polluted environments. In response to toxic concentrations of heavy metals, many aquatic organisms, including microorganisms, can develop tolerance. Some bacteria, representing different phylogenetic groups involved in As-transformation use processes such as reduction, oxidation and methylation mechanism to tolerate arsenic [8-11]. In general, two metabolic pathways are known for arsenate reduction, one serving for detoxification purposes while the other, respiratory pathway can be used for energy production. The detoxification pathway has been well studied and characterized. In brief, the *ars* system confers resistance to arsenic in prokaryotes, it is composed of up to 5 genes, *arsRDABC*, but at least three are required: *arsR*, encoding a transcriptional repressor, *arsB*, a transmembrane efflux pump, and *arsC*, an arsenate reductase. When present, the proteins encoded by *arsA* and *arsD* help the efflux pump encoded by *arsB* [12-15]. In the respiratory pathway, sometimes called dissimilatory arsenate respiration, a respiratory arsenate reductase consisting of two subunits (ArrA and ArrB) is responsible for the reduction of arsenate. The reductase is encoded by the *arr* operon, which always includes the *arrA* and *arrB* genes, with some strains containing an additional membrane subunit ArrC [13].

On the other hand, microbial arsenite oxidation is a well-known process and many microorganisms are able to do it by means of an arsenite oxidase protein, encoded by *aio* system [16-21]. *aio* genes have been identified in bacteria isolated from various arsenic-rich environments [22]. Arsenite-oxidizing bacteria include chemolithoautotrophic and heterotrophic microorganisms, such as *Acidiphilium acidophilum*, *Acidithiobacillus ferrooxidans*, *Cenibacterium arsenoxidans*, *Alcaligenes faecalis*, *Cupriavidus necator*, *Hydrogenophaga* sp., *Sinorhizobium* sp., *Stenotrophomonas* sp. MM-7, *Ancylobacter dichloromethanicus* As3-1b [23-27] and some *Pseudomonas* spp. [28-30], as well as the thermophiles *Thermus aquaticus*, *Thermus thermophilus*, A*noxybacillus flavithermus* TCC9-4 [31-33]. In addition, a novel arsenite oxidase gene, *arxA*, was identified in the

genome of the Mono Lake (California, USA) isolate *Alkalilimnicola ehrlichii* MLHE-1, a chemolithoautotroph that couples arsenite oxidation to nitrate reduction [19].

Microorganisms mediate many important processes in the aquatic environment, including self-purification, nutrient recycling and development of heavy metal tolerance. The microbial community might even allow these important functions to be maintained despite the input of heavy metals into the environment [7, 9].

In northern Chile, As is leached out of volcanic materials naturally present in watershed areas of the Andes, resulting in high concentrations of As (0.1–1.5 mg L^{-1}) in waters [28, 34-37], representing a serious health concern for populations using waters from Camarones river for both human consumption and agricultural activities. The role of microorganisms in the mobilization and speciation of arsenic has been studied, and several As-resistant bacteria from this river have been previously reported [28, 37-39].
In this work the taxonomic and functional characterization of bacterial communities associated with metal-rich sediments, along a natural gradient of As concentrations were evaluated using a combination of approaches. Phylogenetic diversity of Bacteria has been investigated by the next generation sequencing technique (16S rDNA Illumina sequencing), a molecular approach which allows to simultaneously reveal a large number of individuals and their affiliation. Arsenic resistant isolates were characterized and investigated for the presence of genes coding for enzymes involved in the oxidation (*aio*) and reduction (*arsC*) of arsenic.

MATERIALS AND METHODS

Sample collection

Sediment samples were collected from three sites at Camarones river: the first (M1) is located close to Illapata, at the river upper course, where the flux of water is turbulent (18º56´53.88” S - 69º30´45.64” W, 2,144 m above sea level); the second (M2) is located 38 km West of Illapata close to Camarones village (19º00´31.65” S - 69º51´37.64” W, 709 m above

sea level); the third (M3), located 47 km west M2, is close to Caleta Camarones at the mouth of the river (19º11´06.93" S - 70º16´06.93" W, 13 m above sea level) (Fig. 1).

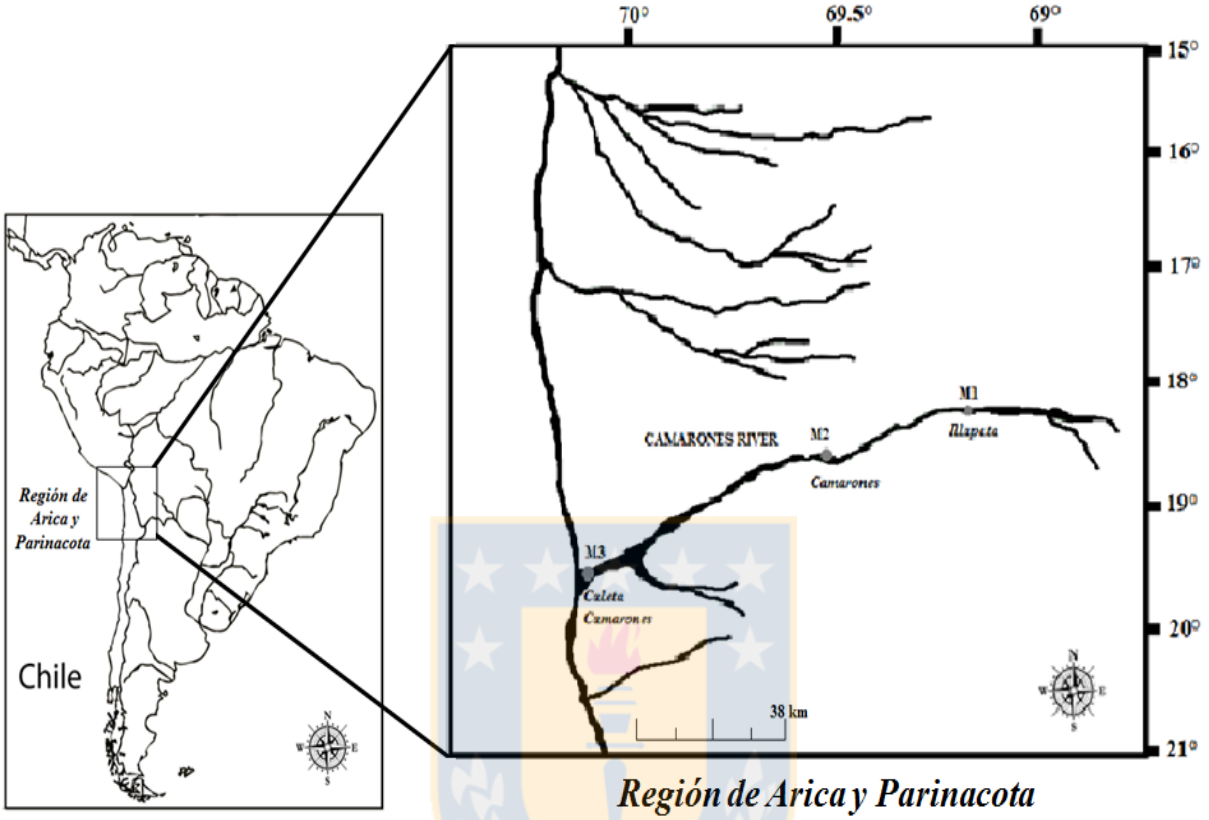

Fig 1. Map of the Chilean Arica y Parinacota Region and location of M1, M2 and M3 sampling sites along the Camarones river

No permissions are required to collect samples at the sites. No endangered or protected species are present at the sampling places. Triplicate samples were obtained from the surface up to a depth of 10 cm using 15 cm long and 3 cm diameter cores. After collection, all samples were kept at 4°C during transport to the laboratory for further processing. After arriving to the laboratory, the superficial 2 cm (aerobic fraction) of each sediment sample were mechanically homogenized under sterile conditions in a laminar flood hood (ZHJH-C 1109C, Zhicheng, Korea).

Physicochemical characterization of sediment samples

Environmental parameters

The pH and temperature were measured on superficial sediments, using the multi-parameter tester Hanna HI98195 (Hanna Instruments). Total organic matter (TOM) was determined gravimetrically following the weight loss by ignition technique [40]. This assay was done in triplicate and the results expressed as % of organic matter.

Total carbon (TC) was determined by combustion at 1300 ºC using a LECO CR-12 equipment. To measure total organic carbon (TOC), carbonates were eliminated acidifying the samples with HCl (1:1) and the remnant organic carbon was determined using a LECO CR-12 equipment.

Inorganic carbon was determined as the difference between TC and TOC. Total nitrogen (TN) was determined using the micro-Kjeldhal technique modified by Branstreet [41]. Certified sediment standards, in accordance with the National Institute of Standards and Technology (NIST), were used for the calibration of carbon and nitrogen analyses. Carbon and nitrogen analyses were performed in duplicates and the results expressed as % in the sample.

Heavy metals concentration

Concentrations of copper (Cu), zinc (Zn), lead (Pb) and cadmium (Cd) were quantified using atomic absorption spectrophotometry, after microwave digestion of samples. Briefly, 0.5 g of sieved and dried sediment was added into 9 ml concentrated nitric acid *plus* 3 ml concentrated hydrochloric acid at 175 °C for 10 min (US EPA 2007). After cooling down, the extracts were centrifuged at 3000 rpm for 5 min. Supernatant was analysed using an AA800 atomic absorption spectrophotometer (PerkinElmer).

Total As was determined in each sample using high performance liquid chromatography (HPLC). HPLC was coupled to a system of gaseous arsine formation and the As detection was achieved by atomic absorption in a quartz bucket (HPLC/HG/QAAS) [42].

Prokaryotic cell abundance

Total prokaryotic cell counts (TC) were determined using 4′,6-diamidino-2-phenylindole (DAPI) staining (1µg ml^{-1}, final concentration). Triplicates of sediment suspensions (fixed with formalin at a final concentration of 2%) were sonicated three times for 1 min to detach cells from particles. Cells were collected on black polycarbonate membrane filters (0.2-µm pore size, 25 mm diameter, Nuclepore Corporation, Pleasanton, USA) and counted using epifluorescence microscopy (Motic BA310, at ×1000 magnification).

Total bacterial community composition analysis

Genomic DNA extraction

The genomic DNA was extracted in triplicate from each sediment sample using PowerSoil DNA Isolation Kit (MO-BIO Laboratories, Inc., USA) according to the manufacturer's direction. The extracts were subsequently pooled. Quality and concentration of DNA were checked by UV/Vis spectroscopy (NanoDrop ND-1000, Peqlab, Erlangen, Germany). DNA was used as template for the high-throughput sequencing.

16S rRNA gene massive sequencing (Illumina MySeq)

The V1-V2 region of the 16S rRNA genes was amplified using the modified universal primers 27f (5'-AGAGT TTGA TCCT GGCT CAG-3') and 338r (5'-GCTG CCTC CCGT AGGA GT-3'), at the "The Greehey Children's Cancer Research Institute" (Greehey CCRI). The Illumina MiSeq Platform was used to generate V1-V2 amplicon reads in a paired-end sequencing run with read length among 200 and 300 bp, at the UT Health Science Center in San Antonio (USA).

Analyses of bacterial communities

The raw data were analysed using the bioinformatics analysis software Mothur (version 1.35.1) with the default options, unless otherwise stated. Reads shorter than 200 bp were discarded.

Reads were denoised using the "pre.cluster" command in Mothur platform to remove sequences that were likely due to errors and assemble reads which differed only by 2bp

[43]. Chimeric sequences were identified and removed, and the remaining sequences classified against the SILVA database [44]. Alpha diversity measures (richness for observed species and Shannon diversity) were calculated on the OTU table obtained from all good quality sequences. To compare the bacterial community compositions across groups of samples, Bray–Curtis similarity analyses were performed and similarity matrices were used to obtain CLUSTER graph, by using PRIMER 6.1.18 (Primer-E, Ltd).

As-resistant strains

Bacterial isolation and tolerance levels to arsenic

For the isolation of As-tolerant bacteria, homogenized sediment (1 g) was added to a sterile solution of 10 mL 0.85% NaCl and agitated at 100 rpm for 5 min. Serial dilutions (0.1 mL aliquots) were cultured on R2A supplemented with 0.5 mM As(III) (as sodium arsenite) or 0.5 mM As(V) (as sodium arsenate) and incubated during 48 h at 25 ºC [45]. Colonies were randomly isolated from As-amended R2A and then screened for As-tolerance, as follows. As-tolerance of each isolate was determined using the agar dilution technique on plates of Luria Bertani (LB) agar containing from 0.5 to 100 mM sodium arsenite or sodium arsenate. Plates were inoculated with approximately 3×10^{7} CFU mL^{-1} cells obtained from each As tolerant strain and cultured at 25 ºC for 24 h. Agar plates containing bacteria but no metalloid were used as controls [46]. According to Rokbani et al. [47], isolates able to grow in the presence of at least 7 mM As (III) (arsenite) or 20 mM As (V) (arsenate) were considered as resistant.

As oxidation and reduction rate

The strains were grown in chemically defined medium (CDM) [45] supplemented with 0.5 mM arsenite or arsenate. Cultures were incubated at 30ºC for 48 h and samples taken every 12 h to monitor As-conversion and bacterial growth (measured by absorbance at 600 nm). Oxidation of As(III) to As(V) and reduction of As(V) to As(III) were determined from supernatants of cultures filtered using sterile 0.22 µm filters (Millipore). As(III) and As(V) were measured by HPLC/HG/QAAS [42]. Samples (500 µL) were introduced to the HG system by means of an automatic injection valve. Samples were transported to the T-joint manifold using 10% hydrochloric acid as carrier and there continuously mixed with a solution

of sodium borohydride to obtain AsH_3. Gaseous arsine was separated from the liquid waste in a gas–liquid separator and carried, by a continuous flow of argon, to the atomizer. Atomic absorption signals were processed using commercial AA Winlab software (PerkinElmer). Quantification limit was 0.5 μg L^{-1} with a linear response up to 20 μg L^{-1} [28, 48].

Identification of As-resistant strains

Genomic DNA from isolates was obtained using UltraClean Microbial DNA Isolation Kit, following the manufacturer's instructions (Mobio Laboratories, Inc.). The 16S rRNA genes were amplified by PCR using universal bacterial primers GM3 and GM4 (5'-AGAG TTTG ATCM TGGC-3' and 5'-TACC TTGT TACG ACTT-3', respectively) according to Brito-Echeverría et al. [49].

Amplified fragments were sequenced using the Dyenamic ET terminator kit (General Electric), in a 3100 Avant genetic Analyser (Applied Biosystem), following the manufacturer's instructions. A nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) was performed to obtain sequences with the greatest significant alignment.

Detection of detoxifying arsenate reductase *arsC* gene

To detect the *arsC* gene, isolates were cultured for 12 to 18 h at 25ºC in LB supplemented with 0.5 mM NaH_2AsO_5 until reaching 10^8 CFU mL^{-1}. The genomic DNA of each isolate was extracted using the UltraClean Microbial DNA Isolation Kit (Mobio Laboratories, Inc.) to serve as template for PCR amplification. Primers used for the PCR technique were arsC-1-F (5'-GTAA TACG CTGG AGAT GATC CG 3') and arsC-1-R (5'-TTTT CCTG CTTC ATCA ACGAC-3'), which correspond to the *ars* operon of *Escherichia coli* [50]; and arsC-1-F (5'-AGTC CTGT TCAT GTGY AC-3') and arsC-1-R (5'-TGGC GTSG AAYG CCG-3') described for the *ars* operon of *Pseudomonas aeruginosa* and *Pseudomonas putida* [11, 51]. PCR products were separated by electrophoresis in a 1.2 % agarose gel and visualized in an UV transilluminator after staining with ethidium bromide [23].

Detection of arsenite oxidation *aio* gene

Strains were cultured in LB supplemented with 0.5 mM NaH_2AsO_3, and DNA was extracted as described above. PCR was performed using the primers 69F (5'-TGYA TYGT NGGN

TGYG GNTA YMA-3') and 1374R (5'-TANC CYTC YTGR TGNC CNCC-3') according to Valenzuela et al. [38]. *E. coli* S17-1 was used as positive control [52], while *E. coli* K-12 was used as negative control. Separation of amplified products was achieved by 0.8% agarose gel electrophoresis followed by ethidium bromide (0.5 µg mL^{-1}) staining and the bands were observed on an UV transilluminator. The QIA quick PCR purification kit (Qiagen), following the manufacturer's instructions, was used to purify the products obtained by PCR and these PCR products were sequencing by Macrogen (Korea). A homology search of 16S rRNA gene sequences was performed using the NCBI Basic Local Alignments Search Tool (BLAST) using the algorithm of the Nucleotide Blast program.

Diversity indices and statistical analysis

The experimental data were recorded with three replications (n=3) and data were expressed as mean ± SD. The data were analysed through Student's t tests using the MINITAB version 15 (USA) software. P values <0.05 were considered as statistically significant [53]. Associations between variables were calculated by Pearson's correlation. Diversity indices and Fisher's exact test was carried out in R software version 3.1.0 [54].

RESULTS

Physical and chemical parameters

The pH, TOM, TOC, TN, As and heavy metals (Cu, Zn, Pb and Cd) measured at each sampling site (M1, M2 and M3) along the Camarones river are reported in Table 1. Samples M1 and M2 showed low values of TOM (<2.5%), TOC (< 0.8%) and TN (< 0.1%). TOM values increased at site M3 and were significantly higher than those of M1 ($p<0.001$) and M2 ($p<0.001$).

The total As concentration in the sediment samples, measured by HPLC/HG/QAAS, decreased as the Camarones river approached the Pacific Ocean. Total As was 498 mg kg^{-1} at the Camarones rhithron zone (M1), it was 245 mg kg^{-1} at the medial zone (M2), and 128 mg kg^{-1} at the mouth of the river (M3) (Table 1).

Prokaryotic cell abundance

The abundance of total prokaryotic cells (TC), measured after DAPI staining, was similar at the three sites, ranging from 8.1 (M1) to 8.9×10^8 cells g^{-1} (M2) (Table 1)

Table 1. Physical and chemical properties, metal composition and total prokaryotic cells (TC) in M1, M2 and M3 samples collected from Camarones river. TOM:total organic matter; TOC: total organic carbon; TN: total nitrogen; As: arsenic; Cu; copper; Cd: cadmium; Zn: zinc; Pb: lead.

	Physical and chemical properties				Metal composition					TC
Site	pH	TOM (%)	TOC (%)	TN (%)	As (mg/kg)	Cu (mg/kg)	Cd (mg/kg)	Zn (mg/kg)	Pb (mg/kg)	(cells $g^{-1} \times 10^8$)
M1	7.18±0.3	2.1	0.6	0.08	498±0.9	56.6±0.4	1.27±0.03	167.9 ± 1.7	47.6±4.2	8.1±1.0
M2	7.44±0.2	2.4	0.7	0.09	245±0.7	37.7±0.5	1.13±0.01	136.5±1.6	45.1±2.7	8.9±2.1
M3	7.49±0.1	3.9	1.3	0.14	128±0.6	45.0±0.4	0.82±0.14	87.6±1.9	32.6±3.2	8.8±1.2

Total bacterial community composition analysis

Sequencing data and diversity estimates

The Illumina-based analysis of the universal V1-V2 region of the 16S rRNA genes for Bacteria produced a total of 3,051,116 sequences across all samples. After quality check within the RDP pyrosequencing pipeline and removing chimeras, 3,032,898 (99.4%) high quality sequences remained.

M1 showed the highest number of quality reads (1,163,206), and the highest number of OTUs was retrieved from sample M3 (26,512) (Table 2). M2 and M3 showed the highest's Shannon diversity indexes. Non-parametric Chao1 and ACE estimators predicted that the highest richness was in M3, whereas the lowest was in M2.

Table 2. Sequencing information, diversity index (H'), estimator of richness (Chao1 and ACE) obtained by Ilumina sequencing from sediment samples (M1, M2 and M3) collected from Camarones river. OTUs: operation taxonomic units.

	M1	M2	M3
Number of reads	1,170,450	767,028	1,113,638
Number of high quality reads	1,163,206	763,126	1,106,566
Unique reads	138,797	127,315	157,358
% Unique reads	11.93	16.68	14.22
Shannon (*H´*)	3.82	6.13	6.10
OTUs at 97% genetic similarity	20,742	22,350	26,512
Chao 1	184,996	146,951	197,054
ACE	183,660	142,632	196,356

Bacterial diversity

Retrieved OTUs were classified in a total of 31 different bacterial phylogenetic groups, of which 26 were common to all samples. Sediment at the upper river site (M1) was, at phylum level, less rich (27 groups) than the other two sites, since four taxa were absent there (*Deferribacteres*, *Elusimicrobia*, *Thermotogae* and SR1) (Table S1 in the supplemental material).

Overall, sequences of the dominant taxonomic groups (abundances ≥1%) across all sediment samples were affiliated with *Proteobacteria* (range 40.3-47.2% of total bacterial sequences), *Firmicutes* (8.4-24.8%), *Acidobacteria* (10.4-17.1%), *Actinobacteria* (5.4-8.1%), *Chloroflexi* (3.9-7.5%), *Planctomycetes* (1.2-5.3%), *Gemmatimonadetes* (1.2-1.5%), and *Nitrospirae* (1.1- 1.2%). However, the relative abundance at phylum level varied considerably across samples, and changes in abundance of phylotypes determined different bacterial assemblages (Fig. 2).

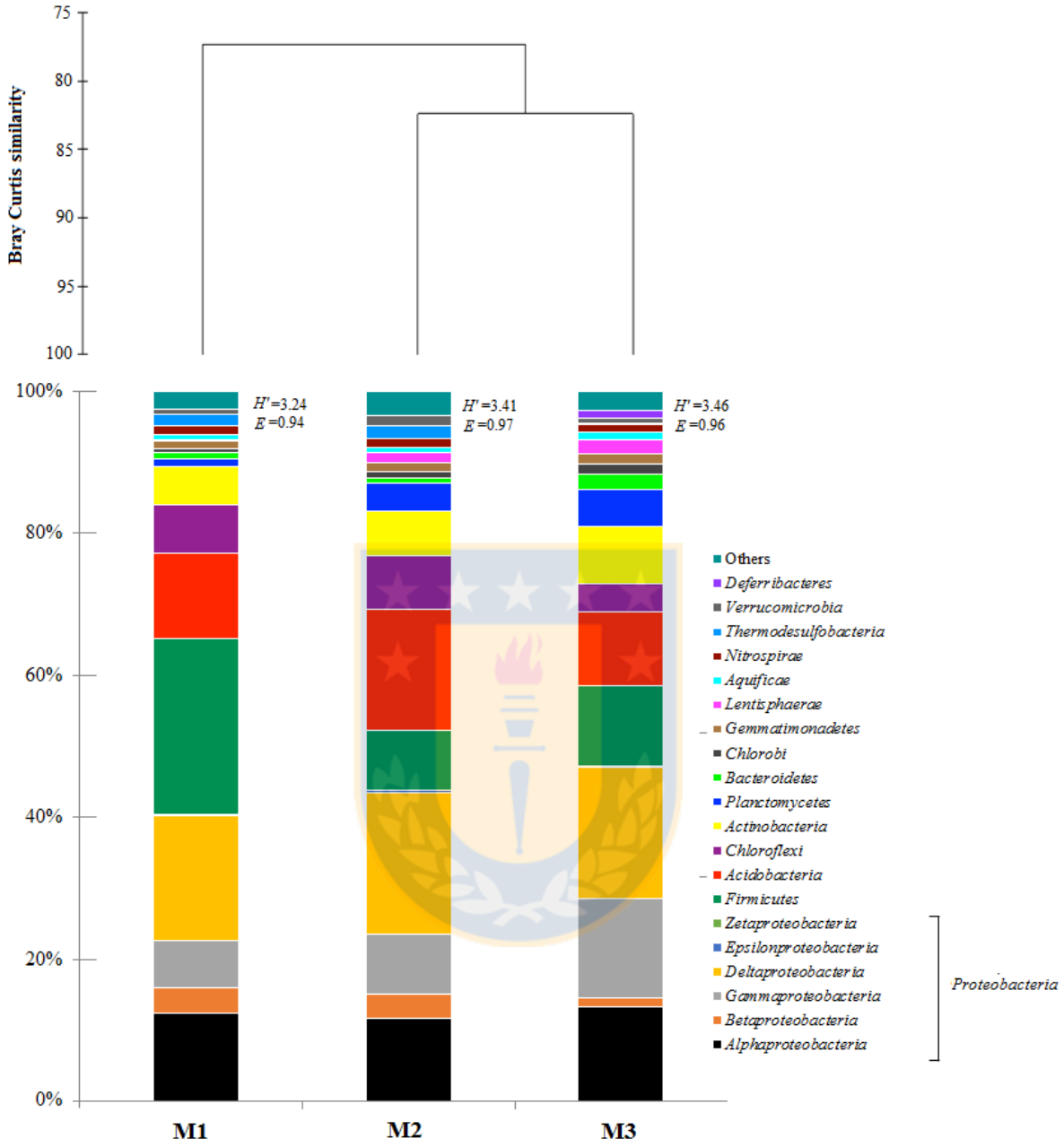

Fig 2.Cluster diagram and relative abundance of sequences (percentage) assigned to bacterial phylogenetic groups and proteobacterial subclasses from sediment samples (M1, M2 and M3) collected from Camarones river. H': Shannon diversity index, E: Evenness index.

Different bacterial communities composition was observed in sediments because sequences affiliated with *Proteobacteria*, *Firmicutes* and *Acidobacteria* covered almost all classified bacterial sequences in M1. *Acidobacteria* were predominant in M2, and *Bacteroidetes*, *Chlorobi*, *Aquificae* and *Deferribacteres* were the dominant groups only in M3, representing minor components in M1 and M2.

Although *Deltaproteobacteria* (range 17.6-20.0%) represented the most abundant proteobacterial class, differences in relative abundance were also observed for sequences affiliated to the other classes: *Alphaproteobacteria* prevailed in M1 and M2, while *Gammaproteobacteria* prevailed in M3. Sequences referred to *Epsilonproteobacteria* and *Zetaproteobacteria* represented a minor component across all samples.

Relatively high abundant phyla in M1 and M2 included also *Thermodesulfobacteria*, but it constituted a minor component in M3. *Lentisphaerae* was dominant in M2 and M3, but not in M1, and finally *Verrucomicrobia* was dominant only in M2.

Among phyla whose abundance was ≤1%, sequences affiliated to BRC1, *Armatimonadetes*, *Cyanobacteria*/*Chloroplast*, *Fusobacteria*, *Deinococcus-Thermus*, *Caldiserica*, *Spirochaetes*, *Chlamydiae*, OD1, *Synergistetes*, TM7 and WS3 occurred across all sediments, even if at different relative abundances. Sequences related to *Tenericutes* were retrieved in M1 and M3, whereas those of *Eleusina* and *Thermotogae* were recovered in M2 and M3, but not in M1. Sequences affiliated with SR1 were found only in M3 (Table S1).

Cluster diagram, representing similarities in the bacterial community composition (phyla and proteobacterial classes) of the studied sediments, because of the great differences among their respective bacterial communities grouped M2 and M3 together, whereas M1 did not clustered with them (Fig. 2).

From all samples, 934 genera were retrieved. The highest number of genera was observed in M3 (716), when compared to M2 (646) and M1 (436). The dominant bacterial genera (40), present in ≥ 1% of the total bacterial sequences at least in one of the three samples, are shown in Table 3.

Diversity at genus level slightly increased from M1 to M3 sites. A total number of 20 dominant genera were retrieved for M1, 19 for M2, and 25 for M3. A minor part of dominant

bacterial genera (8/40) was ubiquitous in all samples: *Anaeromyxobacter*, *Desulfobacca*, *Hippea* (within *Deltaproteobacteria*), *Bacillus* (*Firmicutes*), Gp6 (*Acidobacteria*), *Sphaerobacter* (*Chloroflexi*), *Gemmatimonas* (*Gemmatimonadetes*) and *Nitrospira* (*Nitrospirae*). However, different abundant genera were unique for each site.

The most abundant genus in M1 was *Bacillus*, whereas *Paenibacillus*, *Tumebacillus* and *Vulcanibacillus* were unique genera within *Firmicutes* for M1. Moreover, *Byssovorax*, *Desulfovirga* and *Sorangium* (*Deltaproteobacteria*), and Gp3 (*Acidobacteria*) were retrieved as unique dominant genera in M1. Four dominant genera were unique in M2: *Beggiatoa* (*Gammaproteobacteria*), *Geothermobacter* and *Smithella* (*Deltaproteobacteria*), and Gp2 (*Acidobacteria*), which also was the most abundant genus in M2.

Most of the dominant genera identified in M3 (13/40) were not retrieved in the other samples, and were related to *Alphaproteobacteria* (*Pelagibius*, *Afifella* and *Bauldia*), *Gammaproteobacteria* (*Ectothiorhodosinus*, *Methylohalomonas* and *Thiococcus*), *Deltaproteobacteria* (*Desulfarculus*, *Desulfocurvus* and *Syntrophorhabdus*), *Acidobacteria* (Gp 21 and Gp22), *Chlorobi* (*Ignavibacterium*), and *Actinobacteria* (*Actinotalea*).

The dominant genera common to M1 and M2 were related to *Dongia* (*Alphaproteobacteria*), Gp4 (*Acidobacteria*), *Caldilinea* (*Chloroflexi*) and *Caldimicrobium* (*Thermodesulfobacteria*).

Genus Gp10 (*Acidobacteria*) was the only genus common to M1 and M3. Finally, dominant genera affiliated with *Planctomycetes* and *Lentisphaerae* were retrieved in M2 and M3.

Table 3. Bacterial genera retrieved in sediment samples (M1, M2 and M3) from Camarones river.

Phylum	Class	Genus	M1	M2	M3
Proteobacteria	*Alpha-*	*Afifella*			
		Bauldia			
		Dongia			
		Pelagibius			
	Gamma-	*Beggiatoa*			
		Ectothiorhodosinus			
		Methylohalomonas			
		Thiococcus			
	Delta-	*Anaeromyxobacter*			
		Byssovorax			
		Desulfarculus			
		Desulfobacca			
		Desulfocurvus			
		Desulfovirga			
		Geothermobacter			
		Hippea			
		Smithella			
		Sorangium			
		Syntrophorhabdus			
Firmicutes		*Bacillus*			
		Paenibacillus			
		Tumebacillus			
		Vulcanibacillus			
Acidobacteria		Gp2			
		Gp3			
		Gp4			
		Gp6			
		Gp10			
		Gp21			
		Gp22			
Chloroflexi		*Caldilinea*			
		Sphaerobacter			
Actinobacteria		*Actinotalea*			
Planctomycetes		*Phycisphaera*			
		Singulisphaera			
Chlorobi		*Ignavibacterium*			
Gemmatimonadetes		*Gemmatimonas*			
Lentisphaerae		*Victivallis*			
Nitrospirae		*Nitrospira*			
Thermodesulfobacteria		*Caldimicrobium*			

≥8%
≥5%
≥3%
≥2%
≥1%
<1%

Arsenic-resistant isolates

Strains isolation and identification

Based on their ability to grow at increasing concentrations of arsenic, from 0.5mM to 100mM of As(III) or As(V), a total of 150 bacterial strains were isolated from M1, M2 and M3 sediments. As-resistant strains were defined as able to grow on agar containing 7 mM As(III) or 20 mM As(V) [52]. Of the total bacterial strains, 13 As-resistant strains were obtained from M1, 15 from M2, and 23 from M3 (Table 4). Overall, the 51 strains were capable to grow at concentrations of arsenite greater than 7mM (from 8 to 64 mM). Additionally, all the strains were capable of growth at the maximum concentration of arsenate tested (100 mM).

Seventeen out 51 As-resistant strains (33.3%) were able to oxidize As(III), 47 strains (90.2%) were able to reduce As(V), and 10 strains (19.6%) were able to both oxidize and reduce arsenic. All the 51 As-resistant strains were able to grow at the highest As(V) tested concentration (100mM) and 22 strains (43%) were able to grow up to 64mM As(III).

The percentage of As(III)-oxidation was highest (90-100%) in strains from M1, ranged from 68 to 84% in strains from M2, and varied from 80% to 93% in strains from M3 (Table 4). The highest percentage of As(V) reduction was observed in strains from M1 (55-95%), and the lowest percentage in strains from M3 (10-40%) (Table 4). Pearson's analysis showed a significant positive correlation between total As concentration and the capacity of strains from each sediment to oxidize or reduce the metalloid (data not shown).

The levels of 16S rRNA sequence identity (99.1–100%) of As-resistant strains isolated from Camarones river to the most closely related species are reported in Table S2. As-resistant strains were mainly identified as members of *Proteobacteria* (49 strains), including *Alpha*-(4 strains), *Beta*-(8 strains) and *Gammaproteobacteria* (37 strains), and *Firmicutes* (2 strains) (Table 4).

Detection of *aio* and *arsC* genes

The presence of genes codifying for the enzymes arsenite oxidase (*aio*) and arsenate reductase (*arsC*) was investigated in all 51 As-resistant strains, via PCR. As(III)-oxidizing strains (17/51) presented a PCR product of approximately 1,200 bp, as expected size fragment corresponding to arsenite oxidase genes. The *aio* gene was more frequently detected in isolates from site M1 (41.2%) than from those of M2 (23.5%) or M3 (35.3%) (Table 4). In addition, As(V)-reducing strains (50/51) presented different PCR products, in relation to the presence of different arsenate reductases. The *arsC* gene was detected in all As-resistant strains, with the only exception of strain VC-17 from M1.

Table 4. As-resistant strains isolated from M1, M2 and M3 samples collected from Camarones river.

Strain	As^{3+} (1)	As^{5+} (2)	*arsC* (3)	*aio* (4)	O/R (5)	Strain	As^{3+} (1)	As^{5+} (2)	*arsC* (3)	*aio* (4)	O/R (5)
		M1				**VC-65**	>40	>100	+	+	79/64
VC-02	<40	>100	+	-	0/80	**VC-68**	<10	>100	+	-	0/68
VC-05	<10	>100	+	-	0/70	**VC-71**	>40	>100	+	+	84/65
VC-07	<40	>100	+	+	90/95			**M3**			
VC-08	<10	>100	+	-	0/75	**VC-72**	<10	>100	+	-	0/25
VC-11	<10	>100	+	-	0/98	**VC-73**	<10	>100	+	-	0/36
VC-17	>40	>100	-	+	100/0	**VC-74**	<10	>100	+	-	0/40
VC-19	>40	>100	+	+	96/95	**VC-77**	<10	>100	+	-	0/32
VC-21	>40	>100	+	+	95/0	**VC-79**	<10	>100	+	-	0/28
VC-23	>40	>100	+	+	93/75	**VC-81**	>40	>100	+	+	80/25
VC-29	<10	>100	+	-	0/65	**VC-86**	<10	>100	+	-	0/15
VC-33	>40	>100	+	-	0/70	**VC-87**	>40	>100	+	+	93/0
VC-39	>40	>100	+	+	95/55	**VC-88**	<10	>100	+	-	0/25
VC-41	>40	>100	+	+	90/70	**VC-89**	>40	>100	+	-	0/30
		M2				**VC-90**	<10	>100	+	-	0/11
VC-42	<10	>100	+	-	0/58	**VC-95**	>40	>100	+	+	87/10
VC-44	<10	>100	+	-	0/55	**VC-97**	>40	>100	+	+	85/0
VC-45	>40	>100	+	-	0/60	**VC-102**	>40	>100	+	-	0/20
VC-48	>40	>100	+	+	75/63	**VC-114**	>40	>100	+	-	0/28
VC-50	>40	>100	+	-	0/53	**VC-119**	>40	>100	+	+	91/0
VC-51	<10	>100	+	-	0/67	**VC-123**	>40	>100	+	+	85/20
VC-52	<10	>100	+	-	0/64	**VC-131**	<10	>100	+	-	0/20
VC-53	<10	>100	+	-	0/60	**VC-139**	<10	>100	+	-	0/22
VC-56	>40	>100	+	+	68/65	**VC-141**	<10	>100	+	-	0/28
VC-57	<10	>100	+	-	0/68	**VC-143**	<10	>100	+	-	0/13
VC-59	<10	>100	+	-	0/63	**VC-145**	<10	>100	+	-	0/30
VC-61	<10	>100	+	-	0/60	**VC-146**	<10	>100	+	-	0/10

(1) Tolerance levels to As(III), values expressed in mM of arsenite; (2) Tolerance levels to As(V), values expressed in mM of arsenate; (3) PCR detection aox gen; (4) PCR detection arsC gen; (5) 0/R: Oxidation percentage and/or reduction percentage.

DISCUSSION

The northern Region of Chile, especially the Atacama Desert area, has been described as a naturally arsenic-rich environment. Minerals of metallic sulfides containing arsenic are dissolved in the Andes Mountains, affecting superficial and ground waters that cross the Atacama Desert which are used as drinking water sources. Since 1970, drinking water is treated to remove arsenic in all the large cities of the Atacama Region, such as the city of Antofagasta. However, inhabitants of several small rural villages remains exposed to arsenic in drinking water.

The inhabitants of the towns of Camarones and Illapata (Atacama, Chile) use mainly natural water from the Camarones river for both human consumption and agricultural activities. Waters of Camarones river contain a total As concentration exceeding 1.0 mg L^{-1}, mainly in the form of arsenate (As [V]). This contamination has chronically affected the rural populations living near the river, generating a variety of health problems [34, 55].
In this study, sediments (M1, M2 and M3) collected from Camarones river showed high total arsenic concentration which decreased (from 498 to 128 mg kg^{-1}) as the river approached to the Pacific Ocean, since several inputs of springs and groundwater lacking As input into the lower zone of river (M3) [42]. Other authors reported similar total arsenic levels for this same river [34, 42]. Low values of total organic matter (TOM) were measured in M1 and M2 and may be due to the slopes and fast-flowing pattern of the Camarones river at these sites, dragging the organic matter. M1 and M2 showed also the highest concentrations of heavy metals Cu, Cd, Zn and Pb, attributed to the lithogenic characteristics of the river.

Metal-contaminated sediments are inhabited by extremely complex and well adapted microbial community, that play a fundamental role in degrading organic matter and in biogeochemical cycles [56]. Microbial diversity, here investigated by a massive parallel sequencing (Illumina), revealed a great diversity of bacterial communities, detecting also bacteria occurring at very low abundance (≤0.01%), that would have been masked by dominant populations if techniques with lower resolution had been applied. As evaluated by diversity indices, bacterial communities associated with sediments from Camarones river possessed moderate diversity, which slightly increased across the river, from M1 to M3 sites,

according to the increasing of TOC levels and the concomitant decreasing of arsenic concentrations. As generally accepted, moderately disturbed conditions often result in high diversity of microbial communities, like those occurring at the mouth of Camarones river (site M3).

Sequences of the dominant taxonomic groups (abundances ≥1%) across all sediment samples were affiliated with *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* and *Nitrospirae*, but their relative abundances differed in each sample, resulting in different bacterial assemblages. The bacterial community from M2 and M3 were more diverse, since sequences affiliated with *Proteobacteria*, *Firmicutes* and *Acidobacteria* covered almost all classified bacterial sequences in M1. *Actinobacteria*, *Firmicutes*, *Gemmatimonadetes* and *Nitrospirae* have been reported as dominant in highly contaminated soils, and their functional genes indicated that these phyla could tolerate high concentration of arsenic [57].

Previous studies on prokaryotic diversity about microbial communities associated with metal-contaminated sediments in eutrophic environments also reported *Proteobacteria* as predominant phylum, but with *Betaproteobacteria* as dominant subclass, followed by *Bacteroidetes* as major components [56, 58]. Differently from these studies, bacterial sequences here retrieved were mainly related to the *Deltaproteobacteria* subclass, and *Firmicutes* in oligotrophic conditions, such as those of M1 and M2 sites of Camarones river.

Bacteroidetes, together with *Firmicutes* and *Planctomycetes* are well-known biofilm forming bacteria, which in this form are able to resist various physico-chemical stresses, as the typical turbulence of the riverine waters, and to increase arsenic resistance. Members of *Firmicutes*, mainly represented by *Bacillus* genus, were the most abundant in M1. To date, several *Bacillus* spp. have been isolated as dissimilatory As (V)-reducing bacteria, and *B. selenatarsenatis* was reported to be able to release As from contaminated soils [14].

A great diversity was found within *Deltaproteobacteria*, mainly represented by anaerobic members involved in the metal reduction, and particularly in respiring arsenate through a detoxification pathway encoded by *ars* operon [59]. Sulphate-reducing bacteria are key

mediators of anaerobic carbon cycling in sediments and some genera, such as those related to *Anaeromyxobacter*, *Desulfobacca* and *Geobacter*, have been reported to play an important role in the arsenate reduction [60].

High abundances of *Acidobacteria* and particularly those of GP6, retrieved across all samples, and of GP2, the most abundant in M2 site, may be related to their high metal resistance capability [61]. Sequences related to the genus *Caldimicrobium* (*Thermodesulfobacteria*), grouping extremely thermophilic, strictly anaerobic, sulphur-oxidizing bacteria, widely distributed in other hot environments [32], were dominant in the upper sites (M1 and M2) of Camarones river.

Differently to the other sites, genera identified in M3 were characteristically related to *Gammaproteobacteria*, and principally to *Ectothiorhodosinus*, an anoxygenic phototroph that may contribute to the primary production in halophilic conditions.

A total of 51 heterotrophic As-resistant strains, that can obtain energy from oxidation of As(III) or reduction of As(V), were isolated and identified as member of *Alpha*-, *Beta*- and *Gammaproteobacteria*, and *Firmicutes*. All arsenite (As III) resistant strains (17), possessing the *aio* gene, presented a great As(III)-oxidative capacity (68-100%), particularly those from M1 (90-100%), where the highest concentration of total arsenic was registered, confirming data previously reported in the same riverine zone [37, 38]. *Pseudomonas arsenicoxydans*, isolated From M1 and firstly described from sediment of the same area, represents and indigenous species in Camarones river with the highest ability to oxidize As (III) [36].

Almost all As(V)-resistant strains (50 strains) possessed the *arsC* gene, explaining the high percentage of bacteria resistant to arsenic along the river sediments. Escalante et al. [39] also reported that the major part of As-resistant strains isolated from Camarones river were able to the reduce arsenic. Overall, a significant correlation was observed between the environmental As distribution and the capacity to oxidize or reduce the metalloid present in the sediments of Camarones river.

The diversity of strains possessing *arsC* gene greatly increased as the total As concentration decreased in the river. As(V)-resistant strains isolated from M1, presenting the highest arsenic reducing capacity, were identified as species of the following genera: *Acinetobacter*, *Aeromonas*, *Pantoea*, *Pseudomonas* and *Sphingomonas*, with all of them that have been previously isolated from samples collected from the same riverine zone [36]. In contrast, *Fusibacter paucivorans*, isolated from M2, a thiosulfate-reducing bacterium, has not been described before as As(V)-reducing bacterium.

Moreover, the co-presence of *aio* and *arsC* genes could confer higher resistance to arsenic than bearing *arsC* alone [22]. As-resistant strains possessing both genes in the sediments of Camarones river were: *Aquabacterium* sp., *Alcaligenes* sp., *Burkholderia cepacia*, *Pseudomonas marginalis*, *P. stutzeri*, *P. vancouverensis*, *P. putida*, *Xanthomonas* sp. and *Shewanella* sp. Of them, *Burkholderia cepacia* and *P. marginalis* were previously isolated from a natural biofilm associated to volcanic rocks of the Atacama Desert [37]. *Aquabacterium* sp., a well-known biofilm forming bacterium, *Bacillus* sp. AS-38 and *Xanthomonas* sp. are here reported for the first time as able to oxidize and also reduce arsenic. Moreover, this is the first report describing the presence of *arsC* gene and As(V)-reducing capability in *P. marginalis*, *P. stutzeri*, and *P. vancouverensis*.

Arsenate reduction and arsenite oxidation under aerobic conditions have been reported as detoxification mechanisms in several aerobic bacteria isolated from different As-contaminated sites [62, 63], suggesting that As (As[V]/As[III]) resistance plays an important role in the biogeochemical cycling of this element in nature [64]. Microbial species that bio-transform arsenic between oxidation states with differing environmental behaviours are able to control the release of adsorbed arsenic from sediments into water and therefore they may be potentially utilized for bioremediation purposes. Resistant strains able to transform As(III) into As(V) with a high rate of efficiency, such as *P. arsenicoxydans* strain VC-17, will be further investigated for its biotechnological potential, to detoxify waters from the Camarones river.

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SUPPORTING INFORMATION

Table S1. Relative abundances of bacterial phylogenetic group from sediment samples (M1, M2 and M3) collected from Camarones river. Dominant phylogenetic groups (≥1% of total classified sequences) common to sediments (M1, M2 and M3) are represented in bold.

M1		M2		M3	
Phylogenic group	**Relative abundance (%)**	**Phylogenic group**	**Relative abundance (%)**	**Phylogenic group**	**Relative abundance (%)**
Proteobacteria	40.31	***Proteobacteria***	43.81	***Proteobacteria***	47.17
Alpha-	12.41	*Alpha-*	11.57	*Alpha-*	13.18
Beta-	3.60	*Beta-*	3.44	*Beta-*	1.41
Gamma-	6.65	*Gamma-*	8.56	*Gamma-*	13.91
Delta-	17.58	*Delta-*	19.95	*Delta-*	18.48
Epsilon-	0.06	*Epsilon-*	0.26	*Epsilon-*	0.15
Zeta-	≤0.01	*Zeta-*	0.03	*Zeta-*	0.03
Firmicutes	24.82	***Acidobacteria***	17.12	***Firmicutes***	11.34
Acidobacteria	12.02	***Firmicutes***	8.42	***Acidobacteria***	10.37
Chloroflexi	6.88	***Chloroflexi***	7.50	***Actinobacteria***	8.09
Actinobacteria	5.37	***Actinobacteria***	6.24	***Planctomycetes***	5.29
Thermodesulfobacteria	1.64	***Planctomycetes***	3.93	***Chloroflexi***	3.94
Nitrospirae	1.25	*Thermodesulfobacteria*	1.89	*Bacteroidetes*	2.19
Gemmatimonadetes	1.16	***Gemmatimonadetes***	1.34	*Lentisphaerae*	1.98
Planctomycetes	1.16	*Lentisphaerae*	1.34	***Gemmatimonadetes***	1.46
		Verrucomicrobia	1.32	*Chlorobi*	1.29
		Nitrospirae	1.23	***Nitrospirae***	1.14
				Aquificae	1.06
				Deferribacteres	1.02
Bacteroidetes	0.90	*Chlorobi*	0.84	*Verrucomicrobia*	0.73
BRC1	0.84	*Bacteroidetes*	0.8	*Synergistetes*	0.62
Aquificae	0.77	*Aquificae*	0.75	*Spirochaetes*	0.38
Verrucomicrobia	0.64	*Armatimonadetes*	0.75	*Cyanobacteria/Chloroplast*	0.35
Armatimonadetes	0.58	BRC1	0.55	WS3	0.24
Chlorobi	0.42	*Caldiserica*	0.45	*Thermodesulfobacteria*	0.18
Cyanobacteria/Chloroplast	0.26	*Cyanobacteria/Chloroplast*	0.35	*Chlamydiae*	0.17
Fusobacteria	0.23	*Spirochaetes*	0.35	*Elusimicrobia*	0.15
Deinococcus-Thermus	0.19	*Synergistetes*	0.26	*Fusobacteria*	0.15
Caldiserica	0.16	TM7	0.15	BRC1	0.14
Lentisphaerae	0.10	*Deinococcus-Thermus*	0.13	*Deinococcus-Thermus*	0.12
Spirochaetes	0.10	WS3	0.13	*Caldiserica*	0.09
Tenericutes	0.06	*Chlamydiae*	0.09	OD1	0.08
Chlamydiae	0.03	*Deferribacteres*	0.09	*Thermotogae*	0.08
OD1	0.03	*Elusimicrobia*	0.07	TM7	0.06
Synergistetes	0.03	*Fusobacteria*	0.04	*Armatimonadetes*	0.05
TM7	0.03	OD1	0.03	*Tenericutes*	0.05
WS3	0.03	*Thermotogae*	0.03	SR1	0.02

Table S2. Closest GenBank match to the relative sequences of the strains isolated from the three samples (M1, M2 and M3) collected from the Camarones river.

Strain	Closest sequence relative	% ID	GenBank accession	Strain	Closest sequence relative	% ID	GenBank accession
	Sediment M1			VC-65	*Pseudomonas putida* **	100	AF094743
VC-2	*Sphingomonas* sp. **	100	AY584571	VC-68	*Rahnella aquatilis*	99.9	GU171376
VC-5	*Acinetobacter* sp.	100	AY576723	VC-71	*Xanthomonas* sp. *	99.9	AY689031
VC-7	*Pseudomonas stutzeri*	99.9	AJ270458		Sediment M3		
VC-8	*Aeromonas salmonicida* **	99.7	AF200329	VC-72	*Citrobacter freundii*	99.1	DQ444289
VC-11	*Pantoea agglomerans* **	99.9	EU304255	VC-73	*Enterobacter cloacae*	100	DQ202394
VC-17	*Pseudomonas arsenicoxydans*	100	NR117022	VC-74	*Aeromonas salmonicida* **	99.7	AF200329
VC-19	*Pseudomonas marginalis*	99.9	DQ232737	VC-77	*Fusibacter paucivorans* *	99.8	AF050099
VC-21	*Pseudomonas putida* **	100	AF094743	VC-79	*Aquabacterium* sp. **	100	AF089858
VC-23	*Aquabacterium* sp **	99.1	AF089858	VC-81	*Xanthomonas* sp. *	100	AY689031
VC-29	*Aeromonas hydrophila*	99.6	AB472904	VC-86	*Enterobacter aerogenes* *	99.9	AJ251468
VC-33	*Pseudomonas fluorescens* **	100	AY973265	VC-87	*Bacillus sp. AS-38*	100	AJ391199
VC-39	*Alcaligenes* sp *	99.9	DQ993330	VC-88	*Erwinia sp.*	99.9	AM117463
VC-41	*Pseudomonas vancouverensis* *	99.9	AM293568	VC-89	*Rhizobium sp.*	99.5	AB069652
	Sediment M2			VC-90	*Shigella boydii*	99.9	AY696668
VC-42	*Serratia marcescens* *	99.9	AB061685	VC-95	*Pseudomonas putida* **	100	AF094743
VC-44	*Serratia odorífera* *	99.7	AJ233432	VC-97	*Burkholderia. cepacia* *	98.9	AY741354
VC-45	*Sphingomonas* sp. **	100	AY584571	VC-102	*Sphingomonas sp.* **	100	AY584571
VC-48	*Burkholderia cepacia* *	99.9	AY741354	VC-114	*Pseudomonas fluorescens* **	99.1	AY973265
VC-50	*Pseudomonas fluorescens* **	99.9	AY973265	VC-119	*Alcaligenes* sp *	99.6	DQ993330
VC-51	*Aeromonas salmonicida* **	99.7	AF200329	VC-123	*Shewanella sp*	100	DQ2117634
VC-52	*Fusibacter paucivorans* *	99.6	AF050099	VC-131	*Serratia marcescens* *	99.9	AB061685
VC-53	*Aquabacterium* sp. **	99.9	AF089858	VC-139	*Serratia odorífera* *	99.7	AJ233432
VC-56	*Pseudomonas vancouverensis* *	99.9	AM293568	VC-141	*Rahnella aquatilis*	99.9	GU171376
VC-57	*Enterobacter aerogenes* *	99.9	AJ251468	VC-143	*Pantoea agglomerans* **	100	EU304255
VC-59	*Pantoea agglomerans* **	100	EU304255	VC-145	*Yersinia intermedia*	99.8	GQ451990
VC-61	*Erwinia* sp.	99.7	AM117463	VC-146	*Comamonas* sp.	99.7	HM365951

* Strain present in two sediments samples, ** Strain present in three sediments samples

CAPÍTULO V: Changes in bacterial communities composition accompanying a pronounced Arsenic shift in Loa river, elucidated by Illumina sequencing technology. (submitted to PLoS ONE)

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ABSTRACT

Loa river has unique conditions, such as its extreme desert climate, high evaporation rates, high salinity, neutral to alkaline pH of waters, high Boron concentrations and some of the highest concentrations of arsenic reported in rivers in the world, whose presence is attributed to natural sources and anthropogenic sources, principally mining waste. The Bacteria communities shift along a gradient of As-concentration of sediment, from both upstream and downstream of Sloman dam presented in Loa river was evaluated by Illumina sequencing. Bacterial communities possess moderate abundance and high diversity, which is influenced by the presence of the dam and the physical and chemical parameters (such

as an organic matter, particle size, redox potential, Fe, total dissolved solids, temperature, salinity, conductivity, dissolved oxygen, pH, SO4 and arsenic concentrations of the sediments. Sequences of the dominant taxonomic groups (high abundance ≥1%) across all sediment samples were affiliated with *Proteobacteria* (mainly represented by *Deltaproteobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria*), *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, *Lentisphaerae*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia*, and *Chlorobi*. Changes in their relative abundances from high to low abundant phylotypes or vice-versa resulted in different bacterial assemblages along the river. Highest diversity of dominant genera was found within *Deltaproteobacteria*, *Bacteroidetes* and *Chloroflexi* Phylum, mainly represented by anaerobic members and that are well known for for their capability to reduce S, Fe and As in diverse environments, suggesting that bacterial communities play an important role in the detoxification of the arsenic compounds and they may be potentially utilized for bioremediation purposes.

INTRODUCTION

Loa river rises from an active volcanic area of the Andes mountain, at an altitude over 4000 m above sea level. It runs for approximately 440 km along the Atacama Desert receiving waters from several tributaries (mainly San Pedro, Salado, and San Salvador rivers) before reaching the Ocean. Its main tributaries provide water for domestic, industrial and irrigation uses in this arid region. Loa river has unique conditions, such as its extreme desert climate, high evaporation rates, high salinity, neutral to alkaline waters, high Boron concentrations and some of the highest concentrations of arsenic (from 200 to 4400 μg As L^{-1}) reported in rivers in the world (Smedley & Kinniburgh, 2002).

The presence of arsenic in Loa's water is attributed to two main sources: i) natural sources, associated to its tributaries, mostly the Salado river which is As-enriched by waters from the geothermal springs of El Tatio, with levels up to 27 mg As L^{-1} (Romero et al., 2003), and ii) anthropogenic sources, principally mining waste (e.g., the Chuquicamata cooper mine), smelter emissions and enriched arsenic effluents from water treatment plants (e.g., The Cerro Topater plant from 1978 to 2000) .

As-contamination of natural waters is a particular health concern, since arsenic is a toxic and carcinogenic metalloid (Fatmi et al., 2009; Nguyen et al., 2009 ; Kavcar et al., 2009), where individual chemical species of inorganic arsenic (As(III) and As(V)) are capable of inhibit protein function and cell metabolism, through disturbing disulfide bonds and ATP synthesis, respectively.(Hughes et al., 2011; Tawfik and Viola, 2011).

The high concentration of As, associated with high salinity and Boron, compromises the use of the water in the region of Antofagasta as a safe drinking water (Smith et al., 1998). In the urban supply, the As is removed to a large extent (<50 mg L^{-1}), by conventional treatment processes such include coagulation, filtration, lime softening, activated alumina adsorption, ion exchange, reverse osmosis, electrodialysis reversal and nanofiltration (Campos et al., 2009), but not in the supplies to rural areas or in the water used for irrigation. In addition, adverse health effects by chronic arsenicism as a consequence of As exposure through water and soil, have been recognized in rural populations of this region since 1962, and include As-induced skin lesions and cancers of bladder and lung (Smith et al., 1998; Ferreccio et al., 2000; Hopenhayn-Rich et al., 2000).

Prokaryotes play an important role in the dissolution of arsenic-containing minerals and in transforming arsenic speciation by adsorption, oxidation and reduction processes in As-contaminated aquatic environments. Bacteria inhabiting these environments, represented by different phylogenetic groups, can use arsenic compounds as electron donors, electron acceptors or possess arsenic detoxification mechanisms (Silver and Phung, 2005; Stolz et al. 2002).

The As-rich environment of Loa river, represent an interesting field where to investigate Bacteria communities shift along a gradient of As-concentration by Illumina sequencing technique.

MATERIALS AND METHODS

Description of study area

Along the Loa river, three sections with specific chemical properties can be defined: Upper, Middle and Lower Loa (Romero et al., 2003). The Upper Loa extends from its origin at the foot of Miño Volcano to the confluence with the Salado river near to Chiu-Chiu village. The Middle Loa comprises the zone between the confluence with the Salado river and the confluence of Loa–San Salvador river in the Chacance village. Finally, the Lower Loa comprises the zone downstream of the Medium Loa-San Salvador confluence and the discharge of Loa river into Pacific Ocean (Romero et al., 2003; Bugueño et al., 2014).

Sloman dam and Quillagua village are located in the Lower Loa (Fig. 1). Sloman dam has a surface area of 0.1 km2 and is located at 920 meters above sea level (masl). A dam was built on this part of the river in the 1920s with the purpose of electricity generation for supply energy to saltpeter mining industry. Since, then sediments have been accumulated into the dam. Extremely elevated As concentrations (up to 30 mg l-1) in reservoir waters, have been reported during flood events, occurred during the Altiplano Winter (storm events concentrating annual rainfall between December through March, also known as Bolivian Winter), with great impact on the fluvial and marine ecosystems downstream, as well as on the supply of water for human activities (Arroyo et al., 1999).

Quillagua village (806 masl) is an oasis located 23 km downstream from the Sloman dam, It has a surface area of 3 km2 and is a natural wetland with abundant aquatic plants that covers the river bed and banks. The indigenous community of Quillagua is facing a socio-environmental conflict with mining companies due to the drying and contamination of the Loa river. Since 1997 the waters of the Loa river have been severely polluted by the Sloman mining waste (mainly heavy metals and xanthate), seriously affecting the Loa river basin, and generating the irreversible destruction of the Quillagua Oasis and the local economy causing a massive people migration (Larrain, 2012).

Sample collection

Sediments were collected from three sites at the Lower Section of Loa river: the first is located upstream from Sloman dam (L1) (21°51’14.32"S - 69°30’52.77"W); the second (L2) is located downstream from Sloman dam (21°51’6.44”S - 69°30'53.46"W); the third (L3) is located 23 km downstream from Sloman dam, close to Quillagua Oasis (21°39’32.26"S - 69°32'9.76"W) (Fig. 1). No permissions are required to collect samples at the sites. No endangered or protected species are present at the sampling places. Surface sediment samples were collected.

At each sampling site, water samples (1 liter) were collected at the surface by sterile pyrex bottles. Surface sediment samples were collected using sterile Corning tubes (15 cm long and 3 cm diameter). After collection, all samples were kept at 4°C during transport to the laboratory for further processing. After arriving at the laboratory, the superficial 2 cm (aerobic fraction) of each sediment sample were mechanically homogenized under sterile conditions in a laminar flood hood (ZHJH-C 1109C, Zhicheng, Korea).

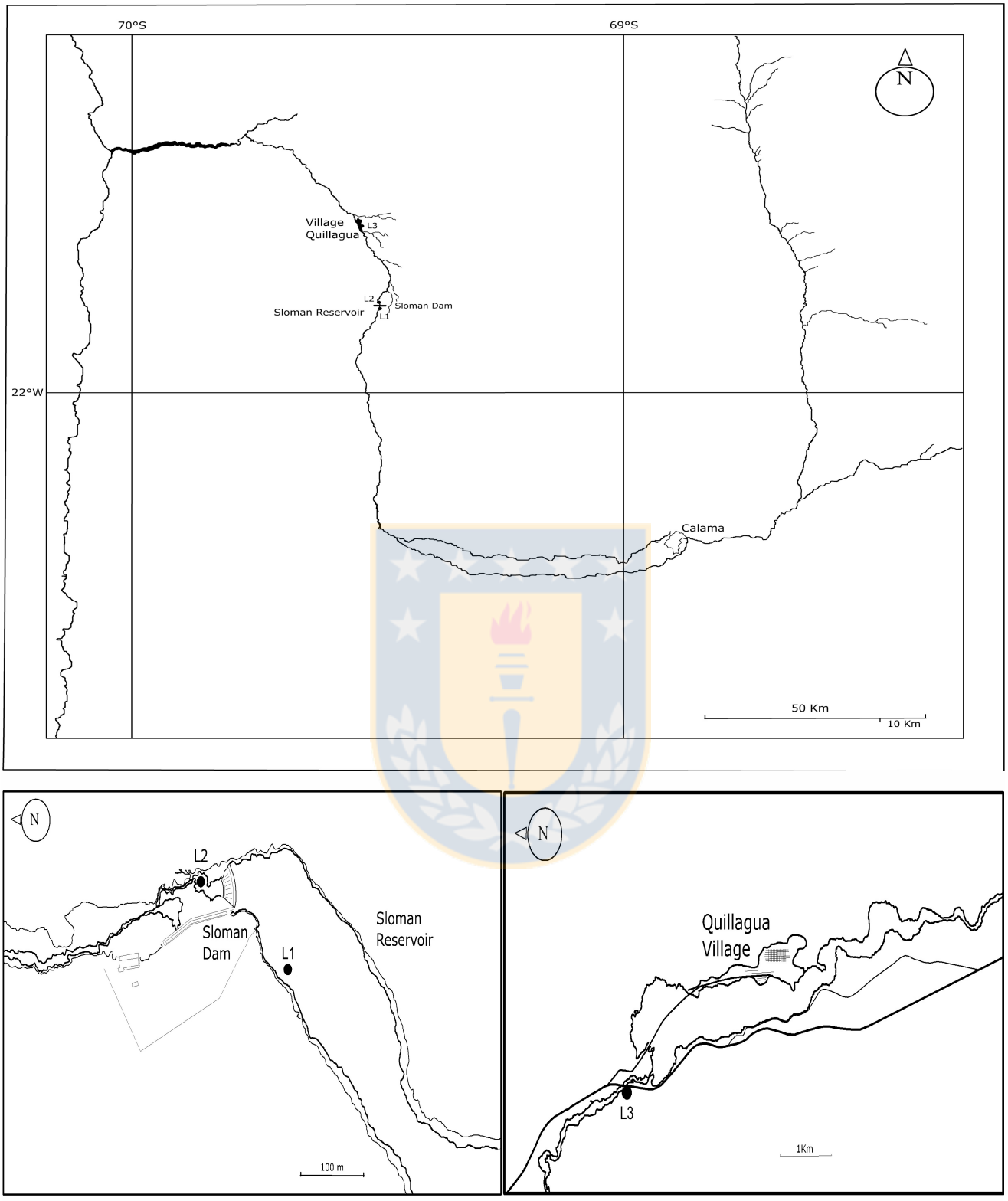


Fig. 1. The Loa river basin (Antofagasta, Chile), Sloman dam reservoir (L1 and L2) and the Quillagua study sites (L3).

Physical and chemical parameters of sediment

The pH, temperature (ºC), conductivity (mS cm^{-1}), total dissolved solids (g L^{-1}), salinity (g L^{-1}) and redox potential (mV) was measured in situ by a multi-parameter tester Hanna HI9025 (Hanna Instruments). Humidity (%), dissolved oxygen (g L^{-1}), total organic matter (%) and carbonates (%) in each sample were estimated by the loss on ignition (LOI) technique following the method described by Heiri et al.(2001). Total iron and sulfate were measured with Merck Spectroquant kits (Merck KGaA, Darmstadt, Germany) and quantified in a spectrophotometer (Spec- troquant NOVA 60; Merck KGaA).

Particle size distributions on selected samples were determined by taking approximately 0.2 g subsamples which were shaken with 5 ml hydrogen peroxide in a 10 ml tube. Samples were washed four times with sterile water, before analysis on a Malvern Mastersizer 2000E laser granulometer. For analysis the dispersed slurry was then added dropwise to a Malvern Hydro 2000MU wet sample dispersion unit (pump speed 1500 rpm) until a laser obscuration value of 10–12% was achieved. Sample data were calculated from the mean of three separate scans. Particle size data were analysed using the Gradistat v8.0 (Blott 2010).

Heavy metal concentration in water and sediment samples.

Arsenic concentration in sediment was quantified using atomic absorption spectrophotometry, after microwave digestion of samples. Briefly, 0.5 g of sieved and dried sediment was added into 9 ml concentrated nitric acid plus 3 ml concentrated hydrochloric acid at 175 °C for 10 min (US EPA 2007). After cooling down, the extracts were centrifuged at 3000 rpm for 5 min. Supernatant was analysed using an AA800 atomic absorption spectrophotometer (PerkinElmer). Total As was determined in each sample using high performance liquid chromatography (HPLC). HPLC was coupled to a system of gaseous arsine formation and the As detection was achieved by atomic absorption in a quartz bucket (HPLC/HG/QAAS) (Campos et al., 2010).

Prokaryotic cell abundance

Total prokaryotic cell counts (TC) were determined using 4′,6-diamidino-2-phenylindole (DAPI) staining (1 μg ml^{-1}, final concentration). Triplicates of sediment suspensions (fixed with formalin at a final concentration of 2%) were sonicated three times for 1 min to detach

cells from particles. Cells were collected on black polycarbonate membrane filters (0.2-µm pore size, 25-mm diameter, Nuclepore Corporation, Pleasanton, USA) and counted using epifluorescence microscopy (Motic BA310, at ×1000 magnification).

Enumeration of Bacteria

Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes was used to estimate the abundance of microorganisms referred to domains Bacteria.

The target groups, probes sequences and specific hybridization conditions used in this study was EUB338 probe. FISH technique was performed according to Leon et al. (2012).

Total bacterial community composition analysis

Genomic DNA extraction

The genomic DNA was extracted in triplicate from each sediment sample using PowerSoil DNA Isolation Kit (MO-BIO Laboratories, Inc., USA) according to the manufacturer's direction. The extracts were subsequently pooled. Quality and concentration of DNA were checked by UV/Vis spectroscopy (NanoDrop ND-1000, Peqlab, Erlangen, Germany). DNA was used as template for the high-throughput sequencing.

16S rRNA gene massive sequencing (Illumina MySeq)

The V1-V2 region of the 16S rRNA genes was amplified using the modified universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 338r (5'-GCTGCCTCCCGTAGGAGT-3'), at the "The Greehey Children's Cancer Research Institute" (Greehey CCRI). The Illumina MiSeq Platform was used to generate V1-V2 amplicon reads in a paired-end sequencing run with read length among 200 and 300 bp, at the UT Health Science Center in San Antonio (USA).

Analyses of bacterial communities

The raw data were analyzed using the bioinformatics analysis software Mothur (version 1.35.1) with the default options, unless otherwise stated. Reads shorter than 200 bp were discarded.

Reads were denoised using the “pre.cluster” command in Mothur platform to remove sequences that were likely due to errors and assemble reads which differed only by 2bp (Schloss et al., 2009). Chimeric sequences were identified and removed, and the remaining sequences classified against the SILVA database (Quast et al., 2013).

Alpha diversity measures (richness for observed species and Shannon diversity) were calculated on the OTU table obtained from all good quality sequences.
To compare the bacterial community compositions across groups of samples, Bray–Curtis similarity analyses were performed and similarity matrices were used to obtain CLUSTER graph by using PRIMER 6.1.18 (Primer-E, Ltd).

RESULTS

Physical and chemical parameters

The particle size, redox potential, humidity, dissolved oxygen, total organic matter, carbonates, pH, temperature, conductivity, total dissolved solids, salinity, Fe, SO_4 and As measured at each sampling site (L1, L2 and L3) along the Loa river are reported in Table 1 and Table 2. Samples L1 and L2 showed high values of total organic matter (>8.44%), redox potential (> -12.9 mV), total dissolved solids (> 8.6 g L^{-1}), temperature (>26.2 ºC), salinity (>7.39 g Kg^{-1}), conductivity (>12.92 mS cm^{-1}) and SO_4 (> 910 mg kg^{-1}) values is increased at site L1. The total As concentration in the sediment samples, measured by HPLC/HG/QAAS, increased as the Loa river sediments go downstream from Sloman dam. Total As was 325.95 mg kg^{-1} at the upstream from Sloman dam (reservoir zone) (L1), it was 434.30 mg kg^{-1} at downstream Sloman dam site (L2), and 864.76 mg kg^{-1} at the Quillagua Oasis (L3) (Table 1 and 2).

Table 1. Physical and chemical properties, in L1, L2 and L3 sediment samples collected from Loa river.

Site	Physical and chemical properties									
	pH	T (ºC)	Eh (mV)	Humidity (%)	Conductivity (mS cm-1)	Salinity (g L^{-1})	TDS (g L^{-1})	DO (g L^{-1})	TOM (%)	Carbonate (%)
L1	6.12	26.2	-12.9	67.1	13.02	7.47	8.7	8.2	17.5	24.51
L2	6.79	26.6	-12.1	53.38	12.92	7.39	8.6	10.7	13.98	23.69
L3	6.42	30.7	-8.2	44.73	15.92	9.7	10.8	4.3	8.44	10.41

Table 2. Elemental composition, granulometry, total cell counts (TC) and Bacterial cell counts (BC), in L1, L2 and L3 sediment samples collected from Loa river.

Site	Element Composition			Granulometry		TC	BC
	As (mg kg^{-1})	Fe (mg kg^{-1})	SO_4 (mg kg^{-1})	Media (µm)	Texture classification[a]	Log 10 cells g^{-1}	Log 10 cells g^{-1}
L1	325.95±158.01	0.03	990	13.0	Fine silt	7.42±0.09	7.33±0.08
L2	434.30±125.26	0.04	910	31.6	Medium silt	7.45±0.47	7.34±0.48
L3	864.76±320.87	0.11	900	60.2	Coarse silt	7.49±0.26	7.38±0.24

Principal component analysis (PCA) was performed to evaluate Physical and chemical parameters in the whole datasets for Loa river sediment samples, as presented in Figure 2. The cumulative percentage of variation explained by the two main axes was 98%. Therefore, further discussions will be limited to the highly meaningful components 1 and 2, which explained 82% and 16%, respectively, of total data variance. Component 1 was especially marked by values of total organic matter, particle size, redox potential, Fe, total dissolved solids, temperature, salinity, conductivity and arsenic concentration (more positive score) and dissolved oxygen and carbonate (more negative score). On the other hand, component 2 was mainly determined by two other groups of variables, including pH (positively) humidity and

SO_4 (negatively) (Figure 2). The PCA further revealed that L2 and L3 sites were similar to each other, but significantly distinguished from that of site L1.

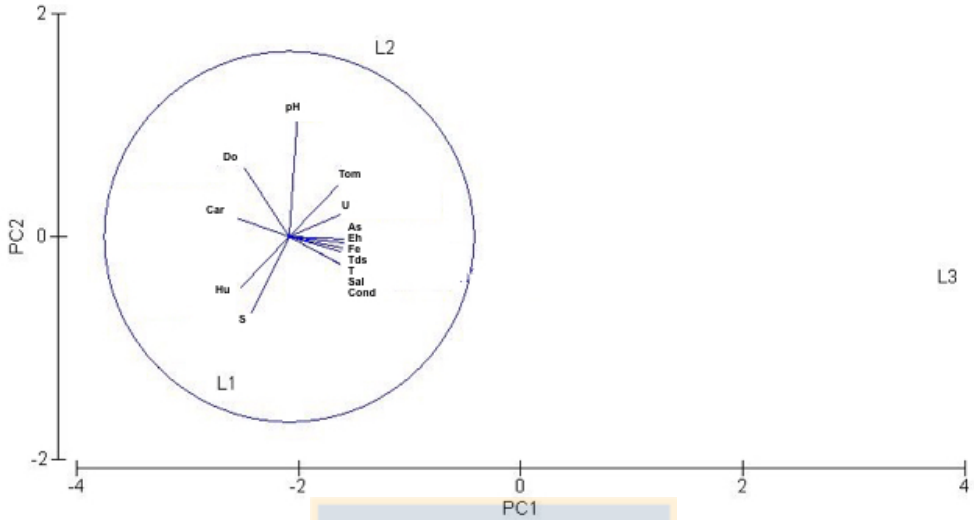


Figure 2. Principal Component Analysis (PCA) of physico-chemical characteristics in L1, L2 and L3 sediment samples collected from Loa river (The pH=pH; temperature=T; conductivity=Cond; total dissolved solids=Tds; salinity=Sal; redox potential=Eh; Humidity=Hu; dissolved oxygen=Do; total organic matter=Tom; carbonates=Car; Iron=Fe; Sulfate=S; arsenic=As; and particle size =U.

Prokaryotic cell abundance

The abundance of total prokaryotic cells (TC), measured after DAPI staining, was similar at the three sites, ranging from 7.42 Log10 cells g^{-1} (L1) to 7.42 Log10 cells g^{-1} (L3) (Table 2). Whereas, the abundances of cells hybridized with probes for Bacteria are reported in Table 2. The number of Bacteria in the sediment samples not show significantly different ($p > 0.05$), ranging from 7.33 Log 10 cells g^{-1} (L1) to 7.38 Log10 cells g^{-1} (L3).

Total bacterial community composition analysis

Sequencing data and diversity estimates

The Illumina-based analysis of the universal V1-V2 region of the 16S rRNA genes for Bacteria produced a total of 3,976,130 sequences across all samples. After quality check within the

RDP pyrosequencing pipeline and removing chimeras, 3,951,730 (99.4%) high quality sequences remained.

L3 showed the highest number of quality reads (1,536,478), and the highest number of OTUs (47,430) (Table 3). L2 and L3 showed higher Shannon diversity indexes than L1. Non-parametric Chao1 and ACE estimators predicted that the highest richness was in L1, whereas the lowest was in L2.

Table 3. Sequencing information, diversity index (H'), estimator of richness (Chao1 and ACE) obtained by Ilumina sequencing from sediment samples (L1, L2 and L3) collected from Loa river. OTUs: operation taxonomic units.

	L1	**L2**	**L3**
Number of reads	1,326,494	1,103,768	1,545,868
Number of high quality reads	1,318,962	1,096,290	1,536,478
Unique reads	173,285	170,638	217,548
% Unique reads	13.14	15.57	14.16
Shannon (*H´*)	6.57	7.34	7.34
OTUs at 97% genetic similarity	32,459	34,662	47,430
Chao 1	200,165	142,164	180,804
ACE	182,272	134,376	170,538

Bacterial diversity

Retrieved OTUs were classified in a total of 34 different bacterial phylogenetic groups, of which all were common to all samples (Table S1 in the supplemental material). Overall, sequences of the dominant taxonomic groups (abundances ≥1%) across all sediment samples were affiliated with *Proteobacteria* (range 39.9-42.9% of total bacterial sequences), *Firmicutes* (13.6-17.1%), *Bacteroidetes* (9.3-14.7%), *Chloroflexi* (2.8-9.9%), *Actinobacteria* (3.3-5.7%), *Lentisphaerae* (2.9-3.4%), *Planctomycetes* (2.3-3.1%), *Spirochaetes* (2.2-2.4%), *Verrucomicrobia* (1.3-2.1%), and *Chlorobi* (1.4-1.9%). However, the relative abundance at

phylum level varied considerably across samples and changes in abundance of phylotypes determined different bacterial assemblages (Fig. 3).

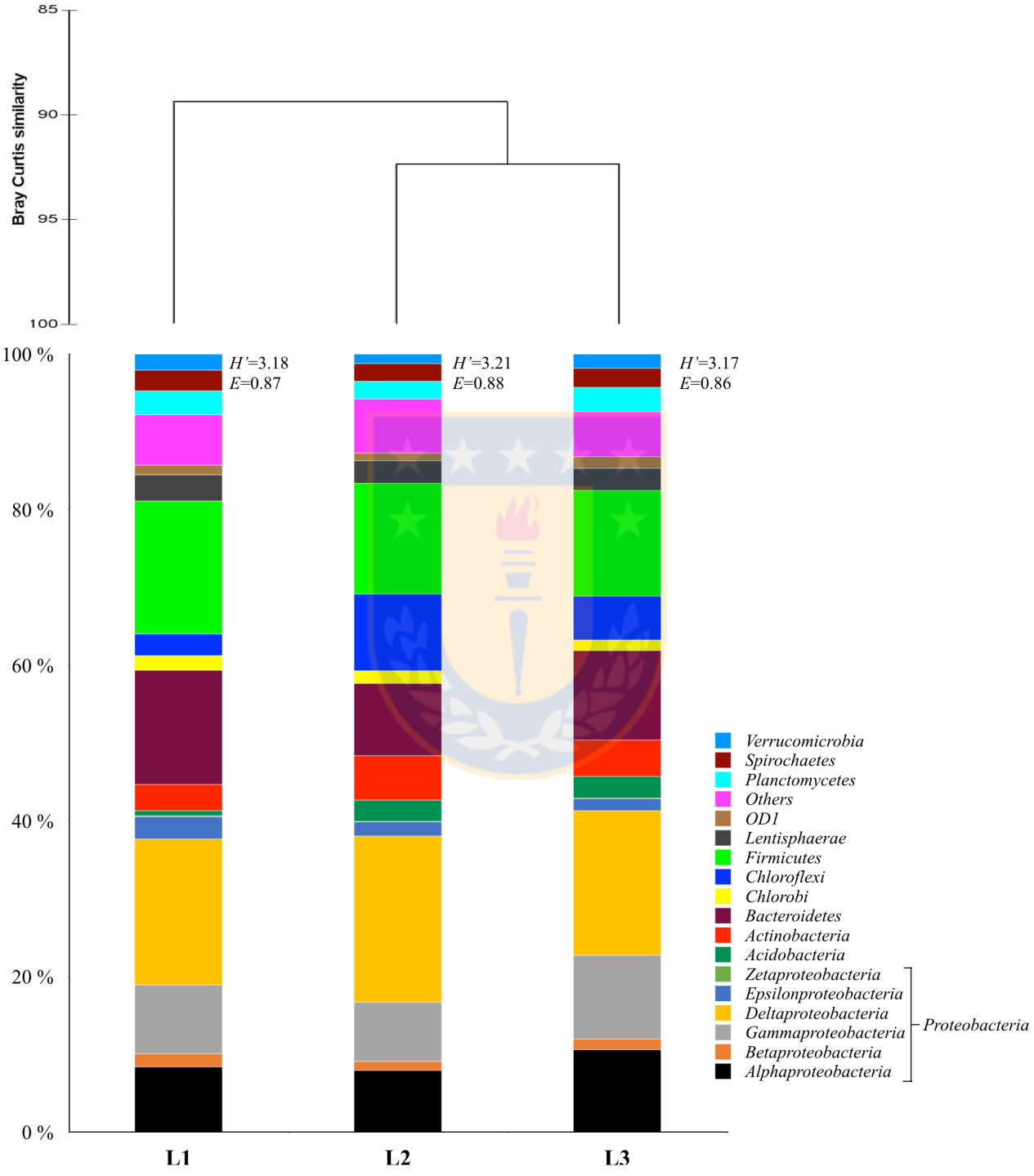


Figure 3. Cluster diagram and relative abundance of sequences (percentage) assigned to bacterial phylogenetic groups and proteobacterial subclasses from sediment samples (L1, L2 and L3) collected from Camarones river. *H':* Shannon diversity index, *E:* Evenness index.

Different bacterial communities composition was observed in Loa river sediments since sequences affiliated with *Proteobacteria*, *Firmicutes* and *Bacteriodetes* covered almost all classified bacterial sequences in L1. Bacterial communities composition in L2 and L3 no presented significant difference (ANOVA $p>0.05$) where *Proteobacteria*, *Firmicutes*, *Bacteriodetes* and *Chloroflexi* covered almost all classified bacterial sequences. *Acidobacteria* were predominant in L2 and L3 representing significant minor components in L1 ($p<0.05$ and $p<0.05$, respectively), and *Chloroflexi* was significantly higher in L2 than in L1 ($p<0.001$). Relatively high abundant phyla in L1 and L3 included also OD1, but it constituted a minor component in L2.

Although *Deltaproteobacteria* (range 18.6-21.4%) represented the most abundant proteobacterial class, differences in relative abundance were also observed for sequences affiliated to the other classes: *Alphaproteobacteria*- and *Gammaproteobacteria* prevailed in all samples. Sequences referred to *Betaproteobacteria*, *epsilonproteobacteria* and *Zetaproteobacteria* represented a minor component across all samples. However, *Betaproteobacteria* was significantly higher than *Zetaproteobacteria* ($p<0.05$) in L1.

Among phyla whose abundance was ≤1%, sequences affiliated to *Cyanobacteria*/Chloroplast, *Tenericutes*, *Caldiserica*, *Fusobacteria*, *Gemmatimonadetes*, *Synergistetes*, WS3, *Thermodesulfobacteria*, *Aquificae*, *Thermotogae*, TM7, *Chlamydiae*, SR1, *Deinococcus-Thermus*, *Chrysiogenetes*, BRC1, *Deferribacteres*, *Armatimonadetes*, *Elusimicrobia*, *Fibrobacteres*, *Nitrospirae*, and OP11 occurred across all sediments, even if at different relative abundances (Table S1).

Cluster diagram, representing similarities in the bacterial community composition (phyla and proteobacterial classes) of the studied sediments, since of the great differences among their respective bacterial communities grouped L2 and L3 together, whereas L1 did not clustered with them (Fig. 3).

From all samples, 1354 genera were retrieved. The highest number of genera was observed in L3 (1126), when compared to L2 (1187) and L1 (1010). The dominant bacterial genera (15), present in ≥ 1% of the total bacterial sequences at least in one of the three samples, are

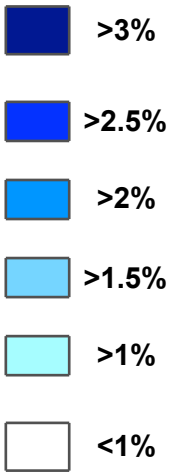
shown in Table 4. Diversity at genus level was higher in L2. A total number of 8 dominant genera were retrieved for L1, 13 for L2, and 10 for L3. A minor part of dominant bacterial genera (5/15) was ubiquitous in all samples: *Meniscus* (within *Bacteroidetes*), *Ignavibacterium* (*Chlorobi*), *Victivallis* (*Lentisphaerae*), *Phycisphaera* (*Planctomycetes*) and *Spirochaeta* (*Spirochaetes*). However, different abundant genera were unique for each site.

The most abundant genus in L1 and L3 was *Victivallis*. Four dominant genera were unique in L2: *Desulfobacca* and *Desulfonema* (*Deltaproteobacteria*), *Thermodesulfobium* (*Firmicutes*) and *Dehalogenimonas* (*Chloroflexi*), which also was the most abundant genus in L2.

Genus *Desulfatiferula* (*Deltaproteobacteria*). was the only genus common to L1 and L2. The dominant genera common to L1 and L3 were related to *Haliscomenobacter* (*Bacteroidetes*) and OD1 genera *incertae sedis* (OD1). Finally, 3 dominant genera affiliated with *Deltaproteobacteria* (*Sorangium*) and *Chloroflexi* (*Bellilinea* and *Levilinea*) were retrieved in L2 and L3.

Table 4. Bacterial genera retrieved in sediment samples (L1, L2 and L3) from Loa river.

Phylum	Class	Genus	L1	L2	L3
Proteobacteria	*Delta-*	*Desulfatiferula*			
		Desulfobacca			
		Desulfonema			
		Sorangium			
Bacteroidetes		*Haliscomenobacter*			
		Meniscus			
Chlorobi		*Ignavibacterium*			
Chloroflexi		*Bellilinea*			
		Dehalogenimonas			
		Levilinea			
Firmicutes		*Thermodesulfobium*			
Lentisphaerae		*Victivallis*			
OD1		OD1 genera *incertae sedis*			
Planctomycetes		*Phycisphaera*			
Spirochaetes		*Spirochaeta*			



DISCUSSION

The Loa river system reflecting the climatic characteristics of the desert region where rain is extremely limited, and is exacerbated by high evaporation rates, which drive the chemical and physical characteristics of these water systems. Sulfate values are relatively high, a common feature in Altiplano environments. Additionally, these systems are particularly sensitive to hydrological changes, which may affect nutrient availability and diminish dissolved oxygen content due to warming during the day, and increased salinity in both water and sediments (Romero et al., 2003; Bugueño et al., 2014).

In this study we investigated the Bacterial communities shift along a gradient of As-concentration in sediments, from both upstream (L1) and downstream (L2 and L3) of Sloman dam, in Loa river. Sediments samples were characterized by pH values near neutral to slightly alkaline. Conductivity, sulfate, Fe and the total dissolved solids showed wide ranges of values along Loa river sites and were consistent with the location of the anthropogenic sources (wastewater and mining activities, principally). On the other hand, the dissolved oxygen at the dam reservoir (L1) and immediately downstream of the dam (L2), were significantly higher than at 23 km downstream from Sloman dam, close to Quillagua Oasis (L3). Reductions in water volume in Sloman dam, following by the water extraction and evaporation results in increases in salinity and nutrient concentrations, due to the concentration of ions and for these reasons, the highest values of temperature, salinity, conductivity and total dissolved solids in L3 sediments, were expected. Additionally, it is well know that solubility of oxygen decreases as temperature increases, and decreases exponentially as salt levels increase, which affect water quality and aquatic life.

Sediments collected from Loa river (L1, L2 and L3) showed high total arsenic concentration which increased (from 326 to 865 mg kg-1) downstream from the Sloman dam reservoir, atribuited to several factors: I) the extreme desert climate wich favors high evaporation rates and high salinity, and the lack of surface tributaries and fresh groundwater contributions impedes dilution. II) the neutral to alkaline pH of water hinders the sorption of As onto solid phases (Romero et al., 2003; Bugueño et al., 2014). III) extremely elevated As concentrations

in reservoir waters have been reported during flood events, with great impact on the fluvial and marine ecosystems downstream (e.g., Quillagua Oasis) (Arroyo et al., 1999). IV) the emissions from the copper smelting plant at Chuquicamata; the mine wastes deposited in the past in the bed and on the banks of the river; the inadequate treatment of the effluents from mining activities, as well the As-rich effluents from the water treatment plant of Calama, represent others potential sources of As (Romero et al., 2003).

High values of total organic matter were measured in L2 and L3 attributed to the presence of primary producers such, algae species. The positive association of arsenic content and total organic matters has been reported as an important factor in the geochemical cycle of arsenic. Organic matter (OM) may enhance the release of As from soils and sediments into the soil solution, thereby facilitating As-leaching into the groundwater. The main influencing mechanisms include competition for available adsorption sites, formation of aqueous complexes, and/or changes in the redox potential of site surfaces and As-redox speciation. OM may also serve as binding agents, thereby reducing As mobility (Wang and Mulligan, 2006; Bauer and Blodau, 2006; Moreno-Jiménez et al., 2013). On the other hand, the presence of green algae species (e.g., *Cladophora* sp. and *Chara* sp.) and aquatic plants (*Azolla* sp., *Myriophyllum aquaticum*, *Phylloscirpus* cf. *desserticola*, *Potamogeton pectinatus*, *Ruppia filifolia* and *Zannichellia palustris*) in L3 site could concentrated the arsenic, since these species were classified as accumulator or hyperaccumulator of arsenic by Pell et al., (2013).

The number of Bacteria in the sediment samples ranged from 7.33 Log10 cells g^{-1} to 7.38 Log10 cells g^{-1}. These densities are lower than those calculated for most fresh water environments but consistent with saline environments with comparable physic and chemical properties (e.g, sulfate-reducing saline or hypersaline soda lakes) (Foti et al., 2007) .

The massive parallel sequencing (Illumina) analyses revealed a significant shift in bacterial communities of As-contaminated sediments from Loa river. In addition to *Proteobacteria*, which was dominated in sediment of all the three sites (40.6, 39.9 and 42.9% in L1, L2 and L3, respectively), the other phyla such as *Firmicutes* (13.6-17.1%), *Bacteroidetes* (9.3-14.7%), *Chloroflexi* (2.8-9.9%), *Actinobacteria* (3.3-5.7%), *Lentisphaerae* (2.9-3.4%),

Planctomycetes (2.3-3.1%), *Spirochaetes* (2.2-2.4%), *Verrucomicrobia* (1.3-2.1%), and *Chlorobi* (1.4-1.9%) were also detected in relatively high numbers, suggesting that the members of these phyla might be more active in the Loa river sediments. The relative abundance of these phyla was noticeably affected by redox potential, temperature, conductivity, total dissolved solids, salinity, Fe and SO_4 content (Figure 2), since of the significative differences among their respective bacterial communities grouped L2 and L3 together, whereas L1 did not clustered with them.Which agrees with the lack of water resources or mineral outcrops that could cause significative changes in the study section and the proximity of L2 and L3 sites, located downstream of the Sloman dam.

The highest abundance of *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* phylum may be due to the copiotrophic nature of this group, which thrive in conditions of elevated C availability and exhibit relatively rapid growth rates (Shange et al. 2012, Fierer *et al.*, 2007; Eilers *et al.*, 2010, Fierer et al., 2012). Within the proteobacterial class, *Deltaproteobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* dominated in all sediments (Figure 3). Similar result of bacterial community composition associated to several As-rich samples from the main Andean salts deposits (Salar de Ascotán), Geothermal geyser field (El Tatio) and from incoming rivers ,were reported by Escudero et al. (2013), concluded that *Gamma* and *Epsilonproteobacteria* were dominant in water samples. Whereas, *Alpha*, *Gamma* and *Deltaproteobacteria* were dominant in sediment samples, recently, Farías et al. (2014) reported discrete bacterial communities of an As-rich hypersaline lake in the Salar de Atacama (Laguna Tebenquiche, Chile), were the most abundant bacterial 16S rRNA amplicons resembled with *Bacteroidetes* and *Proteobacteria* phyla *(Alphaproteobacteria and Deltaproteobacteria*, principally). Differently from ours results, previous studies on prokaryotic diversity of microbial communities associated with As-contaminated sediments in saline environments (Northern, Chile), describe *Gammaprotebacteria,* as dominant subclass, followed by *Alphaproteobacteria* subclass (Lara et al. 2012).

In this study, *Deltaproteobacteria* comprised genera: *Desulfatiferula*, *Desulfobacca*, *Desulfonema*, and *Sorangium*, that are well known for resistant to a variety of environmental stresses and for their capability to reduce S, Fe and As in diverse environments, were dominant in the medium site (L2) of Loa river, attributed to the unique physicochemical

characteristics (e.g, higher pH) of these sediments (Lara et al. 2012; Fan et al. 2012; Fashola et al., 2016, Li et al. 2014; Widdel et al. 1983; Burton et al. 2011; Nunes et al. 2011).

The *Alphaproteobacteria*-related dominant sequences (See Table S1) of Loa river sediment samples, clustered in the families *Rhodobacteraceae* (dominant in L1 and L3) and *Rhodospirillales* (dominant in L2) principally (Table S2), that comprise organisms with diverse physiological properties. In this study, *Rhodobacteraceae* are fundamentally, aquatic bacteria that frequently thrive in marine environments and activated sludge from bioreactors in wastewater treatment plants (Daneshvar et al. 2003; Funke et al. 2004; Schweiger et al. 2011). They comprise mainly aerobic photo- and chemoheterotrophs (e.g., *Paracoccus*) but also purple non-sulfur bacteria (e.g., *Rubribacterium*) which perform photosynthesis in anaerobic environments (Kelly et al. 2006; Boldareva et al. 2009) (See Table S3). They are deeply involved in nitrogen, sulfur and carbon biogeochemical cycling and symbiosis with aquatic micro- and macroorganisms. Furthermore, *Paracoccus* species demonstrated its ability to oxidize As(III) under both aerobic and anaerobic conditions, and some strain are interesting for xenobiotic biodegradation (e.g., chlorpyrifos and 3,5,6- trichloro-2-pyridinol), and these properties raise the possibility of using *Paracoccus* species for bioremediation (Li et al. 2011, Xu et al. 2008). Otherwise, some genera in the family *Rhodospirillaceae* grow photoheterotrophically under anoxic conditions in the light and chemoheterotrophically in the dark, while others grow heterotrophically under aerobic/microaerobic conditions and some genera are widely distributed in arsenic contaminated soils (e.g., *Defluviicoccus* and *Pelagibius*, dominant genera in Loa river) (Héry et al., 2015; Gu et al., 2017).

On the other hand, *Orientia* bacterial *genus* in family Rickettsiaceae is widely distributed along all sample sites (Table S3). The only two species know belonging to the *Orientia* bacterial *genus*, *O. tsutsugamushi* and *O. chuto*, are the causative agent of scrub typhus in humans, a febrile illness endemic to the Asia-Pacific and Dubai region, respectively. The bacterium is an obligate intracytosolic organism that is transmitted to humans by the bite of larval trombiculid mites, and is hosted by rodents (Rodkvamtook et al., 2011). Scrub typhus has been reemerging and occurring in new areas of many countries including Japan, Korea, Sri Lanka, China, Maldives, India, Palau, Malaysia, Taiwan, and Australia (Huber et al. 2012). Furthermore, an endemic scrub typhus-like illness has been documented in Chile recently

(Balcells et al. 2011).These observations show an immediate need for an accurate method of detection of waterborne bacterial (e.g, *Orientia* spp.) that are a concern for the human health.

High abundances of *Gammaproteobacteria* subclass, presented Bacterial genera members of *Alteromonadaceae* and *Ectothiorhodospirae* families (eg., *Haliea* and *Ectothiorhodosinus*, respectively) as dominant in L1, and L2 and L3, respectively (Table S4 and S5). Both families collects a diverse set of species, mostly marine in origin and requiring sodium to grow. They have large cells that grow quite fast with minimal nutritional requirements, and although all are obligate aerobic chemoorganotrophs, they display a diverse set of potential substrates and extensive degradative properties. They have large genomes that contain several degradative genes and sometimes secondary metabolites. Ecologically they are often associated to nutrient-rich environments such as particulate material, marine snow, or marine animals (Lin et al., 2015).

The predominance of *Firmicutes* as the second major components in Loa river was expected because these phylogenetic groups have been described as common inhabitants of metal-contaminated sediment (Luo et al., 2014; Costa et al., 2015). The sequences of *Firmicutes* in L1, L2 and L3, were distributed over three families members of Order Clostridiales: *Ruminococcaceae* (15.9 %), *Thermodesulfobiaceae* (9.6 %) and *Lachnospiraceae* (13.8 %), respectively (Table S6). The genera: *Anaerospora*, *Thermodesulfobium*, *Thermohalobacter, Mahella*, *Acetivibrio*, presented along Loa sediment samples (Table S7) are sulfur-reducing, obligately anaerobic halophilic, biogas-producing, anaerobic cellulose-degrading and arsenic tolerant bacteria. However, *Thermodesulfobium* was the dominant genus in L2 (Giloteaux et al. 2013; Stolze et al., 2015; Manyi-Loh et al. 2013, Mori et al., 2003 and Sánchez-Andrea et al., 2011).

Bacteroidetes (e.g., *Haliscomenobacter* and *Meniscus*) together *Planctomycetes* (e.g., *Phycisphaera*) with diverse organoheterotrophic capabilities are well-known biofilm forming bacteria which in this form are able to colonize of a wide variety of ecosystems, ranging from aquatic to terrestrial habitats including several extreme and As-rich environments (Zhu et al. 2015; Costa et al., 2015; Lage et al., 2014 and Fernandez et al., 2016).

Actinobacterial group in relative high abundance across all sediment samples (L1, L2 and L3), are widely distributed in both terrestrial and aquatic ecosystems, where they play a crucial role in the recycling of refractory biomaterials by decomposition (152, 403). Additionally, the *Actinobacteria* phylum was reported to have a high resistance to environmental stress due to its numerous members being Gram-positive with a high G+C content (Barnard et al., 2013), and an important role in the As-methylation process were described (Zhai et al., 2017).

Members of the phylum *Lentisphaerae* are widely distributed in nature, such as ocean, faecal samples, anaerobic sludge, sulfide- and sulfur-rich springs, marine sediments and landfill leachate (Limam et al., 2010) and plays an important role in the recycling of organic carbon particles and the movement of organic matter from the euphotic zone to the deep ocean (Cho et al., 2004). The phylum *Spirochaetes* represented by the dominant genus *Spirochaeta* is composed by a large number of free living non-pathogenic, helical shaped bacteria commonly found in the anoxic mud, marshes, marine sediments, soda lakes and microbial mats, and genome sequence analysis of strain JC230(T) predicted the presence of genes related to heavy metal resistance of cobalt, zinc, cadmium and arsenic. (Shivani et al., 2015). Otherwise, members of the phylum *Verrucomicrobia* show in general a positive correlation between the numbers of genes annotated as sulfatases in genomes and the salinity of the preferred habitat and thus tend to be higher in environments characterized by large amounts of free sulfate (Spring et al., 2016).

Green sulfur bacteria of Loa river sediments members of the phylum *Chlorobi*, are strictly anaerobic obligately photoautotrophic Bacteria, which obtain electrons for anaerobic photosynthesis from sulfide and other reduced sulfur compounds. The genera *Ignavibacterium*, which is highly presented in all sediment samples (L1, L2 and L3) was described as an dominant genera of the Bacterial community of an acid mine drainage in France, with similar physicochemical properties of Loa river sediments, as high concentrations of arsenic, iron, and sulfate (Volant et al., 2014).

Phylum *Chloroflexi* presented in Loa river sediment samples, included *Dehalogenimonas*, *Bellilinea* and *Levilinea* genera, that are described as an anaerobic sulfate reducing bacteria (SRB), presented as a dominant genera in the rhizosphere sediments of macrophyte (Yan et

al., 2015), and considered that *Chloroflexi* was significantly higher in L2 than in L1 ($p<0.001$), we attributed this difference to the abundance of macrophyte. Whereas, Quillagua is a natural wetland with abundant aquatic plants (e.g, *Scirpus americanus*), the Sloman dam is a reservoir lacking almost completely of macrophytes.

Acidobacteria were predominant in L2 and L3 representing significant minor components in L1 ($p<0.05$ and $p<0.05$, respectively), this can be attributed to the the strong negative correlation between the abundance of *Acidobacteria* and concentration of organic carbon in the environment (Kielak et al., 2016). These has led to the conclusion that members of this phylum presented in L1 (with the lesser percentage of TOC) may be oligotrophic bacteria and they are predicted to have lower growth rates, but high efficiency in converting nutrients to biomass as well as high tolerance to toxic compounds (e.g., arsenic compounds), among other characteristics.

Microorganisms mediate many important processes in the aquatic environment, including self-purification, nutrient recycling and development of heavy metal tolerance. The microbial community might even allow these important functions to be maintained despite the input of heavy metals into the environment and therefore they may be potentially utilized for bioremediation purposes.

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SUPPORTING INFORMATION

Table S1. Relative abundances of bacterial phylogenetic group from sediment samples (L1. L2 and L3) collected from Camarones river. Dominant phylogenetic groups (≥1% of total classified sequences) common to sediments (L1. L2 and L3) are represented in bold.

L1		L2		L3	
Phylogenic group	**Relative abundance (%)**	**Phylogenic group**	**Relative abundance (%)**	**Phylogenic group**	**Relative abundance (%)**
Proteobacteria	40.31	***Proteobacteria***	43.81	***Proteobacteria***	47.17
Alpha-	12.41	*Alpha-*	11.57	*Alpha-*	13.18
Beta-	3.60	*Beta-*	3.44	*Beta-*	1.41
Gamma-	6.65	*Gamma-*	8.56	*Gamma-*	13.91
Delta-	17.58	*Delta-*	19.95	*Delta-*	18.48
Epsilon-	0.06	*Epsilon-*	0.26	*Epsilon-*	0.15
Zeta-	≤0.01	*Zeta-*	0.03	*Zeta-*	0.03
Firmicutes	24.82	***Acidobacteria***	17.12	***Firmicutes***	11.34
Acidobacteria	12.02	***Firmicutes***	8.42	***Acidobacteria***	10.37
Chloroflexi	6.88	***Chloroflexi***	7.50	***Actinobacteria***	8.09
Actinobacteria	5.37	***Actinobacteria***	6.24	***Planctomycetes***	5.29
Thermodesulfobacteria	1.64	***Planctomycetes***	3.93	***Chloroflexi***	3.94
Nitrospirae	1.25	*Thermodesulfobacteria*	1.89	*Bacteroidetes*	2.19
Gemmatimonadetes	1.16	***Gemmatimonadetes***	1.34	*Lentisphaerae*	1.98
Planctomycetes	1.16	*Lentisphaerae*	1.34	***Gemmatimonadetes***	1.46
		Verrucomicrobia	1.32	*Chlorobi*	1.29
		Nitrospirae	1.23	***Nitrospirae***	1.14
				Aquificae	1.06
				Deferribacteres	1.02
Bacteroidetes	0.90	*Chlorobi*	0.84	*Verrucomicrobia*	0.73
BRC1	0.84	*Bacteroidetes*	0.8	*Synergistetes*	0.62
Aquificae	0.77	*Aquificae*	0.75	*Spirochaetes*	0.38
Verrucomicrobia	0.64	*Armatimonadetes*	0.75	*Cyanobacteria/Chloroplast*	0.35
Armatimonadetes	0.58	BRC1	0.55	WS3	0.24
Chlorobi	0.42	*Caldiserica*	0.45	*Thermodesulfobacteria*	0.18
Cyanobacteria/Chloroplast	0.26	*Cyanobacteria/Chloroplast*	0.35	*Chlamydiae*	0.17
Fusobacteria	0.23	*Spirochaetes*	0.35	*Elusimicrobia*	0.15
Deinococcus-Thermus	0.19	*Synergistetes*	0.26	*Fusobacteria*	0.15
Caldiserica	0.16	TM7	0.15	BRC1	0.14
Lentisphaerae	0.10	*Deinococcus-Thermus*	0.13	*Deinococcus-Thermus*	0.12
Spirochaetes	0.10	WS3	0.13	*Caldiserica*	0.09
Tenericutes	0.06	*Chlamydiae*	0.09	OD1	0.08
Chlamydiae	0.03	*Deferribacteres*	0.09	*Thermotogae*	0.08
OD1	0.03	*Elusimicrobia*	0.07	TM7	0.06
Synergistetes	0.03	*Fusobacteria*	0.04	*Armatimonadetes*	0.05
TM7	0.03	OD1	0.03	*Tenericutes*	0.05
WS3	0.03	*Thermotogae*	0.03	SR1	0.02

Table S2. Dominant bacterial family in *Alphaproteobacteria* subclasses from sediment samples (L1. L2 and L3) collected from Loa river. Dominant phylogenetic genera (≥1% of total classified sequences) common to sediments (L1. L2 and L3) are represented in bold.

L1		L2		L3	
Family	**Relative abundance (%)**	**Family**	**Relative abundance (%)**	**Family**	**Relative abundance (%)**
Rhodobacteraceae	32.39	***Rhodospirillaceae***	24.16	***Rhodobacteraceae***	24.68
Rhodospirillaceae	14.72	***Rhodobacteraceae***	19.96	***Rhodospirillaceae***	21.41
Hyphomicrobiaceae	7.59	***Acetobacteraceae***	8.40	***Hyphomicrobiaceae***	7.95
Sphingomonadales	7.02	***Rhodobiaceae***	7.61	***Sphingomonadales***	5.96
Sphingomonadaceae	5.55	***Hyphomicrobiaceae***	7.43	***Rhodobiaceae***	5.26
Hyphomonadaceae	5.10	***Sphingomonadales***	4.20	***Hyphomonadaceae***	4.55
Acetobacteraceae	3.62	***Sphingomonadaceae***	3.10	***Sphingomonadaceae***	4.49
Beijerinckiaceae	3.51	***Beijerinckiaceae***	3.10	***Acetobacteraceae***	4.10
Rhodobiaceae	2.15	***Rhizobiales_incertae_sedis***	3.10	***Rhizobiales_incertae_sedis***	3.46
Rickettsiaceae	1.93	*Hyphomonadaceae*	2.92	***Beijerinckiaceae***	2.12
Bradyrhizobiaceae	1.81	*Methylocystaceae*	2.68	***Phyllobacteriaceae***	1.79
Kiloniellaceae	1.70	***Bradyrhizobiaceae***	2.25	***Bradyrhizobiaceae***	1.73
Rhizobiales_incertae_sedis	1.59	***Phyllobacteriaceae***	2.25	***Erythrobacteraceae***	1.41
Erythrobacteraceae	1.47	***Erythrobacteraceae***	1.10	*Rickettsiaceae*	1.35
Anaplasmataceae	1.25			*Kiloniellaceae*	1.09
Methylocystaceae	1.13				
Phyllobacteriaceae	1.02				
Brucellaceae	1.02				

Table S3. Dominant bacterial genera in *Alphaproteobacteria* subclasses from sediment samples (L1. L2 and L3) collected from Loa river. Dominant phylogenetic genera (≥1% of total classified sequences) common to sediments (L1. L2 and L3) are represented in bold.

L1		L2		L3	
Genus	**Relative abundance (%)**	**Genus**	**Relative abundance (%)**	**Genus**	**Relative abundance (%)**
Paracoccus	3.53	***Pelagibius***	7.18	***Pelagibius***	5.73
Camelimonas	2.31	***Defluviicoccus***	3.56	***Defluviicoccus***	3.48
Blastomonas	2.19	***Tistlia***	3.18	***Tistlia***	3.34
Orientia	2.07	*Tepidamorphus*	3.11	***Bauldia***	3.27
Tistrella	2.07	***Bauldia***	2.92	***Tistrella***	2.25
Tistlia	1.95	*Dongia*	2.54	***Telmatospirillum***	1.98
Seohaeicola	1.95	*Prosthecomicrobium*	2.10	***Paracoccus***	1.98
Defluviicoccus	1.83	***Tistrella***	1.84	*Tepidamorphus*	1.91
Telmatospirillum	1.83	*Afifella*	1.78	*Filomicrobium*	1.64
Kiloniella	1.83	*Rhodovibrio*	1.72	*Prosthecomicrobium*	1.57
Rhodobacter	1.71	***Rubribacterium***	1.65	*Blastomonas*	1.57
Zhangella	1.71	*Parvibaculum*	1.52	***Rubribacterium***	1.50
Caenispirillum	1.58	***Telmatospirillum***	1.46	***Orientia***	1.43
Tropicimonas	1.58	***Paracoccus***	1.33	*Pannonibacter*	1.43
Rubribacterium	1.58	*Methylocystis*	1.27	*Zhangella*	1.43
Gemmobacter	1.58	*Tanticharoenia*	1.21	*Parvibaculum*	1.36
Pelagibius	1.46	*Filomicrobium*	1.14	*Dongia*	1.30
Anderseniella	1.46	*Agaricicola*	1.08	*Agaricicola*	1.30
Rubrimonas	1.34	***Orientia***	1.02	***Gemmobacter***	1.16
Pseudorhodobacter	1.34	***Gemmobacter***	1.02	*Kiloniella*	1.16
Sandaracinobacter	1.34	*Agromonas*	1.02	*Afifella*	1.02
Anaplasma	1.22	*Rhodovarius*	1.02	*Woodsholea*	1.02
Pannonibacter	1.22				
Oceanicola	1.22				
Ahrensia	1.22				
Agaricicola	1.22				
Bauldia	1.22				
Hellea	1.10				

Table S4. Dominant bacterial family in *Gammaproteobacteria* subclasses from sediment samples (L1. L2 and L3) collected from Loa river. Dominant phylogenetic genera (≥1% of total classified sequences) common to sediments (L1. L2 and L3) are represented in bold.

L1		L2		L3	
Family	**Relative abundance (%)**	**Family**	**Relative abundance (%)**	**Family**	**Relative abundance (%)**
Alteromonadaceae	13.12	***Ectothiorhodospiraceae***	18.03	***Ectothiorhodospiraceae***	15.21
Ectothiorhodospiraceae	8.83	***Chromatiaceae***	15.97	***Chromatiaceae***	13.59
Chromatiaceae	8.83	***Methylococcaceae***	9.31	***Alteromonadaceae***	10.09
Oceanospirillaceae	7.43	***Alteromonadaceae***	6.65	***Methylococcaceae***	6.80
Piscirickettsiaceae	5.34	***Oceanospirillaceae***	5.26	***Oceanospirillaceae***	6.12
Methylococcaceae	5.11	***Coxiellaceae***	4.52	***Hahellaceae***	5.59
Hahellaceae	4.41	***Piscirickettsiaceae***	3.93	***Coxiellaceae***	4.17
Thiohalobacter	3.95	***Thiohalobacter***	3.26	***Piscirickettsiaceae***	3.50
Thiotrichaceae	3.60	***Hahellaceae***	2.73	***Thiotrichaceae***	3.50
Coxiellaceae	3.25	***Thiotrichaceae***	2.53	***Thiohalobacter***	3.03
Xanthomonadaceae	3.02	***Xanthomonadaceae***	2.40	***Sedimenticola***	2.89
Halomonadaceae	2.67	***Halomonadaceae***	2.40	***Methylohalomonas***	2.62
Marinicella	2.56	***Methylohalomonas***	2.40	***Halomonadaceae***	1.55
Pseudomonadales_incertae_sedis	2.56	***Sedimenticola***	1.93	***Xanthomonadaceae***	1.48
Thiohalomonas	1.74	***Thiohalomonas***	1.60	*Thioprofundum*	1.41
Arenicella	1.51	*Sinobacteraceae*	1.60	*Pseudomonadales_incertae_sedis*	1.41
Pseudomonadaceae	1.51	*Arenicella*	1.46	*Pseudomonadaceae*	1.35
Moraxellaceae	1.39	*Umboniibacter*	1.26	***Thiohalomonas***	1.21
Methylohalomonas	1.16			*Marinicella*	1.01
Halothiobacillaceae	1.16			*Moraxellaceae*	1.01
Sedimenticola	1.05				
Enterobacteriaceae	1.05				
Thiotrichales_incertae_sedis	1.05				

Table S5. Dominant bacterial genera in *Gammaproteobacteria* subclasses from sediment samples (L1. L2 and L3) collected from Loa river. Dominant phylogenetic genera (≥1% of total classified sequences) common to sediments (L1. L2 and L3) are represented in bold.

L1		L2		L3	
Genus	**Relative abundance (%)**	**Genus**	**Relative abundance (%)**	**Genus**	**Relative abundance (%)**
Haliea	8.32	***Ectothiorhodosinus***	9.35	***Ectothiorhodosinus***	8.85
Ectothiorhodosinus	4.05	***Thiococcus***	7.44	***Thiococcus***	6.70
Thiohalobacter	3.93	***Coxiella***	4.48	***Haliea***	6.37
Coxiella	3.24	***Methylococcus***	3.69	***Coxiella***	4.16
Thiococcus	3.01	***Haliea***	3.56	***Kistimonas***	3.69
Kistimonas	2.89	***Thiohalobacter***	3.23	***Thiohalobacter***	3.02
Leucothrix	2.77	***Thioflavicoccus***	2.57	***Methylococcus***	2.95
Marinicella	2.54	*Nitrosococcus*	2.57	*Sedimenticola*	2.88
Dasania	2.54	*Thiorhodospira*	2.44	***Methylohalomonas***	2.61
Methylococcus	2.08	***Methylohalomonas***	2.37	***Leucothrix***	2.55
Neptuniibacter	2.08	***Sulfurivirga***	2.11	***Thioflavicoccus***	2.28
Thiohalomonas	1.73	***Leucothrix***	2.04	***Neptuniibacter***	2.28
Hydrogenovibrio	1.62	***Neptuniibacter***	2.04	*Thiorhodospira*	1.61
Arenicella	1.50	*Sedimenticola*	1.91	*Thioprofundum*	1.41
Marinobacter	1.39	***Thiohalomonas***	1.58	*Dasania*	1.41
Rhabdochromatium	1.27	*Arenicella*	1.45	***Sulfurivirga***	1.34
Methylohalomonas	1.16	*Methylohalobius*	1.38	*Zooshikella*	1.27
Thioflavicoccus	1.16	*Natronocella*	1.25	***Thiohalomonas***	1.21
Aspromonas	1.16	*Umboniibacter*	1.25	*Thioalkalispira*	1.07
Thioalkalimicrobium	1.16	*Alkalispirillum*	1.25	*Halotalea*	1.07
Sulfurivirga	1.16	*Thioalkalispira*	1.25	*Marinicella*	1.01
Halotalea	1.16	***Kistimonas***	1.19		
Sedimenticola	1.04				
Thiofaba	1.04				
Rheinheimera	1.04				
Modicisalibacter	1.04				

Table S6. Dominant bacterial family in *Firmicutes* Phylum from sediment samples (L1. L2 and L3) collected from Loa river. Dominant phylogenetic genera (≥1% of total classified sequences) common to sediments (L1. L2 and L3) are represented in bold.

L1		L2		L3	
Family	**Relative abundance (%)**	**Family**	**Relative abundance (%)**	**Family**	**Relative abundance (%)**
Ruminococcaceae	15.89	***Thermodesulfobiaceae***	9.64	***Lachnospiraceae***	13.82
Lachnospiraceae	9.98	***Ruminococcaceae***	9.11	***Ruminococcaceae***	10.75
Veillonellaceae	9.85	***Veillonellaceae***	9.04	***Veillonellaceae***	8.86
Erysipelotrichaceae	4.74	***Clostridiaceae_3***	6.90	***Thermodesulfobiaceae***	5.72
Clostridiales_Incertae_Sedis_XII	4.37	***Lachnospiraceae***	5.76	***Clostridiaceae_1***	5.13
Acidaminococcaceae	4.00	***Bacillaceae_2***	4.70	***Clostridiaceae_3***	4.43
Clostridiaceae_3	4.00	***Thermoanaerobacteraceae***	4.38	***Clostridiaceae_4***	3.67
Clostridiales_Incertae_Sedis_XI	3.76	***Halobacteroidaceae***	4.27	***Acidaminococcaceae***	3.29
Clostridiaceae_1	3.76	***Acidaminococcaceae***	3.81	***Clostridiales_Incertae_Sedis_XII***	3.24
Thermodesulfobiaceae	2.96	*Bacillaceae_1*	3.45	***Thermoanaerobacteraceae***	2.97
Clostridiales_Incertae_Sedis_IV	2.83	***Peptococcaceae_1***	3.42	***Erysipelotrichaceae***	2.70
Halobacteroidaceae	2.77	***Erysipelotrichaceae***	2.95	***Clostridiales_Incertae_Sedis_XI***	2.70
Peptococcaceae_1	2.71	***Clostridiaceae_1***	2.38	***Halobacteroidaceae***	2.38
Thermoanaerobacteraceae	2.59	***Clostridiales_Incertae_Sedis_XI***	2.24	*Bacillaceae_1*	2.27
Clostridiaceae_4	2.52	*Thermoactinomycetaceae_1*	2.17	***Peptostreptococcaceae***	2.21
Syntrophomonadaceae	2.09	***Clostridiaceae_4***	1.89	***Bacillaceae_2***	2.16
Carnobacteriaceae	2.09	***Carnobacteriaceae***	1.81	*Thermoactinomycetaceae_1*	1.73
Peptostreptococcaceae	2.03	***Peptostreptococcaceae***	1.64	***Carnobacteriaceae***	1.73
Bacillaceae_2	1.72	***Syntrophomonadaceae***	1.57	***Peptococcaceae_1***	1.62
Peptococcaceae_2	1.29	*Incertae_Sedis_III*	1.39	*Incertae_Sedis_III*	1.46
Eubacteriaceae	1.23	***Clostridiales_Incertae_Sedis_XII***	1.35	*Planococcaceae*	1.24
Streptococcaceae	1.17	*Heliobacteriaceae*	1.28	***Clostridiales_Incertae_Sedis_IV***	1.24
Incertae_Sedis_XI	1.05	*Peptococcaceae_2*	1.17	*Clostridiaceae_2*	1.24
		Planococcaceae	1.17	*Gracilibacteraceae*	1.19
		Clostridiales_Incertae_Sedis_IV	1.10	***Syntrophomonadaceae***	1.13
		Clostridiales_Incertae_Sedis_III	1.03	*Clostridiales_Incertae_Sedis_III*	1.13
		Eubacteriaceae	1.00	***Eubacteriaceae***	1.03
		Gracilibacteraceae	1.00	*Pasteuriaceae*	1.03

Table S7. Dominant bacterial genera in *Firmicutes* Phylum from sediment samples (L1. L2 and L3) collected from Loa river. Dominant phylogenetic genera (≥1% of total classified sequences) common to sediments (L1. L2 and L3) are represented in bold.

L1		L2		L3	
Genus	**Relative abundance (%)**	**Genus**	**Relative abundance (%)**	**Genus**	**Relative abundance (%)**
Anaerospora	3.66	***Thermodesulfobium***	9.57	***Thermodesulfobium***	5.63
Thermodesulfobium	2.88	***Thermohalobacter***	3.96	***Anaerospora***	2.87
Fusibacter	2.76	*Bacillus*	2.23	*Parasporobacterium*	2.82
Mahella	2.76	***Acetivibrio***	1.98	*Cellulosilyticum*	2.82
Anaerovorax	2.58	***Allisonella***	1.87	***Thermohalobacter***	2.34
Acetivibrio	2.40	***Succinispira***	1.80	*Fusibacter*	2.23
Sporobacter	2.28	*Thermoflavimicrobium*	1.70	*Clostridium_XlVa*	2.07
Allobaculum	2.22	*Halanaerobaculum*	1.62	***Acetivibrio***	1.91
Robinsoniella	2.10	***Anaerospora***	1.59	*Geosporobacter*	1.91
Succinispira	2.04	*Anaeroarcus*	1.45	*Clostridium_sensu_stricto*	1.86
Thermohalobacter	2.04	*Clostridiisalibacter*	1.41	***Succinispira***	1.65
Subdoligranulum	2.04	***Succiniclasticum***	1.31	*Robinsoniella*	1.59
Saccharofermentans	1.74	*Heliobacillus*	1.27	*Anaerovorax*	1.59
Pelospora	1.56	*Fervidicola*	1.27	*Caminicella*	1.49
Soehngenia	1.50	*Syntrophaceticus*	1.24	*Thermoflavimicrobium*	1.43
Sporanaerobacter	1.38	*Desulfonispora*	1.24	***Allisonella***	1.33
Geosporobacter	1.38	***Saccharofermentans***	1.17	*Anaerosporobacter*	1.33
Oscillibacter	1.38	*Sporosalibacterium*	1.17	*Fervidicola*	1.28
Halanaerobaculum	1.26	*Thermacetogenium*	1.17	*Ethanoligenens*	1.28
Parasporobacterium	1.20	*Acetohalobium*	1.17	*Bacillus*	1.22
Proteiniclasticum	1.20	*Desulfitibacter*	1.13	***Mahella***	1.22
Succiniclasticum	1.14	***Mahella***	1.10	***Succiniclasticum***	1.12
Syntrophaceticus	1.14	*Allobaculum*	1.06	*Tepidanaerobacter*	1.12
Turicibacter	1.08	*Tepidanaerobacter*	1.02	*Soehngenia*	1.12
Allisonella	1.02			*Subdoligranulum*	1.12
Sporotalea	1.02			***Saccharofermentans***	1.06
Anaerotruncus	1.02			*Sporosalibacterium*	1.01
Clostridium_XlVa	1.02			*Pasteuria*	1.01
				Proteiniclasticum	1.01

CAPÍTULO VI: Arsenite oxidation by *Pseudomonas arsenicoxydans* immobilized on zeolite and its potential biotechnological application

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ABSTRACT

Pseudomonas arsenicoxydans has been recently described as a new arsenite oxidizing bacterial species. Arsenite detoxification activity by this species was determined by HPLC/HG/AAS. *P. arsenicoxydans* showed a high rate of As(III) conversion, particularly when immobilized (it oxidizes 100% of 500 µg arsenite present in the medium after 48 of incubation). Arsenite oxidizing activity, mediated by a constitutive periplasmic enzyme, was determined following the transfer of reducing equivalents from arsenite to 2,4-dichlorophenolindophenol (DCIP) showing that approximately 75% (0.173 µmol DCIP min^{-1} mg^{-1}) of the total activity (0.231 µmol DCIP min^{-1} mg^{-1}) was detected in the periplasmic fraction. Using PCR with primers specific for arsenite oxidase gene showed the presence of a gene encoding for arsenite oxidase in *P. arsenicoxydans*. Results show the potential biotechnological application of *P. arsenicoxydans* as a candidate for detoxification of As(III).

Keywords: As(III), arsenite-oxidizing bacteria, arsenite oxidase, *P. arsenicoxydans*, biofilm

INTRODUCTION

Arsenic, a chemical element ubiquitous in the environment, is present in four oxidation states: 0 (elemental), -3 (arsine), +3 (arsenite) and +5 (arsenate). The two soluble forms of arsenic, arsenite and arsenate, are toxic for biological systems, arsenite being considered the most toxic (Salmassi et al. 2005).

Several arsenite-oxidizing bacteria have been isolated from various environments. Among them, gold mines, sewage, hyper-saline lakes, soil, geothermal springs and arsenic-contaminated ground water and sediments can be included (Santini et al. 2000; Oremland and Stolz 2003; Valenzuela et al. 2009; Campos et al. 2010). All of them oxidize arsenite by means of arsenite oxidase enzymes.

Lett et al. (2012) proposed a new nomenclature for the large subunit of the arsenite oxidase, previously known as *aoxB*, *asoA* and *aroA*, naming it *aioA*. The small subunit was designated *aioB*, in accordance with the designation for the two subunits of the dimethyl sulfoxide (DMSO) reductase family of molybdenum-containing enzymes as described by Hille (1996).

Arsenite-oxidizing bacteria include chemolithoautotrophic and heterotrophic microorganisms, such as *Acidiphilium acidophilum*, *Acidithiobacillus ferrooxidans*, *Cenibacterium arsenoxidans*, *Alcaligenes faecalis*, *Cupriavidus necator*, *Hydrogenophaga* sp. (Muller et al. 2003; Silver and Phung 2005) and some *Pseudomonas* species (Campos et al. 2010; Lia et al. 2012), as well as the thermophiles *Thermus aquaticus* and *Thermus thermophilus* (Gihring et al. 2001). In addition, a novel arsenite oxidase gene, *arxA*, was identified in the genome sequence of the Mono Lake isolate, *Alkalilimnicola ehrlichii* MLHE-1, a chemolithoautotroph that couples arsenite oxidation to nitrate reduction (Zargar et al. 2010).

Conventional treatment processes for As removal include coagulation, filtration, lime softening, activated alumina adsorption, ion exchange, reverse osmosis, reversal electro-dialysis and nanofiltration (USEPA 2001). Although these processes are effective and could

remove about 80–95% As from solutions, operating costs are high. Indeed, oxidation of As (III) to As (V) is a prerequisite for all conventional treatment processes (Khoe et al. 1997). However, the oxidation rate of As(III) to As(V) by oxygen is extremely slow and, hence, stronger and costly oxidants such as chlorine, hydrogen peroxide, or ozone, are needed as part of the As removal process (Kim et al. 1999). Since undesirable secondary pollutants may be generated (Gregor 2001), specific legislations in different countries do not allow the use of chlorine, as is the case for Germany (Driehaus et al. 1995).

Thus, cheaper and environmentally friendly options to oxidize As, such as biological treatments, may offer an interesting alternative to chemical routes. In this respect, several bacteria involved in transformation processes comprising reduction, oxidation, and methylation of arsenic species have been described (Valenzuela et al. 2009; Campos et al. 2011). In this context, microbiological methods for As oxidation are regarded as potential options. Oxidized As species can then be removed by any of the conventional methods. In addition, immobilization of the microorganisms mediating these processes offers the advantage of stabilizing them and may also improve their activity by increasing their local concentration (Gikas and Livingston 1993).

Campos et al. (2010), reported a new arsenite oxidizing species, *Pseudomonas arsenicoxydans*, isolated from the Chilean Atacama desert. *P. arsenicoxydans* is able to oxidize at least 500 µg/ml arsenite present in the culture medium after 48 h of incubation under aerobic conditions. The aim of this study was to characterize the As (III) oxidation by *P. arsenicoxydans* and to study the performance of the strain after its immobilization in zeolite for its potential use in the implementation of a biological treatment system for remediation of arsenite contaminated waters.

MATERIALS AND METHODS

P. arsenicoxydans was isolated from sediments of the Camarones River, Atacama desert, Northern Chile, an area characterized by high arsenic concentrations (up to 1100 µg L^{-1}) due to natural leaching from the rocks (Campos et al. 2010). In the present work, *P.*

arsenicoxydans was grown aerobically at 25ºC in a chemically defined medium (CDM) (Battaglia-Bruner et al. 2002), enriched with 1mM or 2mM arsenite (as NaH_2AsO_3). Carbon sources assayed were, all at 1 mM concentration, sodium lactate, sodium citrate, piruvate, acetate and sodium bicarbonate.

Arsenic tolerance of *P. arsenicoydans* was determined by the agar dilution technique on LB agar plates containing 0.5 to 100 mM sodium arsenite or 0.5 to 1000 mM sodium arsenate. The capacity to grow in the presence of 7 mM As(III) (arsenite) or 20 mM As(V) (arsenate) was considered as As resistant, according to Rokbani et al. (2007). Each plate was inoculated with cell suspensions from fresh pre-cultures containing approximately $3x10^7$ CFU mL^{-1} and incubated for 24h at 25ºC. Agar plates without the metalloid was used as control (Campos et al. 2011).

For biofilm formation, cells were cultured until exponential phase was achieved. Zeolite, washed with sterile distilled water, was added to nutrient broth in proportion 5:100 (w/v) and then inoculated with a small aliquot of bacterial inoculum in order to obtain approximately 10^5 CFU/ml. Incubation, with and without arsenite (500 µg/ml), was performed during 30 days at 25ºC with constant low-speed shaking in order to allow the establishment of the biofilm. Cellular counts were made by fluorescence microscopy. For this, *P. arsenicoxydans* biofilm samples developed on zeolite were washed repeatedly with distilled water, fixed with paraformaldehyde (2%) and then stained with DAPI (4,6-diamino-2-phenyl-indol-dihydro-chloride-dilactate) at a final concentration of 1 mg mL^{-1} for 30 min in the last washing step. Samples were obtained in triplicate and counted along the 30 days of incubation.

For scanning electron microscopy (SEM) studies, *P. arsenicoxydans* biofilm samples developed on zeolite were washed repeatedly with distilled water and fixed with a buffer solution of cacodylate (0.067 mol L^{-1}, pH 7.2) with 2.5% glutaraldehyde and 0.015% Ruthenium red. Then, they were washed in the same buffer solution and fixed with 1% osmium tetroxide. Finally, samples were washed again with the buffer solution, dehydrated with alcohol and acetone (Campos et al. 2011), and dried with liquid CO_2 by means of the critical point method. Finally, samples were gold coated in a sputter coater (Edwards S150, Sussex, UK) and examined in an Etec Autoscan SEM (Etec Corp., CA, USA).

For detecting As (III) to As (V) transformation, planktonic and immobilized cells of *P. arsenicoxydans* were grown aerobically at 25ºC in a chemically defined medium (CDM) (Battaglia-Bruner et al. 2002), enriched with 500 µg ml^{-1} sodium arsenite (as NaH_2AsO_3), using sodium lactate as carbon source. The oxidation of As(III) to As(V) was determined from culture supernatants filtered through a sterile 0.22 µm pore size filter (Millipore). Arsenic species were monitored by High Performance Liquid Chromatography coupled to arsine gaseous formation performing the detection by atomic absorption in quartz bucket (HPLC/HG/QAAS) (Campos et al. 2010).

In order to locate the arsenite oxidizing activity, cells extracts of *P. arsenicoxydans* were prepared. For this purpose, cells were grown to late exponential phase in CDM supplemented as above during 20 h at 30 ºC, with shaking. After incubation, cells were harvested and centrifuged at 1,000 x *g* (4°C) for 20 min and the pellet was re-suspended in ice-cold morpholineethanesulfonic acid buffer (MES) (pH 6.0). Cells were disrupted by passing them twice through a French press (103 lb in^{-2}). Unbroken cells were removed by centrifugation at 15,000 x *g* (4ºC) for 20 min and the supernatant constituted the crude extract (Santini et al. 2000). In addition, spheroplasts and periplasmic fractions were obtained. For this, bacterial cells were harvested during the late exponential phase. They were harvested by centrifugation for 20 min at 20,000 x *g* (4°C), and washed once with 40 ml ice-cold 10 mM Tris/HCl, pH 8.0. The periplasmic cell fraction was prepared using a modification of the method of Osborn and Munson (1974). Throughout the procedure, the bacteria were stirred gently (0°C). Cells were resuspended in ice-cold 750 mM sucrose, 30 mM Tris/HCl, pH 8.0 (0°C). The cell suspension was incubated for 5 min at 0°C. Lysozyme was then added to a final concentration of 0.15 mg ml^{-1}, and incubation was continued for another 2 min. Following the addition of 2 vol. 7.5 mM EDTA, pH 8.0 (0 °C), over a period of 10 min, the cell suspension was stirred for additional 10 min (0°C). The suspension was then placed, without stirring, at room temperature for 15 min to permit the formation of spheroplasts. Spheroplasts were separated from the periplasm by centrifugation at 27,000 x g for 20 min. For protein concentration determination, Bradford reagent was used. Bovine serum albumin was used as standard (Bradford 1976).

Arsenite oxidase activity was determined based on the transfer of reducing equivalents from arsenite to 2,4-dichlorophenolindophenol (DCIP). Reduction of DCIP (60μM) was monitored at 600 nm using 200 μM sodium arsenite in 50mM MES, pH 6.0, at 25°C. Total arsenite oxidizing activity units were evaluated on the basis of the amount of DCIP reduced while specific activity was defined as μM of DCIP reduced per min^{-1} (mg of protein)$^{-1}$ (Anderson et al. 1992).

To amplify and sequence the arsenite oxidase gene, genomic DNA was extracted from a bacterial suspension. This suspension was boiled for 10 min and centrifuged for 5 min at 14,000 rpm. The supernatant was used as DNA template for PCR amplification; using the degenerate primers designed by Rhine et al. (2007). PCR amplification and sequencing were performed according to describe by Valenzuela et al. (2009). We used *Escherichia coli* S17-1 carrying the *aio* operon from *Agrobacterium tumefaciens* in the plasmid pDK402 (Kashyap et al. 2006) as positive control (kindly provided by Dr. Timothy McDermott, College of Agriculture, Montana State University, USA) and *E. coli* K-12 as negative control (Rhine et al. 2007). The DNA sequence of *P. arsenicoxydans* encoding for the arsenite oxidase enzyme was deposited in EMBL-GenBank with access number FN824370.

All assays were performed in triplicate. Statistical analysis included Student's t tests using MINITAB version 15 (USA) software, P values < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

P. arsenicoxydans, isolated from sediment samples from the Camarones Valley, Atacama Desert (Lat. 18º57'S, Long. 69º30'W), was able to grow in the presence of most carbon sources assayed (data not shown). Lactate was the carbon source allowing the best growth, even in the presence of 1 and 2 mM arsenite (Fig. 1). Nevertheless, it was unable to grow in the presence of arsenite when sodium bicarbonate ($NaHCO_3^-$) was the unique carbon source. These results showed that this is a heterotrophic bacterial strain.

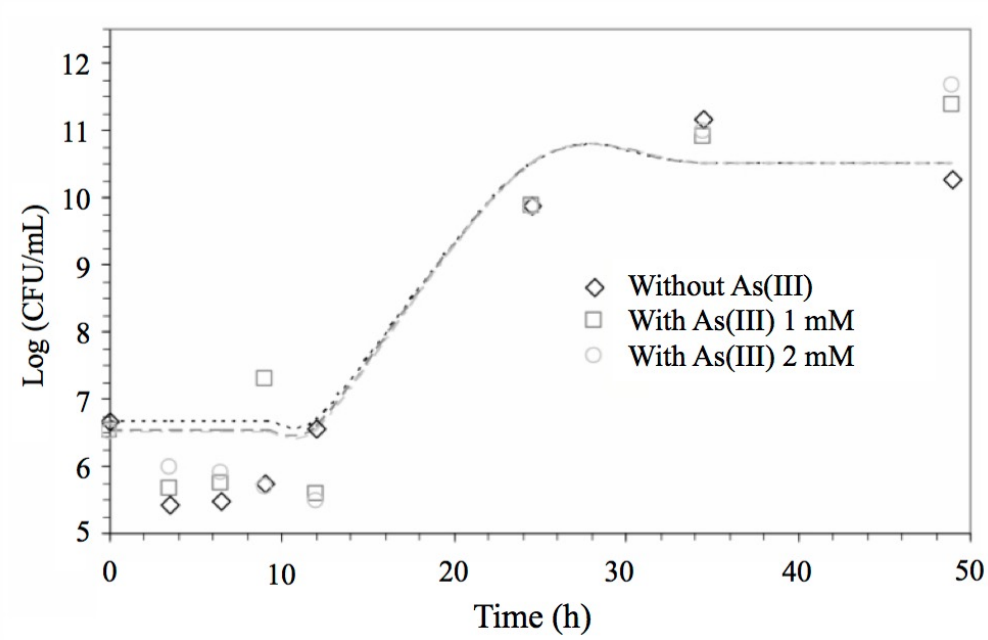

Figure 1. Growth of P. arsenicoxydans in a chemically defined medium with lactate as carbon source and 1 or 2 mM arsenite

The levels of resistance to arsenic were studied. Results showed that *P. arsenicoxydans* was able to tolerate 4 mM of As(III) and 8 mM of As(V). Jackson et al. (2005) isolated 37 arsenic resistant strains, of various bacterial groups, from sediments with low arsenic concentrations (1.33 μM), but they showed high arsenate resistances (up to 400 mM) but most of them showed low levels of resistance to arsenite. Regarding their arsenite resistance, five strains showed resistance up to 5 mM and only one strain was resistant to 10 mM. The remaining strains resisted only between 0 and 2 mM arsenite. They concluded that even some of their strains were able to tolerate high levels of arsenate, the arsenite resistance was usually much lower. In contrast, the *P. arsenicoxydans* strain here reported was able to tolerate both but, similarly as reported by Jackson, arsenate tolerance was higher than arsenite tolerance.

The ability of *P. arsenicoxydans* to adhere to a support was monitored by fluorescence microscopy. *P. arsenicoxydans* cells (10^5 CFU ml^{-1}) growing in the exponential phase were cultured in the presence of zeolite, with or without As(III). The number of bacterial cells per gram of zeolite was evaluated up to day 30. Both, in the presence or absence of As(III), the

strain established a stable biofilm containing about 10^8 cells g^{-1} of zeolite after 4 days of incubation and this count remained constant up to day 30 (Fig. 2).

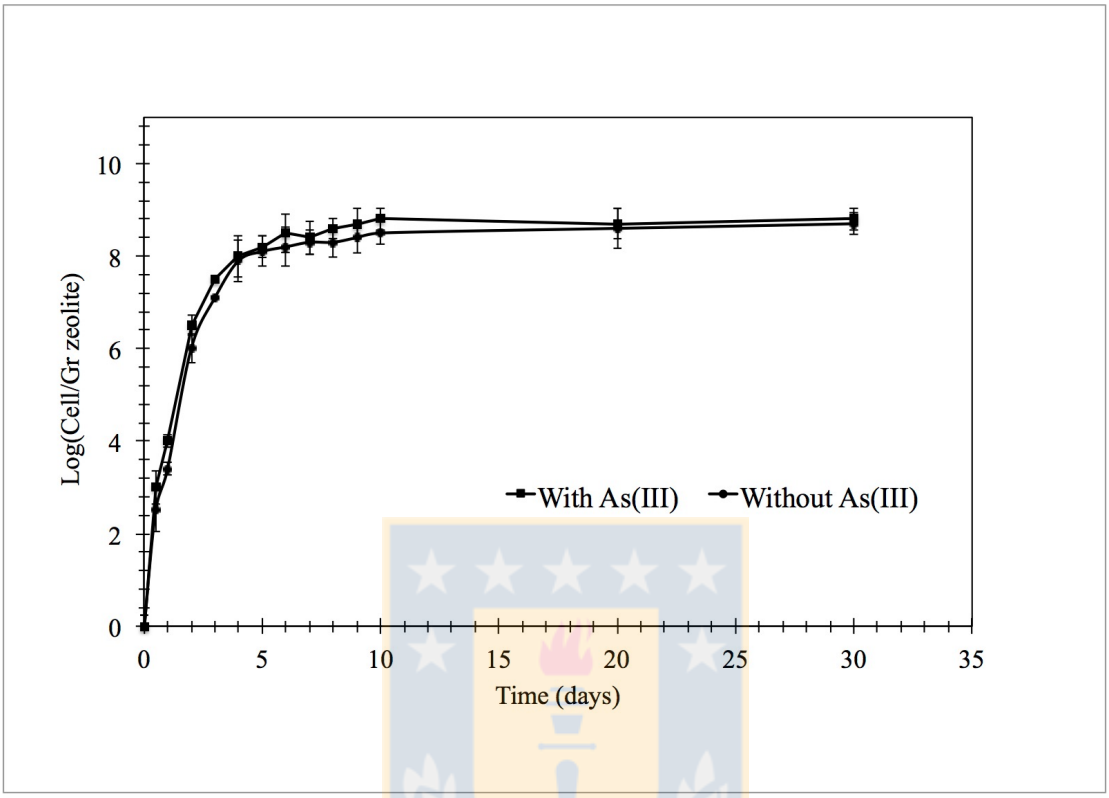

Figure 2. Growth of P. arsenicoxydans on zeolite with and without arsenite.

In addition, *P. arsenicoxydans* biofilm formation on zeolite, in the presence or absence of As(III) in the medium, was monitored by SEM. After 24 h of incubation, with or without As(III) (see Figure 3A and 3E, respectively), small cells were randomly distributed on the surface of zeolite, existing non-colonized spaces. After 48 h of incubation, in the presence or absence of As(III) (see Figure 3B and 3F, respectively), bacteria were anchored to the zeolite surface and to each other by means of fibrils, bridging the space between bacteria. Figure 3C and 3G show a mature biofilm in the presence or absence of As(III), respectively, after 72 h of incubation, with attached bacteria that excreted extracellular polysaccharides covering sessile cells and serving as a matrix for further biofilm formation and remaining stable, as seen by day 30 of incubation in the presence or absence of As(III) (Figure 3D and 3H, respectively). Thus, as revealed by SEM, the presence or absence of arsenite appears not to affect the morphology of the biofilm.

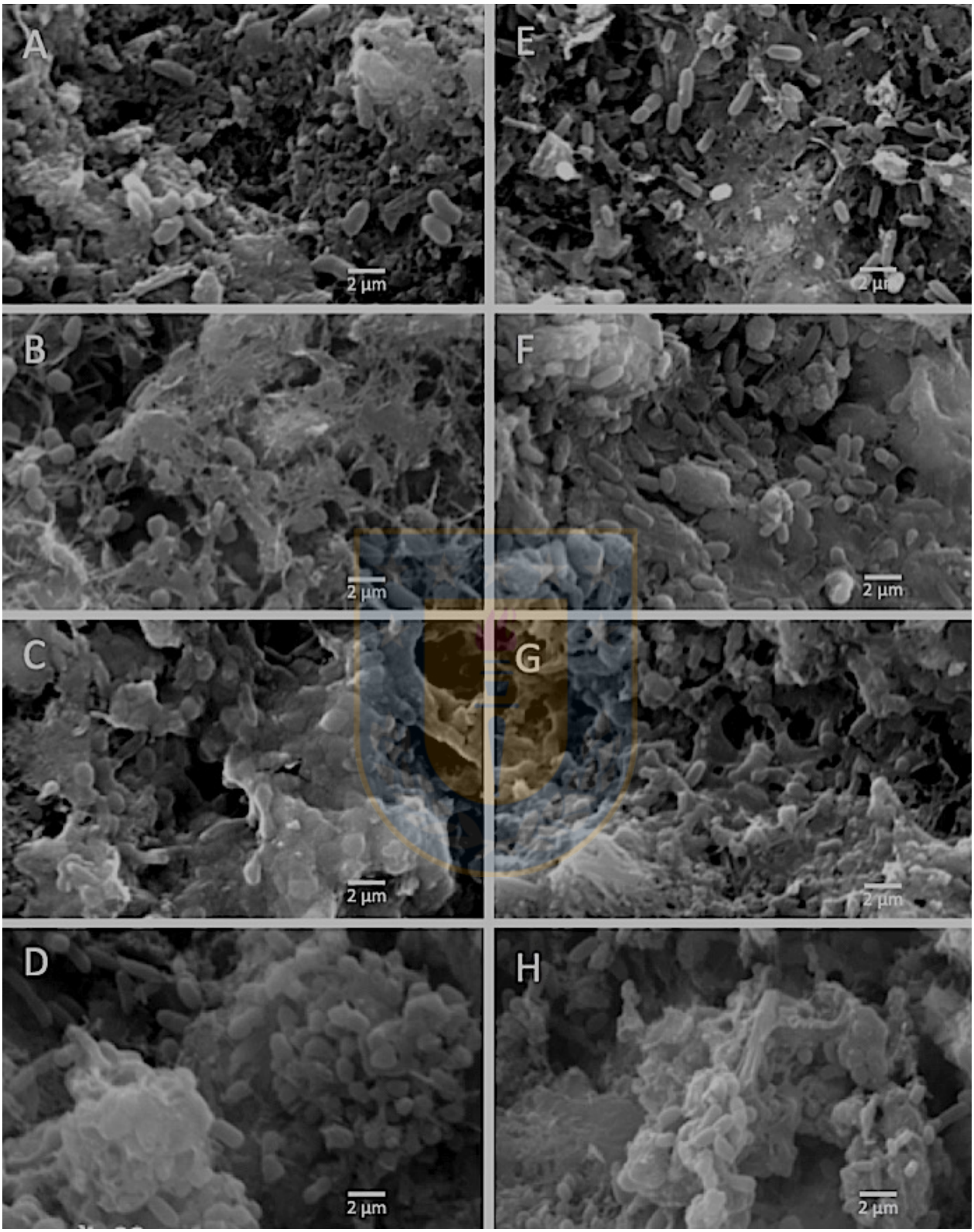

Figure 3. Biofilm formation of P. arsenicoxydans on zeolite in the presence of As(III) after 24 h (3A), 48 h (3B), 72 h (3C) and 30 days (3D) of incubation or in the absence of As(III) after 24 h (3E), 48 h (3F), 72 h (3G) and 30 days (3H) of incubation.

In addition, SEM revealed the presence of thin fibrillar pili at the cell surface (Figure 3A and 3B). These particular pili are similar to curli structures described by Olsen et al. (1989). The curli (pili or micro-fibrillar) seem to be an envelope structure of major importance for surface colonization because it provides stability to the biofilm, allowing strong hydrodynamic support on long term cultures or non-interrupted cultures (Gualdi et al. 2008). As seen in Figure 3C and 3G, bacterial cells are embedded by a matrix probably constituted by extracellular polysaccharides. The polymeric matrix observed appears similar to that previously described for other microorganisms (Lerner et al. 2009).

Arsenite oxidation by planktonic or immobilized *P. arsenicoxydans* cells was evaluated by HPLC/HG/ASS. Immobilized *P. arsenicoxydans* cells were able to oxidize 500 µg ml^{-1} As(III), under aerobic conditions, leading to 100% As(III) conversion into As(V) after 36 hours of incubation, a rate of 12.86 µg mL-1 h-1 (Figure 4). However, planktonic *P. arsenicoxydans* cells, cultured under similar conditions, were able to oxidize 100% As(III) to As(V) after 48 h, a rate of 10.07 µg mL^{-1} h^{-1} (Figure 5). The decrease of As(III) was concomitant with the increase of As(V) in the culture medium for both immobilized and planktonic cells. The immobilized cells were more efficient than the planktonic cells in oxidizing arsenite ($P < 0.05$). Similar results were reported by Simenova et al. (2005) in calcium alginate immobilized *Herminiimonas arsenoxidans* cells, demonstrating the ability of this strain to oxidize high concentrations (100 mg L^{-1}) of As(III) and they also showed that oxidation was faster in immobilized cells.

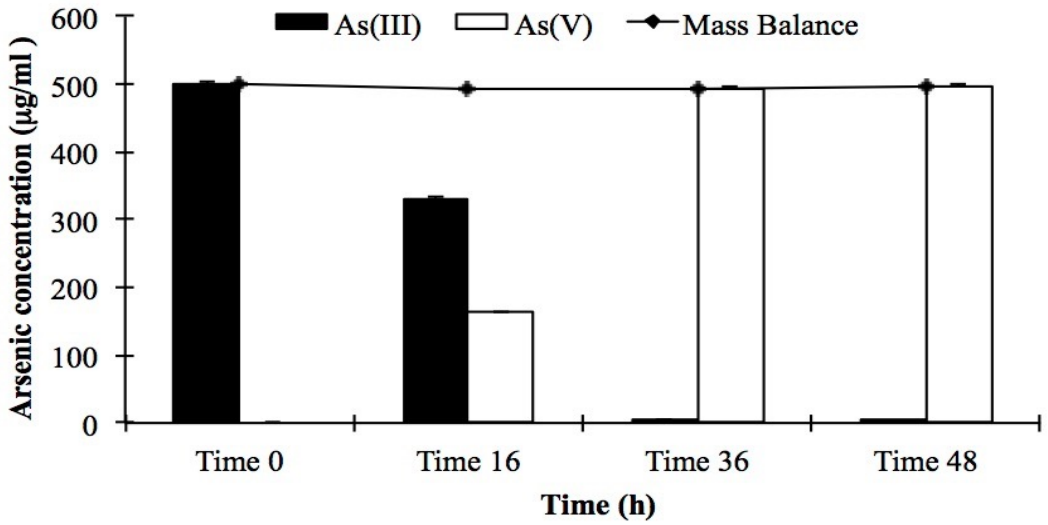

Figure 4. Arsenite oxidation by an arsenite oxidase of immobilized P. arsenicoxydans in a chemically defined medium, using lactate as carbon source.

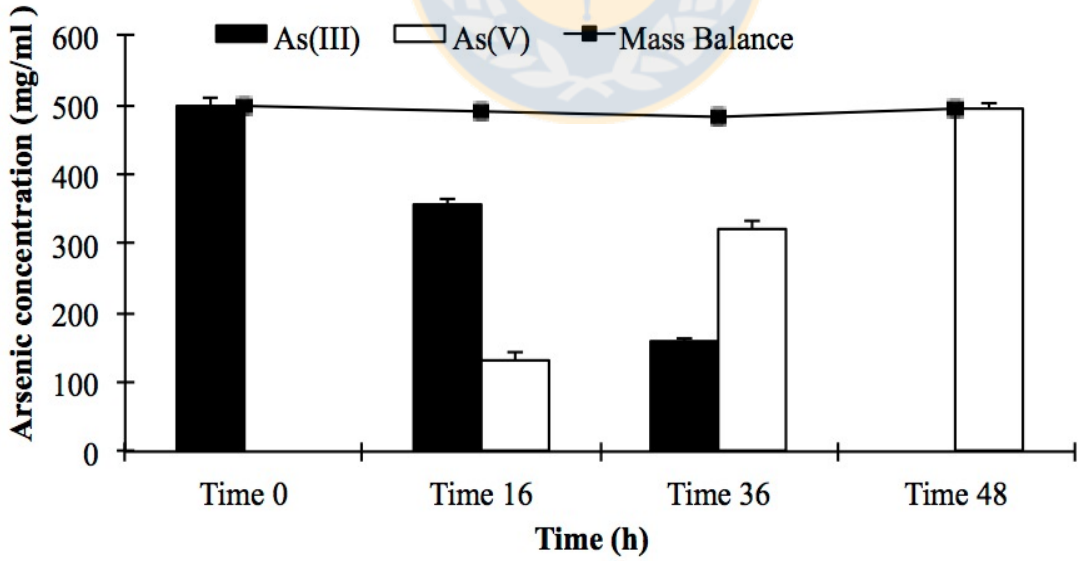

Figure 5. Arsenite oxidation by an arsenite oxidase of planktonic *P. arsenicoxydans* in a chemically defined medium, using lactate as carbon source.

The arsenite oxidizing activity was studied in cells previously cultured in the presence or absence of arsenite, demonstrating that the arsenite oxidizing activity allowing this transformation is constitutive (data not shown). After separation of supernatant and whole-cells by centrifugation, arsenite oxidizing activity was detected only in the pellet (data not shown), indicating that the activity is intracellular. Muller et al. (2003) reported similar results for a metal-resistant β-Proteobacterium arsenite oxidase.

To further localize the arsenite oxidase activity in *P. arsenicoxydans*, three different preparations were undertaken: crude bacterial extract, periplasmic samples and spheroplasts. Specific activity in the crude extract was 0.231 µmol DCIP min^{-1} mg^{-1} (Table 1), and was larger than most so far reported, such as the case of *Alcaligenes faecalis* showing an arsenite oxidase activity of crude extracts nearly 10 times less (Anderson et al. 1992). DCIP reduction showed that the periplasmic preparation had a specific activity of 0.173 µmol DCIP min^{-1} mg^{-1}, while the spheroplasts showed only 0.058 µmol DCIP min^{-1} mg^{-1} (Table 1). These values correspond only to 75% and 25% of the crude extract, respectively. These results demonstrated that the arsenite oxidizing activity is associated to the periplasm.

Table 1. Arsenite oxidase subcellular localization

Step	Total Protein	Total Activity*	Specific activity
	mg/ml	µmol DCIP/min	µmol DCIP/min/mg
Crude extract	605	139.8	0.231
Supernatant (periplasmic)	347	60.3	0.173
Pellet (spheroplats)	257	14.9	0.058

*Activity units are based on the amount of DCIP reduced

Vanden Hoven and Santini (2004) reported similar results in the heterotrophic bacterium *Hydrogenophaga* sp. strain NT-14, showing that the arsenite oxidizing activity is periplasmic. In addition, NT-26, a chemolithoautotrophic strain, possesses a periplasmic arsenite oxidase induced by the presence of arsenite (Santini and Vanden Hoven 2004). Moreover, in *Alcaligenes faecalis*, an inducible membrane-bound enzyme catalyses the arsenite oxidation with an activity, in the crude extract, of 0.023 μmol DCIP min^{-1} mg^{-1} (Anderson et al. 1992). In *Thiomonas* sp., the arsenite oxidase activity was found to be associated with the membrane and in absence of arsenite presents less activity, indicating that the production of this enzyme is induced (Dusquesne et al. 2008).

In order to demonstrate the presence of an enzyme capable of catalyzing arsenite oxidation, PCR analyses using specific degenerated primers for arsenite oxidase genes, as described by Rhine et al. (2007), were undertaken. A 1200 bp product was obtained, coinciding with that reported by Rhine et al. (2007) for an arsenite oxidase. Then, our product was cloned, sequenced and deposited at GenBank with the accession number FN824370. To corroborate that the 1200 bp product corresponded, in fact, to an arsenite oxidase, its sequence was compared with sequences available at GenBank using the BLAST tool. This comparison showed that our sequence had 97 to 99% similarity with other arsenite oxidases available at Gen Bank.

In conclusion, this work describes the ability of *P. arsenicoxydans* to oxidize As(III), using lactate as carbon source, due to the presence of a constitutive highly efficient arsenite oxidase enzyme located in the periplasm. The ability of biofilm formation, the high resistance to As(III) as well as its high oxidation capacity opens the way to further studies on *P. arsenicoxydans* aimed at implementing biological systems to treat arsenic rich wastewaters.

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CAPÍTULO VII:Removal of As(III) and As(V) by arsenic resistant ureolytic-calcifying bacterial strain isolated from natural environments.

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Abstract

Arsenic (As) is a toxic element for humans and it is commonly associated with serious health disruptions. The most common form of massive and chronic exposure is by consumption of contaminated drinking water. Bangladesh, India, Mongolia, China, Taiwan, Mexico, Argentina and Chile are countries where arsenic poisoning appears as a public health problem resulting mainly from consumption of As-contaminated water. Conventional treatment processes for As removal are effective and may remove about 80-95% As from solutions, however, operating costs are high. An effective treatment for water contaminated with arsenic should be directed to the removal of both As(V) and As(III). An alternative technique is Biomineralization based on microbially induced calcite precipitation (MICP). In this context, to objective was isolated and characterize bacterial strain able to mineralize calcium carbonate, and oxidase As(III) to As(V). Bacterial strain resistant to arsenic were isolated from sediment samples from a highly contaminated natural system (Loa River) and ureolytic bacteria (calcifying) able both to oxidase As(II) and resistant to As(V) were selected and characterized microbiologically and molecularly. Once characterized, bacteria were used to batch bioreactor in which the As(III) and As(V) depurative capacity was evaluated using HPLC/Hg/AAS while the elimination of toxicity and genotoxicity was evaluated by the human umbilical vein endothelial cells (HUVEC) assay. A total of 9 bacterial, As(III) and As(V) resistant strains were isolated. Based on their characteristics, 1

ureolytic strain was selected and identified, by 16S rRNA gene sequence, as *P. marginalis*. Strain possessed arsenite-oxidase chromosomal resistance gen. Scanning and transmission electron microscopy analysis showed biomineralization of Arsenic induced by biogenic calcite in the cultures, evidenced by calcite crystals in the presence of Arsenic. HPLC/Hg/AAS analysis demonstrated that as calcite crystals increased along time, the concentration of arsenate in the culture medium decreased to 89% after 72 h of incubation. On the other hand, toxicological analysis using HUVEC cells showed that toxicity decreased in 99,8%. The implementation of treatment systems for arsenic contaminated waters, using ureolytic-calcifying bacteria able to oxidase As(III) and resistant to As(V), is an excellent biotechnological candidate, economically sound and environmentally friendly, decontamination procedure.

Keywords: Arsenite; bio-mineralization, ureolytic-calcifying, detoxification.

INTRODUCTION

Arsenic (As) is a toxic element for humans and it is commonly associated with serious health disruptions. The principal manifestations of arsenicism affecting health are melanosis, keratosis and different forms of cancer (skin, bladder, lung, liver and prostate among others) (Hsu et al., 2013). The most common form of massive and chronic exposure is by consumption of contaminated drinking water. Bangladesh, India, Mongolia, China, Taiwan, Mexico, Argentina and Chile are countries where arsenic poisoning appears as a public health problem resulting mainly from consumption of As-contaminated water (Mandal and Suzuki, 2002).

Northern Chile, especially the Atacama Desert, has been described as an arsenic-rich environment. Minerals of metallic sulfides containing arsenic are dissolved in the Andes Mountains, affecting superficial and ground waters that cross the Atacama Desert which are used as drinking water sources. Since 1970, drinking water is specially treated to remove arsenic, with the concomitant high cost, in all the large cities of the Atacama Region (such as Antofagasta) (Mandal and Suzuki, 2002). However, the population of several small rural

villages remain exposed to arsenic in drinking water. The problem of chronic arsenicism affects around 50000 persons, mainly in rural populations of the Atacama Desert in northern Chile. The affected populations drink water from small waterfalls and rivers with arsenic contents greater than 1000 ug L^{-1} (Yañez et al., 2005; Valenzuela et al., 2009). This situation greatly surpasses both the World Health Organization and the U.S. Environmental Protection Agency recommendations for As concentrations (up to 10 ug L^{-1}) (Smith et al., 2002; EPA, 2001; WHO, 2001).

In general, arsenate (As(V)) is the predominant arsenic species in oxygenated aqueous environments, whereas arsenite (As(III)) species predominate under anoxic or reduced conditions, being 100 times more toxic than As(V) (Al-Abed et al., 2007; Taerakul et al., 2007; Neff, 1997).

Conventional treatment processes for As removal include coagulation, filtration, lime softening, activated alumina adsorption, ion exchange, reverse osmosis, electrodialysis reversal and nanofiltration (Campos et al., 2009). Although these processes are effective and may remove about 80–95% As from solutions, operating costs are high. Indeed, oxidation of As(III) to As(V) is a prerequisite for all conventional treatment processes (Khoe et al., 1997). However, the oxidation rate of As(III) to As(V) by oxygen is extremely slow and, hence, stronger and costly oxidants, such as chlorine, hydrogen peroxide or ozone, are needed as part of the As removal process (Kim et al., 1999). Thus, cheaper and environmentally friendly options to biotransformation As, such as biological action, may offer an interesting alternative to chemical routes. In this respect, several bacteria involved in transformation processes comprising reduction, oxidation, and methylation (arsenic resistant bacteria) of arsenic species have been proposed for the implementation biological treatment system (Heinrich-Salmeron et al., 2011; Kruger et al., 2013; Jareonmit et al., 2012; Sorokin et al., 2012; Osborne and Santini, 2012). In this context, microbiological methods for transforming As are regarded as interesting options. However, all biological systems described are not able to immobilize toxic compounds, or have been designed to remove only one arsenical species.

An effective treatment for water contaminated with arsenic should be directed to the removal of both As(III) and As(V). An alternative technique is biomineralization based on Microbially Induced Calcite Precipitation (MICP) seems to be a promising technique to remediate arsenic from contaminated environments making use of the advantages of bacterial bioremediation (Webb et al., 2006; Pan, 2009). This process is active in almost every environment on earth. Microorganisms can secrete one or more metabolic products that react with ions or compounds in the environment, resulting in the subsequent deposition of mineral particles. Calcite, a biomineralization product, can strongly both adsorb onto its surfaces and incorporate arsenic metalloid ion into its crystal structure (DiBenedetto et al., 2006). Considerable research on bio-calcite precipitation has been performed using ureolytic bacteria (Muynck et al., 2010; Achal et al., 2009). These bacteria are able to influence the precipitation of calcium carbonate by the production of an enzyme, urease. Calcium carbonate precipitation occurs as one of the consequences of bacterial metabolic activity. The ubiquitous presence of MICP and the ability of its products to trap toxic elements may provide a new in situ remediation method for decontaminating natural systems. Most of the studies to remediate heavy metals such as Sr and Pb by MICP have been performed in groundwater (Fujita et al., 2004). To the best of our knowledge, no study has been performed in the past for bioremediating arsenic contaminated water based on MICP.

Based on the fact that most of the bacteria are able to precipitate $CaCO_3$, product of their metabolic processes such as photosynthesis, urea hydrolysis and sulphate reduction, the bioremediation of water contaminated with arsenic by MICP may be a viable alternative (Martin et al., 2012; Hammes et a., 2003). In addition, Achal et al., 2012 described an environmental bacteria able to precipitate calcite (Calcifying bacteria) and resistant to As(III).

In this respect, various bacterial strains able to oxidize As(III) to As(V) have been described. Such activity has been explained as a detoxification mechanism by heterotrophic bacteria, where As(III) is oxidized by a periplasmatic enzyme called arsenite oxidase. In the case of chemolithoautotrophic, arsenic oxidation is coupled to oxygen or nitrate reduction, using the energy to fix oxygen into the organic cellular material (Donahoe et al. 2004).

In this context, the main challenge of this proposal is to characterize bacterial populations resistant to arsenical compounds able to mineralize calcium carbonate which are present in these natural systems, with the purpose of isolating and identifying bacteria strains able to oxidase to As(III) and resistant to As(V) by to remove the Both metalloids from waters contaminated with arsenic by means bio-precipitation calcite.

MATERIALS AND METHODS

Isolation of calcifying As-resistant bacteria

Water and sediments samples were collected from the As-contaminated Loa river. The sample site was located 23 km downstream from Sloman dam, close to Quillagua Oasis (21°39’32.26"S - 69°32'9.76"W). Arsenic resistant ureolytic calcifying bacteria were isolated from sediments. One g of soil was inoculated into 500 mL of Chemically Defined Medium (CDM) (pH 8.0) containing 100 mg L^{-1} arsenic solution (Na_3AsO_3/Na_3AsO_4) and incubated at 30ºC with shaking (130 rpm) for 48 h in darkness (Campos et al., 2009). Bacterial strains were isolated in R2A and CDM agar plates supplemented with arsenite (0.5 mM) and/or arsenate (0.5 mM). The plates were incubated at 30ºC, for 48 h, in darkness. Bacterial isolates tolerating the highest arsenite and arsenate concentrations were selected. Subsequently, the colonies were transferred onto urea agar base, urease selective medium, to check the production of urease (as urease is a key indicator for calcite precipitating microorganisms).

Arsenic levels tolerance

The arsenic levels tolerance of isolates was determined by the agar dilution technique, on R2A agar plates amended with variable concentrations of sodium arsenite between 0.5 to 100 mM and sodium arsenate between 0.5 to 1000 mM. Each plate was inoculated with cell suspensions from fresh cultures to a final density of approximately 10^7 CFU mL^{-1} and incubated for 24 h at 25°C. An agar plate with bacteria and without the metalloid was used as a control. According to Rokbani et al. [47], isolates able to grow in the presence of at least 7 mM As (III) (arsenite) or 20 mM As (V) (arsenate) were considered as resistant.

As oxidation and reduction rate of the isolated strains

For detecting oxidation and/or reduction of As, isolated strain were grown in CDM media supplemented with 0.5 mM of arsenite or arsenate, and then incubated at 30ºC, 130 rpm for 72 h. The transformation of As was determined from culture supernatants filtered through a sterile 0.22 µm filters size (Millipore). Arsenic species were monitored by HPLC/HG/QAAS (Campos et al. 2010).

Identification of As-resistant strains

Arsenic-resistant isolates selected were initially characterized in terms of colony morphology (color, shape, size), basic microscopic observations, and biochemical profiles (RapID ONE System and RapID NF Plus System; Remel). Then PCR amplification of 16S rRNA gene was performed. Total DNA of strains was obtained using UltraClean® Microbial DNA Isolation Kit, following manufactures instructions. PCR was conducted with 16S rDNA bacteria universal primers GM3 (AGAGTTTGATCMTG GC) and GM4 (TACCTTGTTACGACTT). PCR determination followed the procedure described by Ward et al. (1997). Sequencing was conducted on the amplified fragments using the Dyenamic ET terminator kit (General Electric) in a 3100 Avant genetic Analyser (Applied Bio- systems) according to the manufacturer's instructions. Strains were characterized on the basis of sequences stored in the GenBank database using the Basic Local Alignment Search Tool program.

Detection of detoxifying arsenate reductase *arsC* gene

To detect the arsC gene, isolates were cultured for 12 to 18 h at 25ºC in LB supplemented with 0.5 mM NaH2AsO5 until reaching 108 CFU/mL. The genomic DNA of each isolate was extracted using the UltraClean Microbial DNA Isolation Kit (Mobio Laboratories, Inc.) to serve as template for PCR amplification. Primers used for the PCR technique were arsC-1-F (5' GTAATACGCTGGAGATGATCCG 3') and arsC-1-R (5' TTTTCCTGCTTCATCAACGAC 3'), which correspond to the ars operon of Escherichia coli [50]; and arsC-1-F (5' AGTCCTGTTCATGTGYAC 3') and arsC-1-R (5' TGGCGTSGAAYGCCG 3') described for the ars operon of *Pseudomonas aeruginosa* and *Pseudomonas putida* [11, 51]. PCR products were separated by electrophoresis in a 1.2 % agarose gel and visualized in an UV transilluminator after staining with ethidium bromide [23].

Detection of arsenite oxidation aio gene

Strains were cultured in LB supplemented with 0.5 mM NaH2AsO3, and DNA was extracted as described above. PCR was performed using the primers 69F (TGYA TYGT NGGN TGYG GNTA YMA) and 1374R (TANC CYTC YTGR TGNC CNCC) according to Valenzuela et al. [38]. E. coli S17-1 was used as positive control [52], while E. coli K-12 was used as negative control. Separation of amplified products was achieved by 0.8% agarose gel electrophoresis followed by ethidium bromide (0.5 µg mL-1) staining and the bands were observed on an UV transilluminator. The QIA quick PCR purification kit (Qiagen), following the manufacturer's instructions, was used to purify the products obtained by PCR and these PCR products were sequencing by Macrogen (Korea). A homology search of 16S rRNA gene sequences was performed using the NCBI Basic Local Alignments Search Tool (BLAST) using the algorithm of the Nucleotide Blast program.

As oxidation rate of *Pseudomonas marginalis* EM-6.

For detecting oxidation of As (III) to As (V), *P. marginalis*, calcifying As-resistant selected, was grown in CDM media containing 2% urea and 25 mM $CaCl_2$ (from here the name was used as a CDMU media) supplemented with 0.5 mM As_2O_3, and then incubated at 30ºC, 130 rpm for 7 days. The oxidation of As(III) to As(V) was determined from culture supernatants filtered through a sterile 0.22 µm filters size (Millipore). Arsenic species were monitored by HPLC/HG/QAAS (Campos et al. 2010).

Effect of As(III) and As(V) on *Pseudomonas marginalis* growth

P. marginalis were inoculated into CDMU media supplemented with 5 mM of As_2O_3 or 20 mM of As_2O_4, and then incubated at 30ºC, 130 rpm for 24 h. The final pH of CDMU media was adjusted to 7.2. Growth was determined by recording the CFU (colony forming unit) count at regular time interval. The curves obtained were analyzed through mathematical modelling Gompertz. Graphs and models were made using GraphPad Prism version 5.0 (GraphPad software, USA). Determination of arsenic concentration in culture medium was performed at regular time interval by high performance liquid chromatography (HPLC) coupled to arsine gaseous formation performing the detection by atomic absorption in a quartz bucket (HPLC/HG/QAAS) (Campos et al. 2010).

Urease activity and effects into biomineralization of arsenic.

The urease activity of strains was determined at regular time interval by measuring the amount of ammonia released from urea according to the phenol-hypochlorite assay method at different time intervals as described in Achal et al. (2009). One unit of urease is defined as the amount of enzyme hydrolyzing 1 µmol urea min-1. The effect of urease activity was performance by using extrapolating urease production and arsenic concentration data together.

Arsenic biomineralization in bath experiment

The model effluent was performance-using CDMU supplemented with arsenic compounds. The arsenic biomineralization studies were performed at 30ºC in a beaker containing 100 mL of CDMU supplemented with 500 ug/L of As(III) and 200 µL of over night grown of the ureolytic bacteria selected (equivalent to 10^7 CFU mL^{-1}) in CDM media. As control, was also simulated in similar way without addition of bacterial cells. The experiment was terminated in 72 h and samples were analysed for arsenic concentration by means HPLC/HG/ASS and toxicity analysed.

Scanning Electronic Microscopy (SEM), Transmission Electron Microscopy (TEM) and Energy Dispersive Spectroscopy Analysis (EDS)

From arsenic biomineralization experiment were obtained different samples monitoring by SEM, TEM and energy-dispersive X-ray spectroscopy (EDS). For SEM-EDS analyses, the samples were centrifuged and harvested cells were washed thrice with phosphate-buffered saline (PBS, pH 7.4) and layered onto polylysine-coated cover slips. Fixation was done using modified Karnovsky's fixative (2 % paraformaldehyde and 3 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4). Cells were again washed with PBS and distilled water. Fixed cells were dehydrated through a series of ethyl alcohol (30, 50, 70, 90 and 100 %) and finally layered with t-butyl alcohol for freeze-drying and sputter coating. Samples were visualized under a JEOL SEM, Model JSM 6380LV. For TEM-EDS analysed, the samples were centrifuged and harvested cells were washed with isotonic saline and then with distilled water. The pellet was re-suspended in distilled water to prepare a suspension. One drop of this suspension was placed on a copper electron microscope grid, pre-coated with a carbon

film, and air-dried overnight. Then, these grids were examined by using JEOL TEM, Model JEM-1200EX II.

Bioremediation experiment

For bioremediation experiments of contaminated water with As, a batch bioreactor was performed with 400 mL of CDMU media and 200 mL of Loa river water, contaminated with arsenic (1120 µg L^{-1} total arsenic), and ureolytic bacteria selected (equivalent to 10^7 CFU mL^{-1}). The bioreactor was operated during 96 h, at 25ºC, pH 8.2, agitation at 500 rpm and aeration 1 vvm. Samples of bioreactor were obtained at different time and analysed by HUVEC cells toxicity analyse and HPLC/HG/ASS for arsenic detection.

Analysis of toxicity in human umbilical vein endothelial cells (HUVEC)

To study the toxic effect of effluent, human umbilical vein endothelial cells (HUVEC) were isolated using 0.25 mg mL^{-1} *Clostridium histolyticum* Type II Collagenase (Boehringer, Mannheim, FRG) digestion from umbilical cord veins (HUVEC) and cultured up to confluence (González et al., 2004; Casanello et al., 2009). Experiments were performed with normal D-glucose (5 mM) or high D-glucose (25 mM) in the presence or absence (24 hours) of effluent, arsenite and arsenate. After this incubation period, cells were exposed to 1µM of the fluorescent probe 2',7'- dichlorodihydrofluorescein diacetate acetyl ester (DCF, Molecular Probes, Leiden, England) for 30 min, which is sensitive to oxidation by increasing reactive oxygen species (ROS) (La Favor et al., 2014). The fluorescence was determined using a microplate reader (Bio-Rad) at 485 nm excitation and 583 nm emission.

Analytical method for the detection of As(III) and As(V)

One milliliter supernatant was filtered through a sterile 0.22 µm filters size (Millipore). Arsenic species were detected by means of high performance liquid chromatography (HPLC) coupled to arsine gaseous formation performing the detection by atomic absortion in a quartz bucket (HPLC/HG/QAAS) (Campos et al., 2011). Quantitative determination of total arsenic was performed using an atomic absorption spectrometer (AAnalyst 100, PerkinElmer, Norwalk, CT, USA), equipped with a commercial hydride generation system (FIAS-100, PerkinElmer). A quartz cell (15 cm length, 1 cm i.d.) heated with an acetylene flame was used as an atomiser. An electrode-less arsenic lamp externally controlled by an

EDL power supply system (EDL System 2, PerkinElmer) was used as an emission source. 500 µL sample was introduced into the HG system through the automatic injection valve. Hydrochloric acid (10%) was used as the carrier to transport the sample to the T-joint manifold, where it was continuously mixed with sodium borohydride solution to produce AsH_3. The gaseous arsine was separated from the liquid waste in a gas–liquid separator and carried to the atomizer by a continuous flow of argon. The atomic absorption signals were processed using commercial AA Winlab software from PerkinElmer.

RESULTS AND DISCUSSION

A total of 9 bacterial As-resistant strains, were isolated from sediments of the Loa river (EM site), (characterized by high As concentrations, reaching up to 1200 mg mL^{-1}), based on their abilities of aerobic growth in the presence of arsenite (from 0.5 to 100mM) or arsenate (from 0.5 to 1000mM) and according to the criteria of different colony morphologies. Resistance to arsenic was defined as the ability to grow on agar containing either 7 mM As(III) or 20 mM As(V) at 30º C (Rokbani et al. 2007). EM-24, was the only strain susceptible to arsenite, while the remaining strains showed levels tolerance of As(III) to 7 to 10 mM and were classified as arsenite resistant strains. On the other hand, all strains were able to grow in the presence of at least 100 mM arsenate, being are classified as a resistant to this compound (Table 1). Additionally, all strains showed ureolytic capacity (positive urease activity), as determined by the Christensen agar test (Hammes et al., 2003) (Table 1).

Table 1. Levels of tolerance to arsenic and ureolytic activity capacity of strains isolated from the Loa River, Chile.

Strain	Tolerance levels(1)		Ureolytic activity (2)
	As(V)	As(III)	
EM-1	≥100	10	+
EM-3	≥100	7	+
EM-4	≥100	7	+
EM-6	≥100	7	+
EM-19	≥100	10	+
EM-21	≥100	10	+
EM-24	≥100	4	+
EM-29	≥100	7	+
EM-35	≥100	10	+

(1) Tolerance levels to As(III) and As(V), values expressed in mM.
(2) Urease positive reaction

The As(III)-oxidation or As(V)-reduction capability of the isolated strains were characterized (Table 2). Five out 9 As-resistant strains (55.6%) were able to oxidize As(III), 9 strains (100%) were able to reduce As(V), and 4 strains (44.4%) were able to both oxidize and reduce arsenic. In order to identified the isolate strains, the 16s rRNA sequences of all bacterial strains were compared with sequences available in GenBank using the BLAST application (Table 2). Among the 9 isolated strains, 7 were distributed into the Gammaproteobacteria class, 1 to Betaproteobacteria class and 1 to Firmicutes Phylum, and the 44.4% of the total classified in the *Pseudomonas* genus. Proteobacteria and Firmicutes phylum have been reported as dominant in highly contaminated sediments, and their functional genes indicated that these phyla could resistant high concentration of arsenite and arsenate (Heinrich-Salmeron et al., 2011; Escudero et al., 2013; Ye et al., 2017).

The presence of a genetic mechanism for arsenic resistance in strains isolated from Loa river sediments, was detected via PCR amplification of arsenic marker genes, *aio* (As(III)-

oxidation marker gene) and *arsC* (As(V)-reduction marker gene) and the results demonstrated a correlation between the presence of *aoi* or *arsC* genes and the capacity of oxidase or reduce arsenic, respectively (Table 2). However, the capacity of reduce arsenate by *Enterobacter* sp., could be codificated by others genetic mechanism.

Table 2. Bacterial strains identification, arsenic transformation and arsenic marker gene detection of strains isolated from the Loa River, Chile.

Bacterial phylogenetic group	Strain	Closest sequence relative	GenBank	As(III) -Ox (%)	As(V) -Red (%)	*aio* (1)	*arsC (2)*
γ-proteobacteria	EM-21	*Enterobacter* sp.	AY538264	100	0	+	-
	EM-35	*Klebsiella oxytoca*	CP005927	75	100	+	+
	EM-1	*Pseudomonas fluorescens*	KC172063	75	0	-	+
	EM-6	*Pseudomonas marginalis*	JX566610	100	0	+	+
	EM-24	*Pseudomonas* sp.	AJ315068	0	80	-	+
	EM-29	*Pseudomonas putida*	KJ730211	50	100	+	+
	EM-3	*Serratia* sp.	DQ232737	0	100	-	+
β-proteobacteria	EM-4	*Alcaligenes faecalis*	AF094743	90	100	+	+
Firmicutes	EM-19	*Bacillus* sp.	CP003683	0	100	-	+

The gram negative strain EM-6, identified as *Pseudomonas marginalis* and confirmed with 99% similarity at the specie level (access numbers JX566610), was selected to continue with the experiments because presented high As(III)-oxidizing and As(V)-tolerance capacities. Regarding the efficiency of the strain to oxidize arsenite to arsenate, *P. marginalis* was able to oxidize the 97% of the 0.5 mM As(III) presented in the CDM medium, after 72 hours of incubation (Figure 1). Besides, this strain possessed the *aio* gene that encoded the arsenite oxidase, explaining the high As(III)-resistance (Table 2) and the high efficiency rate of As(III)-oxidation (Figure 1). Valenzuela et al. (2009) reported the isolation of a strains *P. marginalis*, from Camarones river (Atacama desert, Chile), able to oxidize 100%

of arsenite of the medium, reporting the similar *aio* genetic mechanism for arsenic resistance. Furthermore, several microorganism isolated from As-rich environments including mine, arsenical pesticide- or smelter- impacted sites and geothermal sites, have been described able to tolerance high concentration of As(III), including chemolithoautotrophic and heterotrophic microorganisms such as, *Acidiphilium acidophilum*, *Acidithiobacillus ferrooxidans*, *Cenibacterium arsenoxidans*, *Alcaligenes faecalis*, *Cupriavidus necator*, *Hydrogenophaga* and some *Pseudomonas* spp. (Campos et al. 2010; Lia et al. 2012; Quéméneur et al., 2010; Heinrich-Salmeron et al., 2011; Escudero et al., 2013; Engel et al., 2013; Ye et al., 2017).

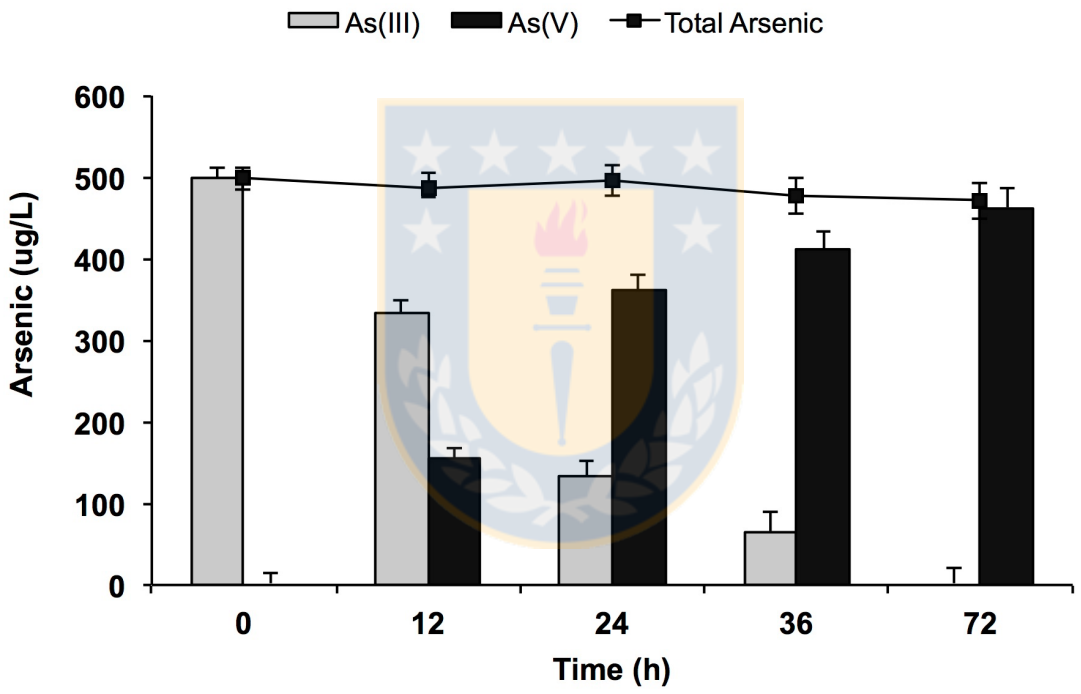

Figure 1. Arsenite oxidation rate of *Pseudomonas marginalis* EM-6 in a chemically defined medium supplemented with 0.5 mM As(III) after 72 hours of incubation.

In order to determine the effect of arsenic concentrations on the growth *P. marginalis,* Growth kinetics (k) were perfomance of ureolytic strains *P. marginalis* EM-6, after 24h of incubation in the presence of 5 mM As(III) or 20 mM As(V) was modeled using the Gompertz equation and compared with a control without As. Where, the k value was 0.07802 in the

presence of 5mM As(III) and 0.07917 in the presence of 20mM As(V), not significantly different when compared to the control showing a k value of 0.08137 ($p>0,05$) (Figure 2).
The similar growth kinetics of *P. marginalis* EM-6 in CDMU media, both with and without arsenic compounds (5 mM As_2O_3 or 20 mM As_2O_4), seem to indicate an adequate adaptation of the strain to a medium containing arsenite, despite the high toxicity induced in the medium. This adaptation could be facilitated by the fact that the strain presented *aoi* and *arsC* genes involved in As-transformation such as As(III)-oxidation and As(V)-reduction that are mechanism to tolerate arsenic (Andres and Bertin, 2016).

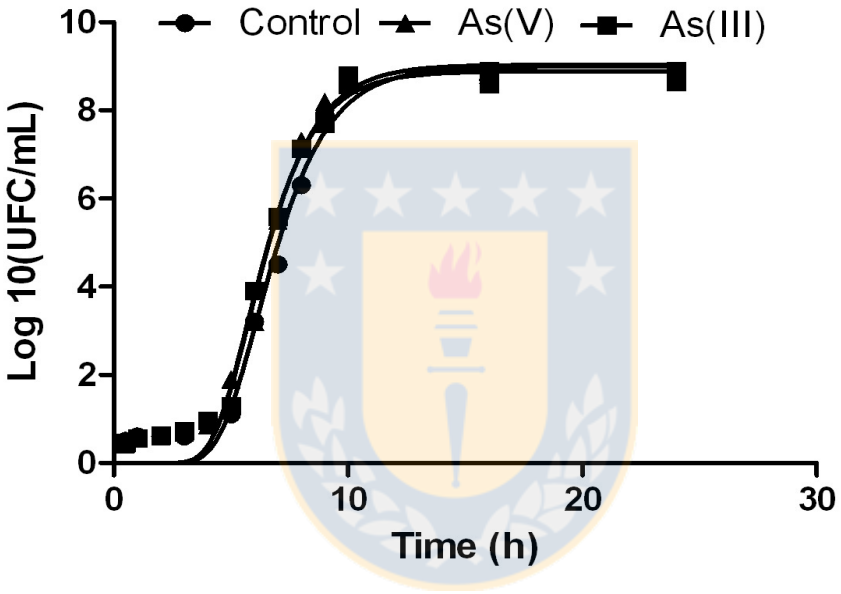

Figure 2. Growth of *P. marginalis* in CDMU media supplemented with 5 mM As_2O_3 or 20 mM As_2O_4, incubated at 30ºC, 130 rpm for 24 h. The final pH of CDMU media was adjusted to 7.2.

For determinate of effects of arsenic on ureasa activity, *P. marginalis* EM-6 was grow in CDMU (Chemical defined Medium plus Urea), in the presence or absence of arsenite and arsenate, and ureolytic capacity was evaluated according to that described by Achal et al. (2009). Results demonstrated that *P. marginalis* EM-6 was able to produced significant amount of urease in CDMU in presence of 5 mM mM of arsenite (389 UmL^{-1}) and 20 mM of arsenate (375 UmL^{-1}), after 72 h of incubation and the urea production was stable up to 120 h of incubation (Fig. 3). Similar results were reported by Achal et al. (2012). Where,

Sporosarcina ginsengisoli CR5, produced significant amount of urease (between 373 U mL $^{-1}$ and 412 U mL^{-1} urease) in NBU media amended with 50 mM As(III). The results were supported by previous studies (Achal et al., 2009; Achal et al., 2011). Urease is a key enzyme that leads to calcite precipitation and has been reported to produce significantly in the media containing urea and calcium source (Stocks Fischer et al., 1999; Achal et al., 2009; Achal et al., 2010). Ureolytic bacteria, such as *Sporosarcina pasteurii* and *Bacillus megaterium,* couple calcification to their metabolic assimilation processes to scavenge protons (McConnaughey and Whelan, 1997). Urease hydrolyses the substrate urea generating ammonia and carbamate. Carbamate spontaneously decomposes to produce another molecule of ammonia and carbonic acid (Mobley & Hausinger, 1989). The two ammonia molecules and carbonic acid subsequently equilibrate in water with their deprotonated and protonated forms, resulting in an increase in the pH. The presence of ammonium ions and the additional release of CO_2 into the surrounding medium increase the pH that accelerates the rate of the urease induced calcite precipitation.

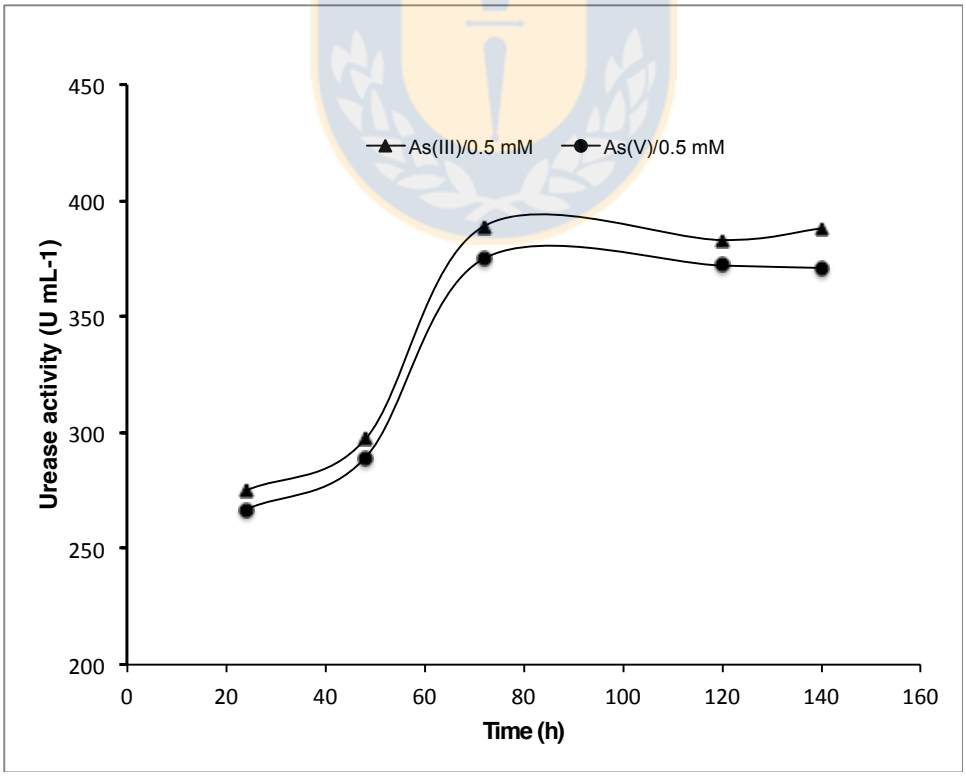

Figure 3. Urease productions by *P. marginalis* in QMD media supplemented with 0.5 mM As(III) and As(V).

The As(III) and As(V) removal means by microbiologically induced biomineralization, using *P. marginalis* EM-6, was evaluated by HPLC/HG/ASS. The results showed that *P. marginalis* EM-6 was able to remove 100% of As(V) and As(III) present in the culture medium CDMU, after 72 h of incubation, using a model effluent contaminated with both metalloid species (Figure 4). Controls incubated in a mineral medium without urea transformed 92% of As(III) into As(V) after 72 h at 25ºC (Figure 1). Control without bacteria not shown biomineralization and transformation of arsenic in the medium. In addition, the balance of mass showing the presence of As(V), due As(III) bio-oxidation, and As(V) were absorb onto its surface and incorporated both arsenic species into its crystal structure of calcite crystal biogenic.

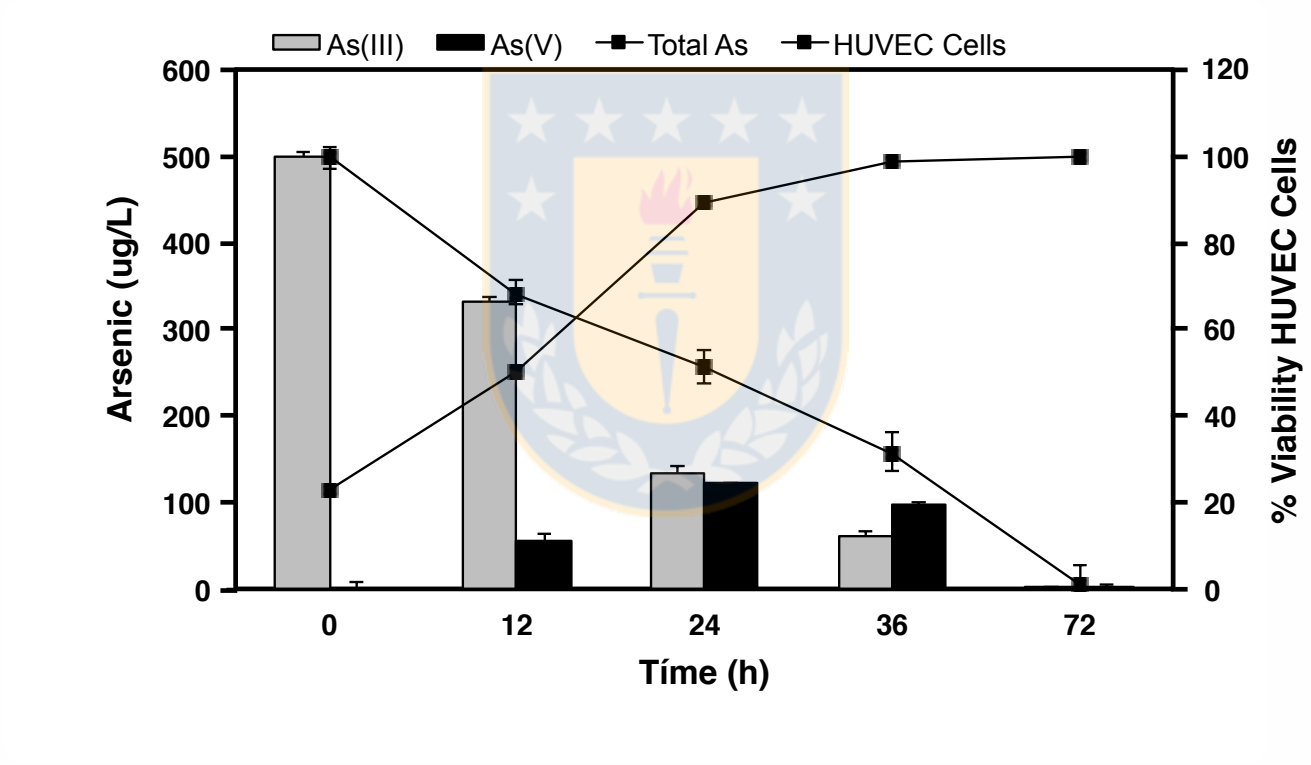

Figure 4. Removal of inorganic arsenic by *Pseudomonas marginalis* EM-6 in CDMU media, after 72 h of incubation.

The capacity of *P. marginalis* EM-6 strain to produce calcite crystals biogenic at pH 7.2 was demonstrated using a scanning electron microscope (SEM) associated to energy-dispersive X-ray spectroscopy (SEM-EDS). The results showed the presence of rhombohedral crystals, characteristic of calcite (Figure 5a-5c). In addition, EDS analyses demonstrated that crystals corresponded to precipitate biogenic calcite associated to As inorganic (Figure 5d and 5e). The results suggested that calcite crystal precipitation could occur as a product of urea hydrolysis. These processes increase the alkalinity, dissolving inorganic carbon present in the environment favouring $CaCO_3$ precipitation. In addition, the negative charge and functional groups of the cell wall facilitates the fixation of As, by means the integration into the calcite structure via substitution of calcium ions in the microenvironment of the mineral precipitate, forming low - strontium carbonate minerals which have very low solubility (Fujita et al., 2000; Smith et al., 2004; Colwell et al., 2005; Mitchell and Ferris, 2006; Warren et al., 2001). The formations of calcite crystals were not observed in the negative controls (Figure 5f and 5g).

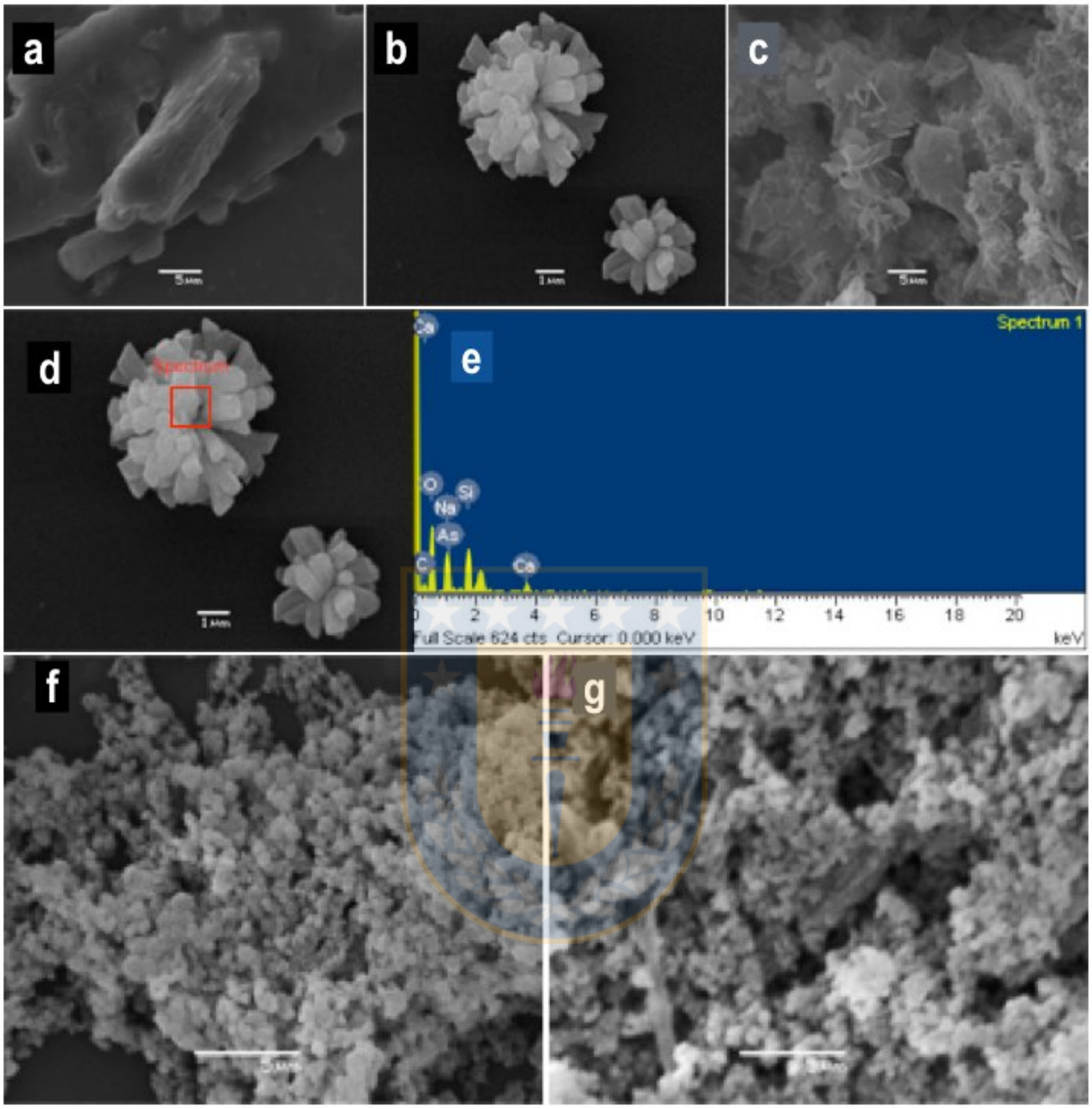

Figure 5. SEM and EDS of $CaCO_3$ crystals obtained from the culture of *P. marginalis* EM-6 strain in CDMU media in the presence or absence of arsenic for 72 h at 30°C. a) rhombohedral calcite crystals without grouping. b) rhombohedral calcite crystals grouping in star. c) poli-rhombohedral calcite crystals. d-e)Detection of arsenic particles incorporated into calcite crystal structure analyzed by EDS. f) Negative control of CDMU media cultured in absence of bacterial inocula for 72 h. g) Negative control of CDMU plus As and cultured in absence of bacterial inocula for 72 h.

Similar results were obtained by TEM, showing sub-micrometric crystals (≤1 um) nucleation, with morphology diamondhedral (Figure 6b, 6d and 6e), corresponding to calcite crystals, according to that described by Guo et al. (2003) and De Söllner et al. (2003). On the other hand, the negative control (without bacteria strain), crystal formation was not detected after 72 h of incubations (Figure 6a). In addition, the structure of crystals was analyses by STEM-HAADF (Figure 6c) and SAED detection (Figure 6e and 6f), demonstrating that the presence of the structure of the calcite crystal.

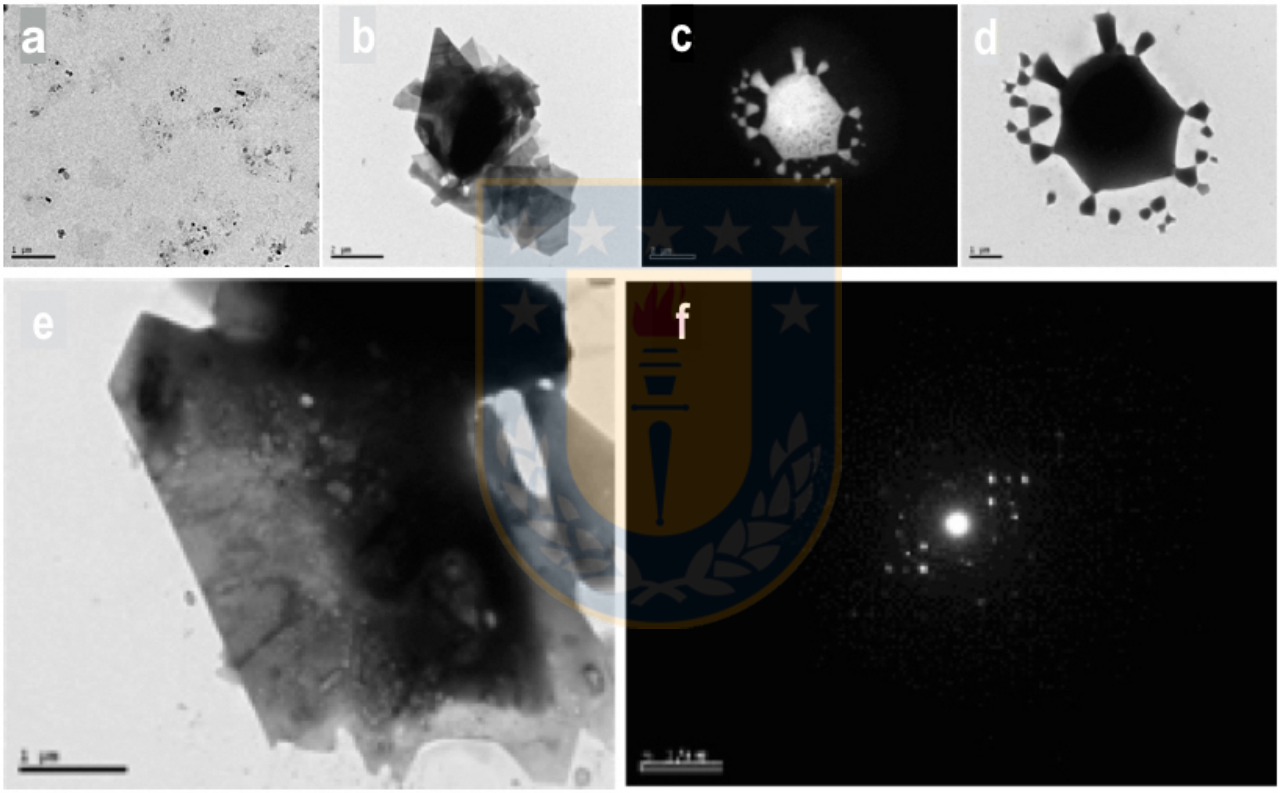

Figure 6. TEM of CaCO3 crystals obtained from the culture of *P. marginalis* EM-6 strain in CDMU media in the presence of arsenic for 72 h at 30°C. a) negative control of CDMU plus As and cultured in absence of bacterial inocula for 72 h. b) TEM of diamond hedral calcite crystals shape. c) STEM-HAADF of rhombohedral calcite crystals. d) TEM of rhombohedral calcite crystals. e-f) SAED detection of poli-rhombohedral calcite crystals.

In addition, the capacity of toxicity removal, by arsenic biomineralization based on microbially induced calcite precipitation (MICP), was evaluated by means HUVECs assays. The results showed that the toxicity was decreasing concomitantly with the decreasing of As(III) and As(V) concentration in the culture medium, product of biomineralization of both arsenic species, after of 72 h of incubation (Fig 4). In addition, in order to analyse the relationships among percentage of toxicity and the presence of As(III) and As(V), a Pearson correlation analysis was done, showing a positive relationship between toxicity and As(III) concentration (Data not shown).

In order to evaluated the efficiency of removal As(III) and As(V) from environmental samples (water samples of Loa river) obtained by means of microbiologically induced biomineralization by using *Pseudomonas marginalis* EM-6, a batch reactor was performance. The removal capacity of arsenic species, from batch reactor was evaluated by HPLC/HG/ASS, demonstrating that *P. marginalis* EM-6 after 48 h of incubation at 30º C and aerobic conditions, was able to removal 100 % of arsenic present in the Loa river water (Fig 7). These results show that there is an increase in arsenic removal rate, which may be due to the presence of calcium carbonate in the water sample of the river ($CaCO_3$ =297.3 mg/L), product of mining activity in the sector, increasing the efficiency of precipitation of arsenic in the biological batch system. In addition, SEM-EDS analyses demonstrated the presence of calcite polymorphous crystal; including arsenic in the crystalline structure (Fig 8), this crystalline structure, associated to As, was similar to obtained using synthetic model effluent.

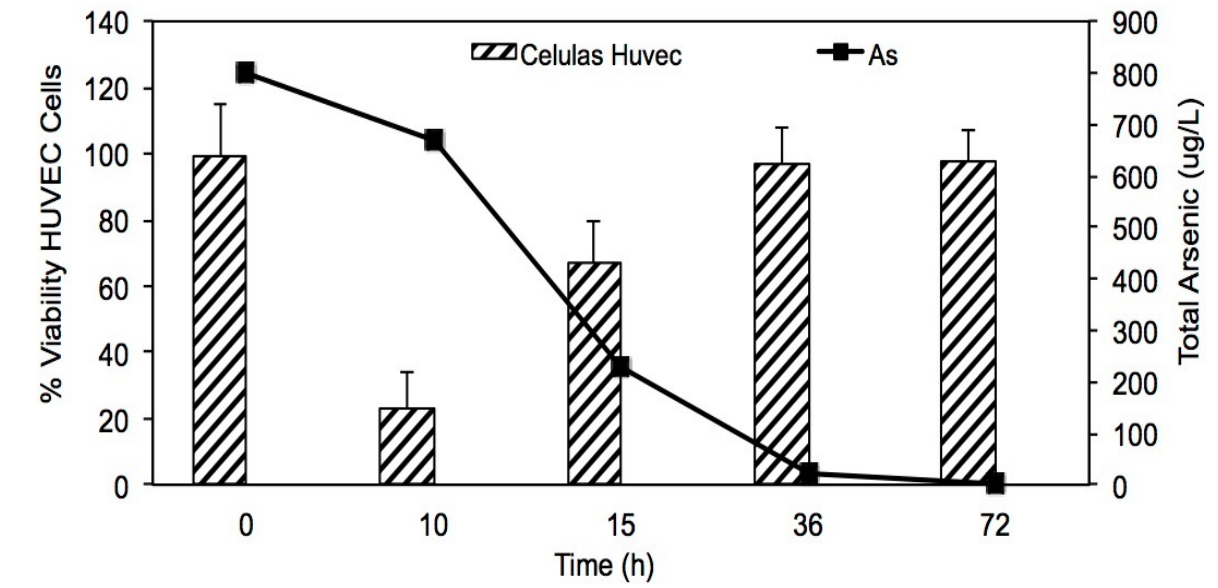

Figure 7. Removal of inorganic arsenic by *Pseudomonas marginalis* EM-6 in batch reactor, after 72 h of incubation.

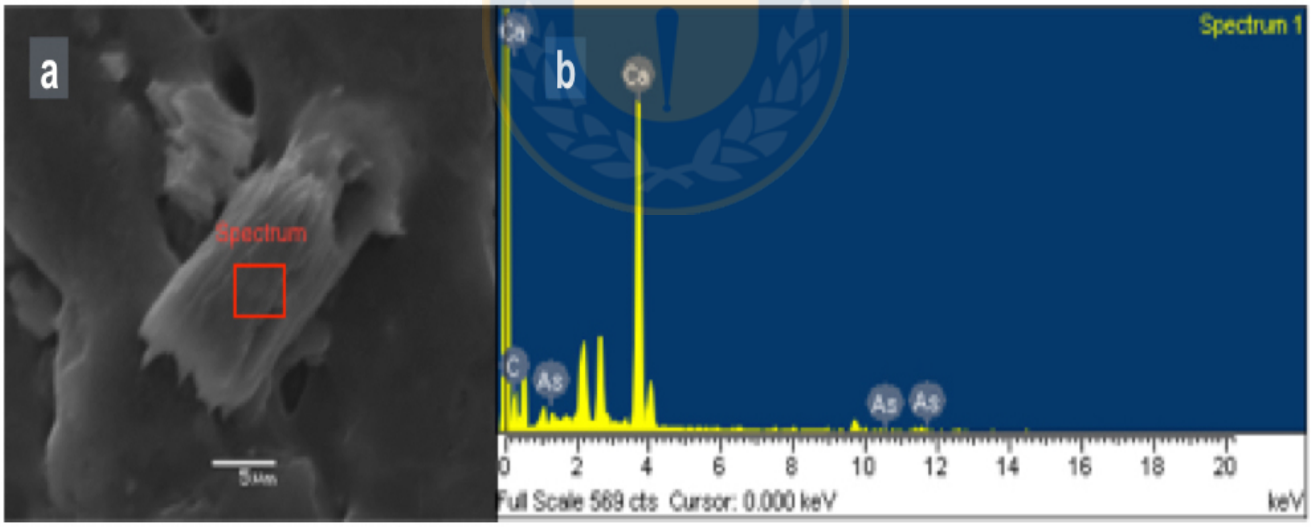

Figure 8. SEM and EDS of $CaCO_3$ crystals obtained from the culture of *P. marginalis* EM-6 strain in CDMU media in the presence or absence of arsenic for 72 h at 30°C. a) rhombohedral calcite crystals. b) Detection of arsenic particles incorporated into calcite crystal structure analysed by EDS.

For evaluate the capacity of removal arsenic-toxicity of batch system, HUVECs assays were performance, demonstrated that the toxicity was decreasing concomitantly with decreasing arsenic concentration in the batch system. Several studies have used urea to promote biogenic $CaCO_3$ precipitation as a remediation strategy. Hammes (2003) used bacterial $CaCO_3$ precipitation to remediate Ca(II)-rich industrial wastewater. Calcium rich wastewater is often a result of bone processing, citric acid production, paper recycling, and landfill leachates (Hammes, 2003). By introducing urea into a reactor system containing a sludge with bacteria present, over 80% of the Ca(II) in the wastewater solution was removed by CaCO3 precipitation (Hammes, 2003):

Most of the studies to remediate heavy metals such as Sr and Pb by MICP have been performed in groundwater. Calcite has been implicated as playing a possible role in the retention and solubility of arsenic in soils and various other environments in the presence of carbonates. Microbial induced biominerals by *Pseudomonas marginalis* EM-6 is a potential alternative for implementation of treatment system, for removal arsenic from water, due to both the efficacy of in situ Biomineralization for sequestration of arsenic as well favourable environmental impacts and low cost.

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CAPÍTULO VIII: DISCUSIÓN GENERAL

La región norte de Chile, especialmente el área del Desierto de Atacama, ha sido descrita como un ambiente naturalmente rico en arsénico. Los minerales de sulfuro metálicos que contienen arsénico se disuelven en la Cordillera de los Andes, afectando las aguas superficiales y subterráneas que atraviesan el desierto de Atacama, que se utilizan como fuentes de agua potable (Yañez y col., 2005; Arriaza y col., 2010; Campos y col., 2011).

Los habitantes de los pueblos, Camarones e Illapata (Atacama, Chile) utilizan principalmente agua del río Camarones (Capítulo IV), tanto para el consumo humano como para las actividades agrícolas. Las aguas del río Camarones contienen una concentración de As total superior a 1000 µg L^{-1}, principalmente en forma de arseniato (As(V)) y esta contaminación ha afectado crónicamente a las poblaciones rurales que viven cerca del río (Steely y col., 2007). Por otra parte, el río Loa nace de una zona volcánica activa en la cordillera de los Andes, a una altitud de más de 4000 metros sobre el nivel del mar. Se extiende por aproximadamente 440 km a lo largo del Desierto de Atacama recibiendo aguas de varios afluentes (principalmente los ríos, San Pedro, Salado y San Salvador) antes de llegar al Océano (Capítulo V). Este rio que proporciona agua para usos domésticos, industriales y de riego en esta árida región, posee condiciones únicas como: clima desértico extremo, altas tasas de evaporación, alta salinidad, aguas neutras a alcalinas, altas concentraciones de Boro y algunas de las concentraciones más altas de arsénico (de 200 a 4400 µg L^{-1} As) en el mundo (Smedley y Kinniburgh, 2002), las cuales se atribuyen a dos fuentes: I) naturales, asociadas a sus afluentes (e.g., río Salado que está enriquecido por las aguas de los manantiales geotérmicos de El Tatio, con niveles de hasta 27 mg As). II) contaminación humana que involucra, desechos mineros (mina de Chuquicamata), emisiones de la fundición de cobre y efluentes enriquecidos con As provenientes de las plantas de tratamiento de aguas (Planta Cerro Topater, desde el año 1978 al año 2000) (Arroyo y col., 1999; Romero y col., 2003; Bugueño y col., 2014).

En este estudio, las muestras de sedimentos recolectadas en el río Camarones (M1, M2 y M3) mostraron una alta concentración de arsénico total (de 498 a 128 mg kg^{-1}), que

disminuyó a medida que el río se acercaba al Océano Pacífico (Capítulo IV). Esta disminución de concentración de As, se atribuyo a la presencia de diversas cascadas y aguas subterráneas ubicadas antes de la zona inferior del río (M3), que permitieron su disolución (Yañez y col., 2005). Otros autores reportaron niveles similares de arsénico total para este mismo río (Yañez y col., 2005; Yañez y col., 2015). En cuanto a los valores de materia orgánica total, se detectaron bajas concentraciones en M1 y M2, lo cual puede ser producto de las pendientes y al patrón de flujo rápido del río Camarones en estos sitios, arrastrando la materia orgánica. Por otra parte, M1 y M2 presentaron las mayores concentraciones de metales pesados (Cu, Cd, Zn y Pb), atribuidas a las características litogénicas del río.

Los sedimentos estudiados en el río Loa (Capítulo V), presentaron altas concentración de arsénico total (de 326 a 865 mg kg^{-1}), el cual aumentó aguas abajo del tranque Sloman, atribuido a varios factores: I) clima desértico extremo que favorece altas tasas de evaporación y alta salinidad, y la falta de afluentes superficiales y contribuciones de agua subterránea que impiden su dilución. II) el pH neutro a alcalino del agua impide la absorción de As en las fases sólidas (Romero y col., 2003; Bugueño y col., 2014). III) concentraciones extremadamente elevadas de As en aguas del embalse Sloman se han reportado durante inundaciones, con gran impacto en los ecosistemas fluvial y marino aguas abajo (L3, correspondiente al oasis Quillagua) (Arroyo y col., 1999). IV) las emisiones de la planta de fundición de cobre en Chuquicamata; los residuos de la mina depositados en el pasado en el rio; el tratamiento inadecuado de los efluentes de las actividades mineras, así como los efluentes ricos en As de la planta de tratamiento de agua de Calama, representan otras fuentes potenciales de As (Romero y col., 2003). Por otra parte, se detectaron altos valores de materia orgánica total en los sitios L2 y L3 atribuidos a la presencia de productores primarios tales como, especies de algas (*Cladophora* sp. and *Chara* sp.) y plantas acuáticas (*Azolla* sp., *Myriophyllum aquaticum*, *Phylloscirpus* cf. *desserticola*, *Potamogeton pectinatus*, *Ruppia filifolia* and *Zannichellia palustris*), que a su vez facilitarían la acumulación de arsénico en esos sitios, por ser descritos por Pell y col. (2013), como especies acumuladoras o hiperacumuladoras de arsénico presentes en el rio Loa.

Por otra parte, se ha descrito que sedimentos contaminados con metales pesados están habitados por comunidades microbianas extremadamente complejas y bien adaptadas, que desempeñan un papel fundamental en la degradación de la materia orgánica y en los ciclos biogeoquímicos (Gillan y col., 2014; Luo y col., 2014; Costa y col., 2015). La diversidad microbiana, fue investigada por secuenciación masiva (Illumina), la cual reveló una gran diversidad de comunidades bacterianas, detectando también bacterias que se presentan en muy baja abundancia (≤0.01%), que habrían sido enmascaradas por poblaciones dominantes si se hubieran aplicado técnicas con menor resolución.

Según la evaluación de los índices de diversidad, las comunidades bacterianas asociadas a sedimentos del río Camarones (Capítulo IV) tuvieron una diversidad moderada, que aumentó ligeramente a través del río, de los sitios M1 a M3 (de 27 a 31 Phylum), según el aumento de los niveles de TOC y la disminución concomitante de las concentraciones de arsénico. Como es generalmente aceptado, condiciones de perturbación moderadas a menudo dan como resultado una alta diversidad de comunidades microbianas, como las que se producen en la desembocadura del río Camarones (sitio M3). Por otra parte, las comunidades bacterianas asociadas a sedimentos del rio Loa (Capítulo V), poseen una diversidad mayor que las del río Camarones. Sin embargo, a diferencia del rio Camarones, su distribución se ve influenciada por la presencia del tranque Sloman, que favorece una variación importante de los parámetros físicos-químicos, tales como materia orgánica, tamaño de partícula, potencial redox, concentración de Fe y SO_4, sólidos disueltos totales, temperatura, salinidad, conductividad, oxígeno disuelto, pH y concentraciones de arsénico, entre los sedimentos ubicados aguas arriba (sitio L1) o aguas abajo del tranque Sloman (sitios L2 y L3). Presentando, un porcentaje similitud de un 93% entre las comunidades bacterianas en los sedimentos L2 y L3, mientras que L1 no agrupo con ellos, lo que concuerda con la falta de recursos hídricos o afloramientos minerales, que podrían causar cambios en los parámetros físicos-químicos de los sitios L2 y L3, situados aguas abajo de L1.

Las secuencias de los grupos taxonómicos dominantes (abundancia ≥1%) en las muestras de sedimento del rio Camarones afiliaron con los Phylum: *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* y

Nitrospirae (Capítulo IV). Sin embargo, una afiliación diferente se detecto para las secuencias provenientes de las muestras del rio Loa (Capítulo V), las cuales fueron clasificadas dentro de los Phylum, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, *Lentisphaerae*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia y Chlorobi*, principalmente. Según la disponibilidad de carbono, la mayor abundancia de *Proteobacteria*, *Bacteroidetes* y *Actinobacteria* phylum puede deberse a la naturaleza copiotrófica de estos grupos, que se desarrollan en condiciones de elevada disponibilidad de C y exhiben tasas de crecimiento relativamente rápidas (Shange y col., 2012, Fierer y col., 2007; Eilers y col., 2010, Fierer y col., 2012). Por otra parte, miembros de los Phylum, *Actinobacteria*, *Firmicutes*, *Gemmatimonadetes* y *Nitrospirae* han sido reportados como dominantes en suelos altamente contaminados por arsénico y en su mayoría presentan genes funcionales para resistir altas concentración de arsénico (Luo y col., 2014; Costa y col., 2015; Zhai y col., 2017). Bacterias pertenecientes a los grupos *Firmicutes*, *Bacteroidetes* y *Planctomycetes* han sido descritas también por formar biofilms, siendo capaces de colonizar una gran variedad de ecosistemas acuáticos y terrestres, incluyendo varios ambientes extremos y ricos en As (Zhu y col., 2015, Costa y col., 2015, Lage y col., 2014; Fernandez y col., 2016). Por ultimo, miembros de los grupos *Chloroflexi*, *Chlorobi*, *Verrucomicrobia*, *Lentisphaerae* y *Spirochaetes* han sido descritos como bacterias reductoras de sulfatos, mediadoras claves del ciclo anaeróbico del carbono en los sedimentos (Limam y col., 2010; Shivani y col., 2015; Spring y col., 2016; Volant y col., 2014; Yan y col., 2015). Lo cual concuerda con la alta presencia de minerales de sulfuro, que contienen arsénico descritos para la zona (Yañez y col., 2005; Arriaza y col., 2010; Campos y col., 2011).

Las subclases: *Deltaproteobacteria*, *Alphaproteobacteria* y *Gammaproteobacteria*, dominaron en todos los sedimentos (Capítulos IV y V). Sin embargo, una mayor diversidad se encontró en *Deltaproteobacteria*, principalmente representados por miembros anaerobios involucrados en la reducción de metales y en particular en la reducción del arseniato a través de la vía de desintoxicación codificada por el operon *ars* (Li y col., 2014). Resultados similares fueron reportados por Farías y col. (2014) y Escudero y col. (2013), para comunidades bacterianas asociadas a muestras de sedimentos ricos en arsénico de salares y ríos, en la region norte de Chile.

El estudio de genes funcionales envueltos en las reacciones redox del arsénico se basan en gran medida en el aislamiento y caracterización de bacterias resistentes a arsénico. En particular, las bacterias pueden transformar el arsénico a través de reacciones de reducción, oxidación y respiración. Las reacciones de reducción implican la presencia del operón *ars* que consta de tres genes (*arsRBC*) o cinco genes (*arsRDABC*). Además de la reducción, las bacterias también pueden oxidar el As(III) a As(V) como un mecanismo de desintoxicación, ya que el As(V) es 100 veces menos tóxico que el As(III). Los genes de oxidación *aioA* y *aioB* han sido identificados en diferentes especies de bacterias. Para la respiración de As las bacterias utilizan la reducción del As(V) en la cadena respiratoria anaerobia. La proteína reductasa tiene dos subunidades codificadas en los genes *arrA* y *arrB*. Por otra parte, se han encontrado variantes adicionales del gen *arr* en la cepa bacteriana *Alkalilimnicola ehrlichii* MLHE-1, que se denominan genes *arx* .El operon en esta bacteria incluye *arxE*, *arxD*, *arxC*, *arxB*, *arxA* y los genes reguladores *arxS* y *arxR* que realizan la oxidación anaeróbica del As(III) (Andres y Bertin, 2016). Sin embargo, como se ha comentado anteriormente, los métodos cultivo-dependientes podrían no ser tan sensibles en la detección de la mayor parte de las bacterias resistentes al arsénico en las comunidades bacterianas, por lo cual en este estudio se propone un enfoque metagenómico independiente de cultivo, para descubrir como se distribuyen los genes funcionales envueltos en las reacciones redox del arsénico, en las comunidades microbianas resistentes al arsénico presentes en muestras de sedimento del rio Camarones (M1, M2 y M3) y del rio Loa (L1, L2 y L3), contaminado por el metaloide. Para lo cual, se usaron secuencias conocidas de genes *ars*, *arr* y *aio* descritas por PCR y secuencias disponibles en la base de datos NCBI, para construir una base de datos personalizadas, para su posterior análisis utilizando el software Picrust, el cual asigno estos genes funcionales a secuencias del gen ARNr 16S basandose en las clasificaciones taxonómicas disponibles en la base de datos del Centro Nacional para la Información Biotecnológica (NCBI). Los resultados de BLAST de nuestra base de datos personalizada del genes *ars* contra las secuencias obtenidas por secuenciación-Illumina se resumen en el Anexo I (Tabla I). Las muestras de ambos ríos presentaron secuencias similares a los genes involucrados en la reducción de As(V), *arsA*, *arsB*, *arsC*, *arsR* y *arsH*. Los sedimentos provenientes del rio Loa muestran una mayor diversidad a nivel de genero (11 géneros) de bacterias As(V)-

reductoras, que los sedimentos del rio Camarones (9 géneros) y una mayor tasa de coincidencias o abundancia. Por otra parte, tanto los géneros del rio Camarones, como del rio Loa, afiliaron con los Phylum: *Proteobacteria*, *Firmicutes y Actinobacteria*, lo cual concuerda con los grupos taxonómicos dominantes en las muestras de ambos ríos, reportados en los Capítulos IV y V.

En el caso de los genes *arr* involucrados en respiración de As(V), se observó un número de coincidencias total mayor en el río Loa. Por otra parte, los genes asociados a la oxidación anaeróbica del As(III) *arx*, presentaron una alta abundancia de secuencias homologas a las secuencias de genes del operon *arx* del genero *Alkalilimnicola* en ambos ríos (Anexo I, Tabla II). Lo que podría indicar una mayor abundancia bacterias As(III) oxidantes en estos ríos.

Por ultimo, secuencias homologas a los genes *aioB* y *aioA* se encontraron en ambos ríos (Anexo I, Tabla III). Para la búsqueda utilizamos genes conocidos disponibles en NCBI de las especies bacterianas, *Roseobacter litoralis* (Koechler y col., 2010), *Thiomonas arsenivorans* cepa DSM-16361 (Kelly y col., 2007) y *Pseudomonas arsenicoxydans* (Campos y col., 2010), los cuales han sido aisladas desde ambientes contaminados con arsénico y que presentan altos niveles de tolerancia a As(III). Cabe señalar, que *P. arsenicooxydans* es una especie única reportada sólo en el desierto de Atacama y contiene una variante única del gen *aio* (Campos y col., 2010). El gen *aioB* de *P. arsenicoxydans* se presentó en ambos ríos, lo que indicaría que esta bacteria forma parte de los ecosistemas acuáticos del desierto de Atacama y que presentaría una alta capacidad genética de resistencia al arsénico.

Con el objetivo de aislar y caracterizar bacterias calcificantes oxidantes desde sedimentos de ríos enriquecidos con compuestos de As, capaces de biomineralizar As(V), con vista a su potencial utilización en sistemas de tratamientos biológicos MICP. Un total de 189 cepas bacterianas fueron aisladas, 149 cepas desde el rio Camarones cepas y 40 cepas bacterianas desde el rio Loa. 143 cepas bacterianas fueron capaces de hidrolizar urea, por medio de la enzima ureasa liberando amoníaco y dióxido de carbono. Por otra parte, en los caldos de cultivo, se detecto un precipitado que se atribuyo a la presencia de calcita, el cual

mediante ensayos preliminares por microscopía óptica se detectaron la presencia de cristales (Anexo I, Figura 1). En los Capítulos IV y VII, se muestran la identificación las cepas ureolíticas aisladas desde el rio Camarones y el rio Loa, respectivamente. De las bacterias ureolíticas identificadas, se estudiaron los niveles de resistencia a As(III) y As(V). La determinación de la resistencia a arsénico se realizó mediante la técnica de dilución seriada en placa (NCCL, 1992). La resistencia a arsénico fue evaluada de acuerdo a la habilidad de crecer en agar, de acuerdo a lo descrito por Rokbani y col. (2007). Los resultados demostraron que 17 cepas bacterianas ureolíticas, aisladas desde el Rio Camarones fueron capaces de resistir concentraciones superiores a 7 mM de As(III) y 29 cepas fueron resistentes a As(V), donde los niveles de resistencia fueron mayores a 20 mM, de acuerdo a lo descrito por Rokbani y col. (2007). Para el rio Loa, los resultados demostraron que una cepa de nueve fue susceptible a arsenito y las 8 cepas bacterianas restantes fueron capaces de tolerar As(III) y As(V) en rangos de 7 a 10 mM y 100 mM, respectivamente. En respuesta a concentraciones tóxicas de metales pesados, muchos organismos acuáticos, incluyendo bacterias, pueden desarrollar mecanismos de resistencia (Oremland y Stolz, 2005; Yamamura y col., 2009; Macur y col., 2004; Stolz y col., 2006). Algunas bacterias, que representan diferentes grupos filogenéticos involucrados en la transformación de As usan procesos como: reducción y oxidación para resistir el arsénico, cuyas vías metabólicas se encuentran codificadas por los genes *arsCBAR* y genes *aio* respectivamente (Paez-Espino y col., 2009; Yamamura y Amachi, 2014)

Se estudiaron los mecanismos genéticos responsables de la resistencia a arsénico (Capítulos IV, VI y VII), detectando que las cepas ureolíticas aisladas desde el rio Camarones, poseían el gen *arsC*, correspondiente a la arsenato reductasa, responsable de la reducción de As(V) a As(III). Solo *Pseudomonas arsenicoxydans* no presentó dicho gen, lo que concuerda con los resultados de reducción química del metaloide. Por otra, para el gen *aoi* que codifica la arsenito oxidasa, responsable de la oxidación de As(III) a As(V), los resultados fueron variados, detectándose el gen principalmente las cepas del genero *Pseudomonas*. Resultados similares fueron obtenidos en las cepas caracterizadas desde el rio Loa, donde se detecto que sobre el 89% presentaron el gen *arsC* y solo el 56% el gen *aoi*. (Capítulo IV y Capítulo VII). La oxidación de arsenito y la reducción de arseniato, bajo condiciones aeróbicas, ha sido reportada como un mecanismo de detoxificación en varias

bacterias aeróbicas aisladas desde diferentes sectores contaminados con el metaloide, sugiriendo que la biotransformación de arsénico, juega un rol importante en los ciclo biogeoquímico de este elemento en el ambiente (Li y col., 2014; Ye y col., 2017). Por otro lado, la capacidad de oxidar y/o reducir arsénico, que presentan las bacterias aisladas desde ambos ríos presentan un gran potencial biotecnológico, para la implementación de sistemas de tratamientos de aguas contaminadas con el metaloide.

Con el objetivo de evaluar las capacidades bacterianas oxidantes y reductoras de arsénico (Capítulos IV, VI y VII), las cepas seleccionadas fueron cultivadas en medio R2A enriquecido con As(III) y/o As(V) a una concentración de 0.5 mM por un periodo 48 horas. La detección de la oxidación y reducción de As fue realizada mediante HPLC/HG/QAAS. Para las muestras del rio Loa, se obtuvo que las bacterias que poseían en el gen *arsC* fueron capaces de reducir As(V) en porcentajes entre un 80% y 100% y las cepas que poseían el gen *aio* fueron capaces de oxidar As(III), entre un 50% y 100%. Por otra parte, las bacterias seleccionadas desde el rio Camarones en su mayoría (98%) fueron capaces de reducir As(V), excepto *P. arsenicoxydans* que presento un 0% de reducción. Sin embargo, *P. arsenicoxydans* presento una alta capacidad de oxidar As(III) (100%). Por lo anterior, esta cepa fue seleccionada y caracterizada, obteniendo resultados interesantes desde el punto de vista biotecnológico en la remoción biológica del As(III), ya que su eficiencia aumento cuando fue inmovilizada en zeolita (Capítulo VI). Por otra parte, 17 cepas bacterianas que poseían el gen *aio* fueron capaces de oxidar As(III), con porcentaje entre un 75% y 100%, confirmando los datos previamente reportados para la misma zona de muestreo, en donde se destacan especies indigenas presentes en el rio Camarones con altas capacidades de oxidar el As(III) (Valenzuela et al., 2009; Escalante et al. 2009).

Se seleccionaron 2 cepas bacterianas resistentes a As(III) y As(V), oxidantes de As(III); *Pseudomonas putida* VC-65, aisladas desde rio Camarones y *Pseudomonas marginalis* EM-6, aisladas desde el rio Loa (Capítulos IV y VII). En la Figura 2 (Anexo I), se muestra el modelamiento de la curva (Gomperz) de la cepa *Pseudomonas putida* VC-65, en la cual se observó un efecto negativo en el crecimiento, en presencia de As(III) y As(V), detectando velocidades de crecimiento (*k*) cercanas a 0,007946 y 0,007680, respectivamente. Estas velocidades de crecimiento fueron menores a las estimadas para el control, el cual presentó

una velocidad de crecimiento (*k*) de 0,008077. Por otra parte, para *Pseudomonas marginalis* EM-6, la cepa aislada desde sedimentos del rio Loa, se demostró que la presencia tanto de As(III) como As(V) no inhibía el crecimiento bacteriano, presentando una velocidad de crecimiento (*k*) para As(III) y As(V) de 0,07802 y 0,07917, respectivamente. Estas velocidades de crecimiento fueron similares ($p>0.05$) a las estimadas para el control (0,08137) (Capítulo VII). Estos resultados, demuestran una adecuada adaptación de la cepa a un medio que contiene arsénico, a pesar de la alta toxicidad inducida en el medio. Esta adaptación podría ser atribuida a que la cepa posee ambos genes implicados en la resistencia a As: genes *aio* y genes *arsC*. Cuya co-presencia podría conferir mayor resistencia al arsénico (Andres y Bertin, 2016). Estos resultados sugirieron que *Pseudomonas marginalis* EM-6 era una buena candidata para ser seleccionada para estudiar el proceso de biorremediación de As(III) mediante el proceso de biomineralización (MICP).

La remoción de As (III) y As (V) mediante biomineralización inducida microbiológicamente, utilizando *P. marginalis* EM-6, se evaluó por HPLC/HG/ASS (Capítulo VII). Los resultados demostraron que *P. marginalis* EM-6 fue capaz de remover el 100% de As (V) y As (III) presente en el medio de cultivo CDMU, después de 72 h de incubación, utilizando un modelo de efluente contaminado con ambas especies metaloides. Los controles, incubados en un medio mineral sin urea (CDM), demostraron que *P. marginalis* EM-6 transformaba el 92 % del As(V) a As(III), después de 72 h de incubación a 25ºC. Basándonos en esto y en el balance de masas de los analitos estudiados, podemos discutir que gran parte del As(V) producido por la transformación de As(III), pudo ser absorbido en su superficie de calcita biogénica, incorporaron ambas especies de arsénico en su estructura cristalina.

Para determinar la presencia de arsénico (As) en los cristales biogénicos de calcita, se realizaron estudios de microscopía FTIR. Los análisis fueron realizados desde el reactor batch en presencia de As (III) (P1) (Anexo I, Figura 3B). El control fue el reactor sin la presencia de As (P2) (Anexo I, Figura 3A). Las Imágenes de microscopía y los espectros FTIR demuestran que en ambos reactores se observan precipitado de calcita del tipo romboédricos sin agrupación, policristales aglomerados y cristales amorfos. Por otra parte, los espectros obtenidos por FTIR entre 400 a 4.000 cm^{-1} de la muestra P1 y P2 (Anexo I,

Figura 3C), revelaron la presencia de picos característicos de componentes celulares bacterianos, los picos 3288 cm^{-1} (P1), 3303 cm^{-1} (P2), lo que representan a los grupos N-H de proteínas (Naumann, 2000), los picos 2927 cm^{-1} y 2960 cm^{-1} (P1 y P2, respectivamente) y los picos 2928 cm^{-1} y 2963 cm^{-1} (P1 y P2, respectivamente), representaron los grupos CH_3 y CH_2 en ácidos grasos, respectivamente (Naumann, 2000). Adicionalmente, ambas muestras poseen picos cercanos a 2350 cm^{-1}, que corresponderían a CO_2.

Por otra parte, los espectros obtenidos microscopía FTIR, de las células bacterianas cultivadas en ausencia y en presencia de As(III), revelaron la presencia de carbonato de calcio (calcita), situada en números de onda cercanos al 1448 cm^{-1}, 1086 cm^{-1}, 879 cm^{-1} y 714 cm^{-1} (Anexo I, Figura 3C). Los picos localizados entre 1250 cm^{-1} al 1550 cm^{-1}, pueden atribuirse a los enlaces C-O de $CaCO_3$ (Chen y Nan, 2011). Los grupos funcionales presentes en las superficies de los microorganismos actúan como sitios de unión para una variedad de especies químicas, en particular metales traza (Fein y col., 2001). Estos grupos también median la adhesión entre las células microbianas y superficies minerales (Burns y col., 2010). Los microorganismos secretan uno o más productos metabólicos que reaccionan con iones o compuestos en el medio resultando en la posterior deposición de partículas minerales (Huang y col., 2011).

Para mejorar el análisis de la interacción absorbato/adsorbente (As/calcita, respectivamente), se realizó espectroscopia FTIR de una agrupación de cristales de carbonato de calcio, obtenida del medio liquido en donde fue cultivada la cepa bacteriana en presencia de arsenito (5mM) durante 72h, observándose picos característicos de calcita (1448 cm^{-1}, 1100 cm^{-1}, 876 cm^{-1} y 714 cm^{-1}) (Anexo I, Figura 3D). Por otra parte, se observo también la formación de una nueva banda (840 cm^{-1}), correspondiente al estiramiento vibracional del grupo As-OH, que está cercano a los reportado en la literatura para el arseniato a pH 9 (858 cm^{-1}) (Goldberg y Johnston, 2001). Lo anterior estaría indicando que el As(V) está unido como un complejo de superficie y no como una fase sólida precipitada.

Cabe destacar que el análisis FTIR de los precipitados de biocalcita (adsorbente) es un método útil para obtener información sobre la presencia de grupos funcionales en la

superficie del adsorbente y estudiar la interacción entre los grupos funcionales superficiales y oxianiones arseniato (absorbato) adsorbidos durante de la biomineralización de Metaloide.

Los resultados obtenidos de los análisis por TEM, del reactor bajo las mismas condiciones, en presencia y ausencia de As, fueron similares a los obtenidos mediante microscopía asociada a FTIR, observándose cristales con morfologías romboedral, con alargamientos c-axial y co-agregaciones de partículas similares a morfologías de estrellas atribuidos a cristales de calcita (Capítulo VII), lo que de acuerdo a lo descrito por Guo y col. (2003) y De Söllner y col. (2003), correspondería a cristales de calcita. Además, los análisis mediante SEM y su posterior análisis mediante espectroscopía de energía dispersiva de rayos X (EDS) del cultivo en Bach en presencia de arsénico, incubado durante 72h, demostró que los cristales corresponden a calcita ($CaCO_3$) y que estos cristales, en su conformación, contenían arsénico. Estos resultados demuestran que *P. marginalis* EM-6 es capaz de producir cristales de calcita en medio de cultivo CDMU y que el arsénico transformado por esta cepa es capaz de biomineralizarse.

Para evaluar la eficiencia de la eliminación As (III) y As (V) de muestras ambientales ricas en arsénico, obtenidas mediante biomineralización de calcita inducida microbiológicamente utilizando la cepa *Pseudomonas marginalis* EM-6, se realizo un reactor Batch, en donde la remoción de las especies arsenicales, se evaluó mediante HPLC/HG/ASS. Demostrando que *P. marginalis* EM-6, tras 48 h de incubación a 30º C y condiciones aeróbicas, fue capaz de eliminar el 100% del arsénico presente en las muestras de agua obtenidas desde el río Loa (Capítulo VII). Estos resultados demuestran que existe un aumento en la tasa de remoción de arsénico, que puede deberse a la presencia natural del carbonato de calcio en la muestra de agua del río ($CaCO_3$= 297.3 mg L^{-1}), producto de la actividad minera en el sector, incrementado la eficiencia de precipitación de arsénico en el biorreactor Batch. Además, los análisis SEM-EDS demostraron la presencia de cristal polimorfos de calcita, incluyendo el arsénico en la estructura cristalina (Capítulo VII), resultados similares a los obtenidos con el efluente del modelo sintético.

Existen fuertes evidencias que vinculan el As con una amplia variedad de enfermedades en el hombre, que van desde una toxicidad aguda hasta enfermedades crónicas (Arsenicismo)

que incluyen lesiones cutáneas (Hiperpigmentación y Hiperqueratosis), hipertensión, isquemia, algunos trastornos vasculares periféricos (e.g., “enfermedad del pie negro"), diabetes, arteriosclerosis severa, neuropatías (Gong y col., 2011; Hall y col., 2009). Por otra parte, el cáncer a la piel, vejiga, riñón, hígado, próstata y pulmón, también han sido asociados a la ingesta de arsénico en humanos (Ferreccio y col., 2000; Liaw y col., 2008, Dauphiné y col., 2013; Hong y col., 2014). Por lo cual, el compuesto ha sido clasificado por la Agencia Internacional para la Investigación del Cáncer (IARC, siglas en inglés), como uno carcinógeno humano de clase I y como un contaminante tóxico prioritario por la Agencia de Protección Ambiental de Estados Unidos (US EPA) y la Unión europea (IARC, 2004; Filella y col., 2009).

Con el objetivo de evaluar la disminución de la toxicidad en las agua tratada con el biorreactor en Batch, se realizaron ensayos de toxicidad en células eucariotas, para lo cual se utilizaron, células endoteliales de vena umbilical humana (Huvec), las cuales fueron obtenidas mediante digestión con colagenasa tipo II (0,2 mg/mL) y mantenidas (37 ºC, 5% CO_2) en medio de cultivo primario suplementado con suero. La toxicidad se evaluó mediante el efecto del compuesto sobre la viabilidad celular de las células Huvecs. Los resultados demostraron que la toxicidad celular disminuye en concomitancia con la disminución de As en el medio, producto de la biomineralización del metaloide. Aumentando los recuentos celulares a través del tiempo (Capítulo VII).

Particularmente, algunos estudios han utilizado la urea para promover la precipitación biogénica de $CaCO_3$ como una estrategia de remediación. Fujita y col. (2004) reportaron la incorporación de estroncio radioactivo (90Sr) en calcita generada por la ureolisis de *Bacillus pasturii*, como estrategia de biorremediación de aguas subterráneas contaminadas con el compuesto. Achal y col. (2012a) estudiaron la capacidad biorremediadora de plomo en suelos, de la bacteria calcita-precipitante *Kocuria flava*, los resultados indicaron que la incubación de suelo contaminado, en presencia de la bacteria podría reducir el Pb activo y estabilizar el suelo contaminado. Por otra parte, la MICP de una *Halomonas* sp resistente a estroncio productora de ureasa, se caracterizó por su potencial papel en la biorremediación de estroncio (Sr) en acuíferos por Achal y col. (2012b). La cepa bacteriana elimino el 80% de Sr de la fracción soluble en la arena. La difracción de rayos X detectó una solución

sólida de calcita-estroncianita (SrCO(3)) en la muestra tratada con indicios de que Sr se incorporó a la calcita. Achal y col. (2013) demostraron la biorremediación de cromato (Cr(VI)) a partir de desechos de la industria de cromo, por una bacteria ureolítica calcificante *Bacillus* sp. CS8 y se reportó que la movilidad del Cr(VI), disminuyo significativamente en la fracción intercambiable del desecho y que posteriormente, la concentración de Cr(VI) incrementó en la fracción carbonatada. Los resultados de estos estudios demuestran que el secuestro a base de MICP de metales pesados solubles a través de coprecipitación con calcita puede ser útil para la biorremediación de metales pesados tóxicos. Hasta ahora no han sido publicados estudios similares en Chile, así como tampoco se han descritos los parámetros para el diseño de un biorreactor especializado en la remoción de As(III) y As(V) bajo un sistema MICP.

Por lo anterior, la biomineralización de calcita inducida microbiológicamente, que presenta la cepa arsenito-oxidante *Pseudomonas marginalis* EM-6 aislada desde sedimentos ricos en arsénico, es una via alternativa para la implementación de sistemas de tratamiento de remoción de arsenito y arseniato de agua de consumo humano.

CONCLUSIONES

Los análisis metagenómicos demostraron que existe una mayor proporción de los Phylum, *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* y *Nitrospirae*, en los sedimentos del rio Camarones, que sustentan los procesos biogeoquímicos de los sistemas acuáticos estudiados.

Las secuencias de los grupos taxonómicos dominantes en las muestras de sedimento del rio Loa, fueron clasificadas dentro de los Phylum: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, *Lentisphaerae*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia y Chlorobi*, predominando el grupo de las reductoras de sulfato debido al impacto antropogénico.

La comunidad bacteriana presente en los sedimentos contaminados con arsénico, esta constituida por una alta diversidad microbiana, capaces de reducir y/o oxidar arsénico, mediante mecanismos de detoxificación del metaloide, a través de la expresión de genes de resistencia, los cuales influenciarían directamente la especiación del metaloide en sistemas acuáticos estudiados.

Pseudomonas arsenicoxydans, cepa aislada desde sedimentos del rio Camarones, presenta un alto potencial biotecnológico debido a su capacidad de oxidar As(III) en condiciones aerobicas. Sin embargo, presenta una baja capacidad ureolítica en presencia de As(III) y As(V), limitando su utilización en la implementación de un sistema de tratamiento basado en MICP.

Pseudomonas marginalis EM-6 aislada desde los sedimentos del rio Loa, presenta una alta capacidad ureolítica que sumado a su capacidad de oxidar As(III) y tolerar As(V) en condiciones aerobicas, la transformaría en un buena candidata para la implementación de un sistema de tratamiento biológico de agua basado en MICP.

Los experimentos de biomineralización de arsénico en un sistema Batch, demostraron que *Pseudomonas marginalis* EM-6 es capaz de producir cristales de $CaCO_3$ biogenica, permitiendo la captura del arsénico (biomineralización) y eliminando la toxicidad en el medio.

La implementación de un sistema de tratamiento, basado en MICP, para agua naturales contaminadas con el metaloide, usando *Pseudomonas marginalis* EM-6, demostraron la capacidad de eliminar la toxicidad, mediante la remoción del 100% del As(III) y/o As(V) presente en la muestra.

Los microorganismos asociados a sedimentos de ríos contaminados con arsénico, capaces de oxidar y/o reducir el metaloide, cumplirían una función importante en la especiación y movilización del arsénico en los sistemas acuáticos. Por otra parte, estos resultados aportarían al conocimiento básico y biotecnológico, con vistas a la implementación de un sistema de tratamiento efectivo para la remoción de arsénico mediante el MICP.

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ANEXO I:
TABLAS Y FIGURAS

Tabla 1. Abundancia de genes implicados en la reducción de As(V) a As(III), operon *ars.*

Sitio	Phylum	Genero	Gen	Abundancia
Rio Loa (L1, L2 y L3)	*Actinobacteria*	*Brevibacterium*	*arsA*	1
	Firmicutes	*Bacillus*	*arsA, arsC*	3
		Sporomusa	*arsA, arsB*	1
		Veillonella	*arsA, arsB*	2
	Proteobacteria	*Acidithiobacillus*	*arsH*	1
		Acinetobacter	*arsA*	2
		Burkholderia	*arsA*	7
		Cupriavidus	*arsA*	1
		E. coli	*arsA, arsD*	4
		Pseudomonas	*arsB, arsC*	5
		Xanthomonas	*arsR*	2
Rio Camarones (M1, M2, M3)	*Actinobacteria*	*Brevibacterium*	*arsA*	1
	Firmicutes	*Bacillus*	*arsA, arsC*	3
		Sporomusa	*arsA*	1
	Proteobacteria	*Acidithiobacillus*	*arsH*	1
		Acinetobacter	*arsA*	1
		Burkholderia	*arsA*	7
		Cupriavidus	*arsA*	1
		E. coli	*arsA, arsD*	4
		Pseudomonas	*arsB, arsC*	5

Tabla 2. Abundancia de genes implicados en la respiración de As(V) operon *arr* y la respiración de As(III), operon *arx.*

Sitio	Phylum	Genero	Gen	Abundancia
Rio Loa (L1, L2 y L3)	*Proteobacteria*	*Alkalilimnicola*	*arxA,arxB, arxC,arxX, arxR,arxS*	565
		Halorhodospira	*arrA*	9
		Shewanella	*arrA*	16
		Thioalkalivibrio	*arrA, arrB*	9
Rio Camarones (M1, M2, M3)	*Proteobacteria*	*Alkalilimnicola*	*arxA, arxB, arxX, arxR, arxS, arxD*	116
		Halorhodospira	*arrA*	2
		Shewanella	*arrA*	1
		Thioalkalivibrio	*arrA, arrB*	1

Tabla 3. Abundancia de genes implicados en la oxidación de As(III) a As(V), operon *aio.*

Sitio	Phylum	Genero	Gen	Abundancia
Rio Loa (L1, L2 y L3)	*Proteobacteria*	*Roseobacter*	*aioA, aioB*	16
		Thiomonas	*aioA, aioB*	9
		Pseudomonas arsenicoxydans	*aioB*	3
Rio Camarones (M1, M2, M3)	*Proteobacteria*	*Roseobacter*	*aioA, aioB*	9
		Thiomonas	*aioA, aioB*	4
		Pseudomonas arsenicoxydans	*aioB*	7

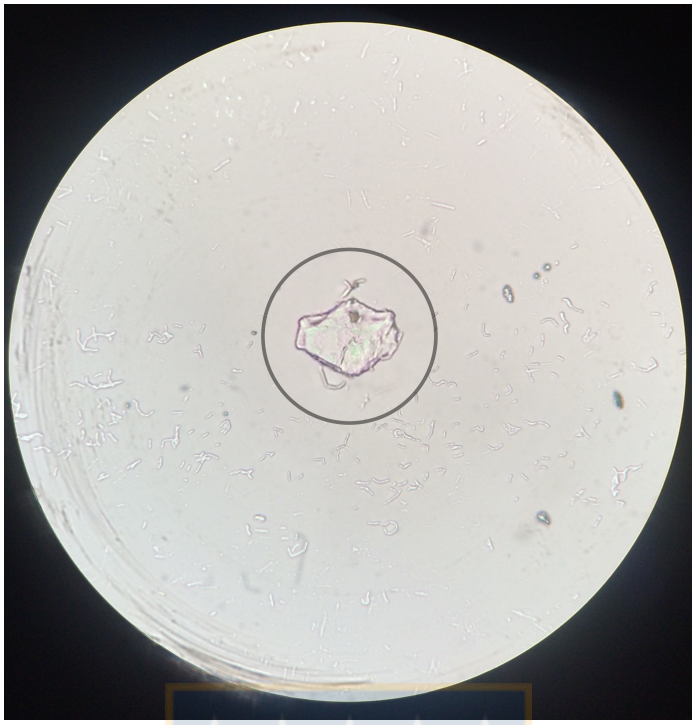

Figura 1. Precipitación de calcita inducida por la cepa EM6, observados por microscopía óptica.

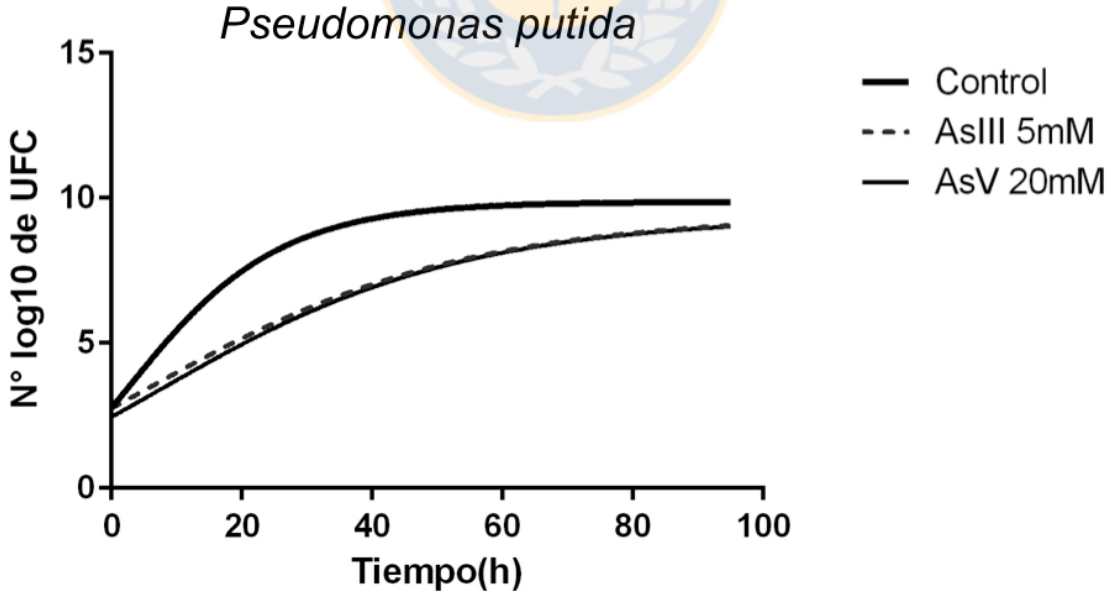

Figura 2. Curva de crecimiento de la *P. putida*, ajustada al modelo de gomperz.

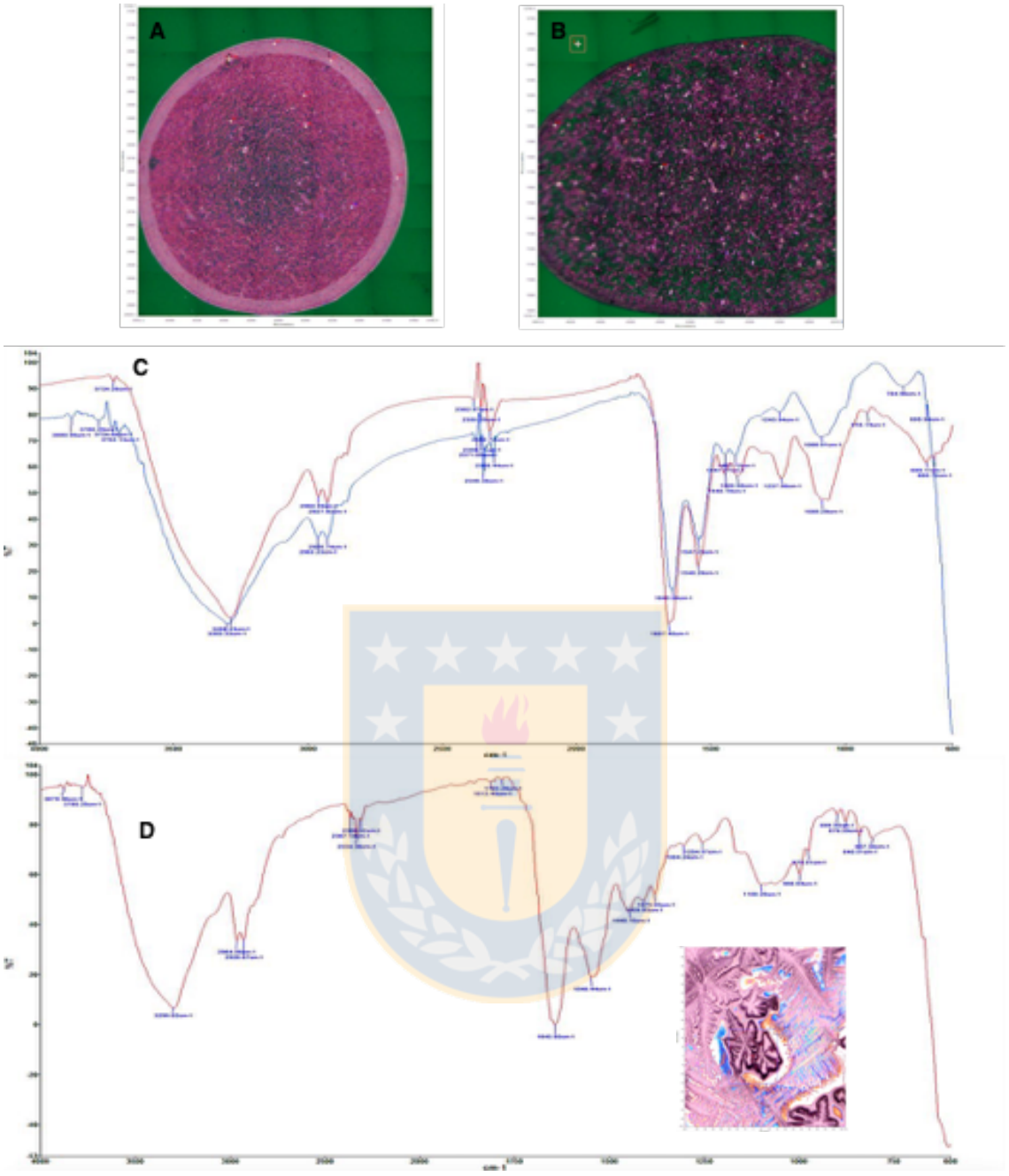

Figura 3. Imágenes de microscopía y espectroscopia FTIR obtenidas del cultivo de la cepa *P. marginalis* EM-6 en medio CDMU en presencia o ausencia de As(III) por 72 h at 30°C. A) imagen de microscopía FTIR de la cepa *P. marginalis* EM-6 incubada en medio CDMU en ausencia de As(III). B) imagen de microscopía FTIR de la cepa *P. marginalis* EM-6 incubada en medio CDMU en presencia de As(III). C) Espectroscopia FTIR de la cepa *P. marginalis* EM-6 cultivada en: ausencia de As(III) (Linea roja) y en presencia de As(III) durante por 72h. D) Espectroscopia FTIR de un cristal de calcita obtenido del cultivo de *P. marginalis* EM-6 en medio CDMU en presencia de As(III).