



FACULTAD DE CIENCIAS NATURALES Y EXACTAS
DOCTORADO INTERDISCIPLINARIO EN CIENCIAS AMBIENTALES

**REGULACIÓN AMBIENTAL Y ANTRÓPICA SOBRE LA
ACUMULACIÓN DE GASES DE EFECTO INVERNADERO (CO₂ CH₄
Y N₂O) Y EL MICROBIOMA BENTÓNICO EN HUMEDALES
COSTEROS DE LA ZONA SEMIÁRIDA DE CHILE CENTRAL**

Tesis para optar al grado de Doctor en Ciencias Ambientales

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DEDICATORIA

Quiero dedicarles esta tesis doctoral a tres personas muy queridas de mi familia. En primer lugar se la quiero dedicar a mi padre Juan Pablo Pozo Ruiz, que nos dejó hace más de siete años. En segundo lugar le dedico esta tesis doctoral a mi madre María Gabriela Solar Silva, quien me ha apoyado durante todo este proceso y por último le dedico esta tesis doctoral a mi tío Juan Sebastián Solar Silva, que al igual que mi padre, nos dejó hace ya más de ocho años.

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RESUMEN

Los humedales son una fuente importante de gases de efecto invernadero (GEI), constituyéndose entre los ecosistemas naturales que globalmente más aportan a las emisiones de metano (CH_4). Diversos gradientes fisicoquímicos presentes en los humedales costeros, incluyendo a la salinidad, influyen de manera relevante sobre procesos microbianos que descomponen la materia orgánica (MO) y reciclan nutrientes, como son la metanogénesis, la oxidación de metano y la nitrificación, que a su vez participan en la producción y consumo de GEI (tales como: dióxido de carbono o CO_2 , CH_4 y óxido nitroso o N_2O). A nivel global, los humedales costeros se han visto gravemente intervenidos por diversas actividades antrópicas, siendo una de las más importantes, el vertido de aguas residuales, por ejemplo, desde plantas de tratamientos de aguas servidas (PTAS). Entre otros impactos, estos vertidos introducen MO alóctona, diversos contaminantes y microorganismos foráneos al ecosistema alterando potencialmente su funcionamiento.

La Zona Semiárida de Chile Central (ZSACHC) presenta una gran variedad de humedales costeros (~120) a lo largo de ~ 400 km de costa $29^{\circ}82'$ - $33^{\circ}77'$ distribuidos en una gradiente de aridez que aumenta de sur a norte. En la zona norte, las cuencas presentan una menor disponibilidad de agua (HAB, high aridity basins tales como Limarí, Elqui y Culebrón) y sus humedales son más salinos (mesosalinos) en comparación a las cuencas de la zona sur (LAB, low aridity basins tales como Maipo, Aconcagua y Mantagua) donde los humedales son mayormente hiposalinos. Además, en áreas cercanas a cuencas LAB se concentran una mayor densidad poblacional y un mayor uso del recurso hídrico asociado a actividades antrópicas, incluyendo utilización doméstica, industrial y agrícola, entre otras. En estas cuencas se destaca el impacto proveniente del vertido de aguas tratadas desde plantas de tratamiento de aguas servidas (PTAS) ubicadas en la Región de Valparaíso, las que producto de su alcance y persistencia han generado relevancia pública. Un ejemplo de este tipo de impactos corresponde a los vertidos en el humedal El Sauce, ubicado en la localidad rural

de Laguna Verde perteneciente a la Región de Valparaíso. Debido a estos motivos, esta tesis doctoral abordó el impacto de gradientes fisicoquímicos naturales en un conjunto de humedales sobre la distribución de GEI, la estructura del microbioma bentónico y la cuantificación específica de grupos funcionales asociados al reciclaje GEI (metanogénicos, amonio oxidantes y metanotróficos) en períodos contrastantes (invierno-verano) a lo largo de un transecto latitudinal (~ 400 km) que incorporó cuencas tipo HAB y LAB y a lo largo del estero El Sauce, un modelo de estudio de un humedal impactado producto de un efluente de PTAS.

Los resultados indican que los humedales poseen una alta variabilidad en sus características físicoquímicas y sedimentarias, en particular destacándose diferencias en la magnitud de la MO durante el verano, siendo mayor en los humedales de HAB en comparación a los de LAB, mientras que en estos últimos presentaron altas concentraciones de nutrientes principalmente en invierno. En todos los humedales, se observó un exceso positivo de CO_2 y CH_4 , a concentraciones de μM y nM , respectivamente, mientras que para N_2O se registró un notorio cambio latitudinal, asociado a un potencial efecto sumidero en los humedales de HAB. Las comunidades microbianas bentónicas fueron taxonómicamente diversas e incluyeron > 60 phyla, representadas en su mayoría por taxas poco frecuentes (< 1%). Los microorganismos bentónicos más abundantes de los humedales se asociaron a bacterias de las clases Gammaproteobacteria, Alphaproteobacteria y Deltaproteobacteria, destacándose dentro de la primera clase, grupos microbianos funcionales claves para el reciclaje de GEI asociados a bacterias nitrificantes y metanotróficas (incluyendo metilotróficas). Basados en el modelo generalizado aditivo (GAM) se identificó que la conductividad fue la variable más explicativa de la distribución de CH_4 y CO_2 . Sin embargo, la adición de variables como la latitud y el pH en el GAM, aumentó la significancia de la predicción. Por otra parte, la concentración de nitrato junto con la latitud fueron las variables que mejor predicen la distribución del N_2O . El modelo de ecuaciones estructurales (SEM) confirmó que factores ambientales como la conductividad, los nutrientes, la MO y la latitud influyen

significativamente sobre los grupos funcionales microbianos (nitrificantes y metano oxidantes) y estos últimos sobre la distribución de los tres GEI medidos en el presente estudio. Este primer resultado de la tesis destaca a la salinidad y concentración de nutrientes, ambos factores relevantes en la prevalencia de grupos funcionales clave asociados al reciclaje del carbono y nitrógeno, como factores que inciden de manera combinada en la distribución de GEI y su potencial intercambio entre el agua y atmósfera en humedales costeros en un gradiente de aridez e impacto antrópico latitudinalmente variable.

En particular el análisis detallado del impacto de las PTAS en el humedal El Sauce, indicó que los vertidos modifican las condiciones fisicoquímicas en el agua, por ejemplo, disminuyendo el pH en 0,5 unidades, aumentando de 4-6°C en la temperatura y la concentración de GEI ($> 600 \mu\text{M CO}_2$, $> 30000 \text{ nM CH}_4$ y $> 3000 \text{ nM N}_2\text{O}$). En los sedimentos, posterior al vertido se observó evidencia de una señal de MO ($\delta^{15}\text{N}$ y $\delta^{13}\text{C}$) de origen más terrestre. Adicionalmente, la estructura de las comunidades microbianas bentónicas cambió espacial y estacionalmente, incluyendo phyla específicos que son característicos de aguas residuales, como Firmicutes (del orden Clostridiales) y Bacteroidetes. Además, los taxa Tenericutes, Lentisphaerae, Synergistetes y LCP-89, fueron únicamente observados en la estación de descarga de la PTAS, en comparación con los otros sitios estudiados de la predescarga y boca del Estero. La cuantificación específica de grupos funcionales indicó un enriquecimiento de metanogénicos asociados al vertido de PTAS y una disminución de metanotrofos y amonio oxidantes, incluyendo comammox particularmente en invierno. A partir de los resultados obtenidos se puede concluir que la descarga de aguas provenientes de las PTAS ejercen un impacto sobre la fisicoquímica del Estero El Sauce, alterando la estructura de las comunidades microbianas bentónicas y su contribución a los ciclos biogeoquímicos que realizan, incluyendo grupos funcionales capaces de mitigar las emisiones de CO_2 y CH_4 y el reciclaje de nutrientes en estos ecosistemas acuáticos.

Palabras claves: Factores ambientales, Estuarios, PTAS, GEI, Ecología microbiana.

ABSTRACT

Wetlands are important sources of greenhouse gases (GHG) becoming one of the natural ecosystems that contribute the highest methane (CH_4) emission globally. Several physicochemical gradients present in coastal wetlands, including salinity, play a key role in microbial processes that degrade organic matter (OM) and recycle nutrients. These processes include methanogenesis, methane oxidation and nitrification, which also regulates the production and consumption of GHG (For instance: carbón dioxide or CO_2 , CH_4 and nitrous oxide or N_2O) in such environments. In recent years, coastal wetlands have been seriously threatened by several anthropic activities. Among those, the discharge of wastewater from wastewater treatment plant (WWTP) has become one of the activities that has raised more concerns around the world, since this discharge introduces allochthonous OM, several contaminants and foreign microorganisms to the ecosystem potentially altering its functioning.

Semiarid zone of central Chile (SAZCCh) has a great diversity of coastal wetlands (~120) along ~400 km of coastline ($29^\circ 82'$ - $33^\circ 77'$) in an aridity gradient that increases from south to north. In the northern zone, basins contained less water (HAB, high aridity basins such as Limarí, Elqui and Culebrón) and their wetlands are more saline (mesosaline) compared to basins of southern zone (LAB, low aridity basins) such as Maipo, Aconcagua and Mantagua, where wetlands are mostly hyposaline. In addition, in areas near LAB basins, there are higher population density and greater use of water resources associated with anthropic activities, including, domestic, industrial and agricultural use, among others. Environmental impacts from discharge of treated water from wastewater treatment plant (WWTP) in those basins located in Valparaíso have been the focus of increasing attention over the last years, primarily because of the persistence and extent of contamination. A good example of this is the effect produced by the discharge in the El Sauce wetland, located in the rural town of Laguna Verde, Valparaíso. In consequence, the present doctoral thesis addressed the impact of natural physicochemical gradients in a set of coastal wetlands on GHG distribution,

the benthic microbiome structure and the specific quantification of functional groups associated with GHG recycling (methanogenic, ammonium oxidizing and methanotrophic) in contrasting seasons (winter and summer) along a latitudinal synoptic transect that incorporated HAB and LAB type bays along the El Sauce Estuary, a study model of a wetlands impacted by a WWTP effluent.

Results indicate that wetlands belonging to SAZCCh have a high variability in their physicochemical and sedimentary characteristics. In particular, HAB wetlands contained higher levels of organic matter during summer, compared to LAB wetlands, while in the latter it was registered they contained higher nutrient concentration mainly in winter. In all the wetlands, a positive excess of CO_2 and CH_4 was registered ranging between 20 – 480 μM and 50 – 1200 nM, respectively. In contrast, N_2O emissions showed a notorious latitudinal shift, associated with a potential sink effect in HAB wetlands. Benthic microbial communities were taxonomically diverse associated with > 60 phyla, represented mostly by low frequent taxa (< 1%). The most abundant benthic microorganisms in the wetlands were associated with bacteria belonging to the Gammaproteobacteria, Alphaproteobacteria and Deltaproteobacteria classes, with the first class mainly affiliated to key microbial functional groups associated with GHG recycling such as nitrifying and methanotrophic (including methylotrophic) bacteria. Based on a generalized additive model (GAM), conductivity was identified as the physicochemical variable accounting for the distribution of CH_4 and CO_2 , however, the addition of other variables, such as latitude and pH, respectively, increased the significance of the prediction. On the other hand, nitrate concentration together with latitude were the variables that best predict the distribution of N_2O . Structural equation modeling (SEM) supported that environmental factors, such as conductivity, nutrient, OM and latitude, significantly influenced microbial functional groups (nitrifying and methane oxidizing) and the distribution of the three GHG measured. In summary, the first result of the thesis highlights that salinity and nutrient concentration were relevant factors accounting for key functional groups associated with carbon and nitrogen recycling, as factors having a combined effect

on the distribution of GHG and its potential water-atmosphere exchange in coastal wetlands in an aridity and anthropogenic impact gradient.

The impact of WWTP discharge in El Sauce wetland was mainly associated with the physicochemical water conditions, for example, by a decrease of pH in up to 0.5 units, and by a temperature increment between 4 to 6 C° and by higher GHG concentration ($> 600 \mu\text{M CO}_2$, $> 30000 \text{ nM CH}_4$ and $> 3000 \text{ nM N}_2\text{O}$). Sediments in the WWTP discharge area recorded typical terrestrial organic matter isotopic signatures ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$). In addition, benthic microbial community structure changed spatially and according to the sampling season, including phyla characteristic of sewage, such as, Firmicutes and Bacteroidetes. Besides, Tenericutes, Lentisphaerae, Synergistetes y LCP-89 taxa, were solely observed at the WWTP discharge area, compared with upstream (predischage area) and the mouth of the estuary. Specific functional groups quantification indicated that there is an enrichment of methanogenes linked to the discharge of WWTP and a decrease of methane and ammonium oxidizing bacteria, including commamox, particularly in the winter. In summary the results indicate that WWTP highly altered the microbial community composition and their contribution to biogeochemical cycles including functional groups able to mitigate CO_2 and CH_4 emissions and nutrients recycling in this aquatic ecosystem.

Keywords: Environmental factors, Estuary, WWTP, GHG, Microbial ecology

ABREVIATURAS

GEI = Gases de Efecto Invernadero

CO₂,= Dióxido de Carbono

CH₄ = Metano

N₂O = Óxido Nitroso

MO = Materia Orgánica

SO₄²⁻ = Sulfato

NH₄⁺ = Amonio

NO₂⁻ = Nitrito

NO₃⁻ = Nitrato

NO = Óxido Nítrico

N₂ = Nitrógeno Molecular

BAO = Bacterias Amonio Oxidantes)

AAO = Arqueas Amonio Oxidantes

AMO = Amonio Monooxigenasa

NIR = Nitrito Reductasa

MCR = Metil-coenzima M Reductasa

IDI = Índice de Perturbación Antrópica

ZSACHC = Zona Semiárida de Chile Central

MMA = Ministerio de Medio Ambiente

DGA = Dirección General de Agua

MOP = Ministerio de Obras Públicas

HAB = High Aridity Basins

LAB = Low Aridity Basins

GAM = Generalized Additive Model

SEM = Structural Equation Model

PTAS = Plantas de Tratamiento de Aguas Servidas

AIC = Criterio de Akaike

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CAPÍTULO I. INTRODUCCIÓN

INTRODUCCIÓN

Los gases de efecto invernadero (GEI) tienen la capacidad de retener calor en la atmósfera y, por lo tanto, controlan la temperatura atmosférica del planeta. En la actualidad los GEI más importantes alcanzan concentraciones de 403 ppm, 1853 ppb y 329 ppb, para el dióxido de carbono (CO_2), el metano (CH_4) y óxido nitroso (N_2O) respectivamente [1]. Sin embargo, el efecto de cada gas en el planeta afecta de acuerdo a su capacidad de retención de calor. Por ejemplo, el N_2O en la atmósfera tiene un potencial de calentamiento global (PCG) 298 veces mayor que el CO_2 en comparación con el CH_4 que solo es 25 veces más que el CO_2 [1]. A nivel global los humedales costeros funcionan como una importante fuente de GEI, entre ellos el CO_2 , el CH_4 y el N_2O [2]. Aunque presentan una dinámica espacial y temporal relacionada a diversos condicionantes ambientales, se ha estudiado mayormente las dinámicas del CO_2 y CH_4 , donde se destacan factores como la profundidad, la inundación y la salinidad, entre otras [3, 4]. Por otra parte, algunos tipos de humedales costeros como manglares, marismas y pastos marinos son considerados dentro de las estrategias de manejo para mitigar las emisiones de GEI [1, 5], por ser sumideros de carbono (C) al registrar una alta producción primaria con bajas tasas de descomposición de la materia orgánica (MO) [6].

La MO es un factor clave dentro del funcionamiento regulatorio de los humedales costeros, ya que a través de su degradación y descomposición, se estimula el reciclaje de nutrientes y GEI [7]. Además, la MO se puede originar desde fuentes alóctonas asociadas a MO proveniente de material terrestre o marino que es transportado por los ríos y el océano, así como también autóctonas, la cual es generada por organismos acuáticos residentes que mueren y entran en procesos de descomposición [8]. El origen y calidad de la MO influye sobre su tasa de degradación [9, 10], así como también factores abióticos y bióticos, en donde la composición y distribución de las comunidades detritívoras y microorganismos juegan un rol fundamental [11, 12]. Con respecto a los factores abióticos, la

salinidad destaca como un factor relevante que incide en la degradación de la MO. Sin embargo, este efecto ha sido reportado en correlaciones directas e inversas. Mientras hay diversos estudios que reportan que la tasa de descomposición de la MO en humedales aumenta junto a un incremento de la salinidad [13, 14], hay otros estudios que registran el mismo efecto asociado a una disminución de esta [15, 16]. Esta discrepancia se atribuye a diferencias en la magnitud y temporalidad de la exposición a la salinidad, como es descrito en el modelo de Neubauer et al. (2013), en donde se sugiere que la descomposición de la MO en ecosistemas de agua dulce es impactada a través de vías directas e indirectas asociadas a una exposición a la salinidad aguda o crónica, respectivamente [17]. Adicionalmente, se ha observado que el aumento de la salinidad inhibe en más de un 20% el porcentaje de degradación y disminuye en 4 unidades la razón carbono:nitrógeno (C:N) en humedales costeros [18].

En los humedales costeros, la salinidad es un factor clave, ya que son considerados ecotonos, es decir, una interfaz entre ecosistemas terrestres y marinos donde el aporte continental y oceánico genera gradientes con condiciones estuarinas [19]. El aporte salino de las mareas influye sobre el reciclaje de los GEI en humedales costeros [20], ya que introduce aceptores de electrones, como el sulfato (SO_4^{2-}) que promueve la sulfato reducción, un proceso metabólico microbiano de mayor eficiencia energética que la metanogénesis [21-23] y por lo tanto influyente sobre el reciclaje del CH_4 . Al igual que con la degradación de MO, existen evidencias contradictorias con respecto a cómo la producción de GEI responde a cambios de la salinidad en humedales costeros. Aproximaciones experimentales han documentado un incremento del CO_2 en respuesta a un aumento de la salinidad [24, 25], mientras que otros estudios *in-situ* y basados en microcosmos indican una disminución del CO_2 a medida que incrementa la salinidad en un gradiente salino [26, 27]. Por otro lado, otros estudios han encontrado que la salinidad no tiene efectos sobre las emisiones de CO_2 [28, 29]. Con respecto al CH_4 , algunas investigaciones registraron una disminución de las emisiones de CH_4 con el aumento de la salinidad en humedales costeros estuarinos y marismas [13, 25, 30, 31], mientras otras investigaciones indican un

incremento de CH_4 a medida que aumenta la salinidad [23, 32]. Similar a lo observado en el CH_4 y el CO_2 , también existen observaciones discrepantes con respecto al N_2O . Este es el caso del registro de mayores flujos de N_2O hacia la atmósfera en zonas con baja salinidad en marismas y estuarios en comparación con la zonas de mayor salinidad [33, 34]. Lo opuesto ha sido observado en estuarios por Li et al., (2019), quienes informan un incremento en las emisiones de N_2O hacia la atmósfera a mayor salinidad [32]. Por lo tanto, según los antecedentes recién mencionados es posible evidenciar que hasta ahora existe una comprensión incompleta acerca de los efectos del aumento de la salinidad sobre la producción y consumo de GEI en humedales costeros, restringiendo así el entendimiento de los procesos biogeoquímicos que toman lugar en estos ecosistemas.

1.1 Procesos metabólicos y grupos funcionales que participan en el reciclaje de GEI

La disponibilidad de oxígeno es clave para determinar la forma en que se degrada la MO en los humedales costeros y en general en el ecosistema [35]. Cuando el ambiente se encuentra bajo condiciones de alta disponibilidad de oxígeno, es decir, a $> 200 \mu\text{M}$ de oxígeno disuelto [36], la descomposición de la MO es aeróbica, generando bioproductos que generalmente están conformados por compuestos inorgánicos como CO_2 y amonio (NH_4^+) [11]. Bajo esas condiciones, el NH_4^+ es un compuesto reducido que puede ser oxidado desasimilativamente actuando como un dador de electrones a través de proceso de respiración, o ser asimilado a través de su incorporación a la biomasa microbiana. La nitrificación es el proceso quimiolitotrófico por el cual el NH_4^+ es oxidado a nitrito (NO_2^-), posteriormente de NO_2^- a nitrato (NO_3^-) y los electrones son utilizados para fijar CO_2 [37]. Adicionalmente, la nitrificación es un proceso que contribuye directamente a la emisión de N_2O como subproducto de la oxidación del NH_4^+ [38]. Por otra parte, bajo condiciones deficientes de oxígeno (sub óxicas y anóxicas; $< 40 \mu\text{M}$) [36], la oxidación de la MO puede ocurrir mediante la reducción desasimilativa del NO_3^- [39] y por la desnitrificación heterotrófica

(canónica). Durante este proceso el NO_3^- es reducido a NO_2^- y posteriormente de NO_2^- a óxido nítrico (NO). Luego el NO es reducido a N_2O , el cual es finalmente transformado en nitrógeno molecular (N_2) [37]. Los productos de la nitrificación (NO_2^- y NO_3^-) son utilizados por comunidades desnitrificantes, por lo que la nitrificación y la desnitrificación son procesos que se encuentran acoplados en el ambiente [40]. Por lo tanto, la desnitrificación heterotrófica al igual que la nitrificación, son procesos biogeoquímicos que pueden influir directamente sobre las emisiones globales de N_2O si la desnitrificación es incompleta, o bien mitigar dichas emisiones, al reducir el N_2O . Finalmente, el CH_4 producido en los sedimentos de los humedales costeros se genera mediante la metanogénesis, un metabolismo estrictamente anaeróbico [41], favorecido bajo condiciones anóxicas en humedales costeros en comparación a otros ecosistemas sedimentarios como lagos, ríos y turberas [42]. La metanogénesis es realizada por arqueas que a través de la oxidación de hidrógeno, acetato o compuestos metilados que producen CH_4 [41], mediante vías hidrogenotrófica, acetoclástica y metilotrófica, respectivamente [43]. Sin embargo, el CH_4 además de ser producido también puede ser oxidado por comunidades metanotrofas, sirviendo de fuente de carbono y energía mediante metabolismos aeróbicos y anaeróbicos [44].

1.2 Ensamblajes microbianos nitrificantes y desnitrificantes

La oxidación aeróbica del NH_4^+ a NO_2^- en humedales costeros es un proceso conducido tanto por bacterias quimiolitóautótrofas conocidas como BAO (bacterias amonio oxidantes) [45], como también por arqueas autótrofas conocidas con el acrónimo AAO (arqueas amonio oxidantes) [46-48]. Las BAO se agrupan dentro del Phylum *Proteobacteria* actualmente asociados a un orden β -*proteobacteria* (β -BAO) y además dentro de las γ -*proteobacteria* (γ -BAO), de acuerdo a la clasificación SILVA 132. Sin embargo, la clasificación de las proteobacterias se encuentra en revisión de acuerdo a nuevos estudios basados en filogenómica (<https://forum.qiime2.org/t/silva-132-for-qiime/3262/12>). Por otra parte, algunos microorganismos nitrato oxidantes del género *Nitrospira* son capaces de oxidar NH_4^+ , por lo tanto ambas etapas de la nitrificación y es

conocida como COMAMMOX (oxidación completa del amonio) [49, 50]. Las AAO se agrupan dentro del Phylum *Thaumarchaeota*, un Phylum que separa a las arqueas del Phylum *Crenarchaeota* [51, 52]. Tanto las AAO como las BAO se han estudiado mediante la caracterización del gen *amoA* que codifica la subunidad α de la enzima amonio monooxigenasa (AMO) responsable de catalizar el primer paso de la nitrificación, que corresponde a la oxidación del NH_4^+ [53]. Mediante el uso del gen *amoA*, se observó una mayor abundancia de comunidades β -BAO en comparación con las comunidades AAO en el estuario “Elkhorn Slough”, California (USA). La mayoría de las BAO se asoció con los géneros *Nitrosospira* y *Nitrosomonas* y las AAO con el género *Nitrosopumilus* [54]. Similares resultados fueron observados en el estuario de la bahía de San Francisco, California (USA), [55]. En contraste con esto, en el estuario del “Río Amarillo” (China), se observó una mayor abundancia de AAO en comparación con las BAO [56].

La desnitrificación heterotrófica (canónica), es realizada por microorganismos heterótrofos que se encuentran distribuidos en una amplia gama de taxones (eucariontes, bacterias y arqueas), dentro los cuales, bacterias pertenecientes a las especies *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, y *Brachymonas denitrificans* ya han sido identificadas en el sedimento de humedales costeros [57]. La enzima nitrito reductasa (*nir*) es una enzima particularmente importante en el proceso de desnitrificación ya que cataliza el primer paso, generando un producto gaseoso el óxido nítrico (NO) [58]. La producción de gases, incluyendo el N_2O , por la desnitrificación junto con otros procesos quimiolitotótrofos que también desnitrifican, es decir, son conducentes a la formación de N_2 , por ejemplo, la oxidación anaeróbica del amonio, anammox, proceso que oxida NH_4^+ anaeróbicamente mediante el uso de NO_2^- como aceptor [59].

1.3 Ensamblajes microbianos metanogénicos y metanotróficos

Las arqueas metanogénicas son microorganismos autótrofos anaerobios estrictos responsables en más del 90% del CH_4 producido en el planeta [60]. Son clasificadas principalmente dentro del Phylum *Euryarchaeota* [61], aunque también

han sido reportadas dentro de los phyla *Crenarchaeota*, *Halobacterota*, *Thermoplasmatota* [62] y *Bathyarchaeota* [63]. Además se han podido agrupar dentro ocho órdenes, incluyendo *Methanobacteriales*, *Methanocellales*, *Methanococcales*, *Methanofastidiosales*, *Methanomassiliicoccales*, *Methanomicrobiales*, *Methanopyrales* and *Methanosarcinales* [61, 64, 65]. Todos estos grupos realizan la misma reacción final que implica la conversión de metil-CoM a CH₄, la cual es catalizada por la metil-coenzima M reductasa (codificada por el gen *mcr*), un complejo enzimático presente en todas las arqueas metanogénicas [66]. Una comparación global de arqueas metanogénicas entre diferentes biomas, que incluyó sedimentos de estuarios, marinos, hidrotermales, lagos, suelos y volcanes, indicó que los estuarios corresponden al tipo de biomas que presenta una mayor riqueza de comunidades metanogénicas, sugiriendo que poseen un entramado ecofisiológico robusto orientado a la producción de CH₄ en diversas condiciones ambientales. En dicho estudio, las metanogénicas más dominantes en el total de estuarios estudiados (N = 14) corresponden a especies pertenecientes a los géneros *Methanobacterium*, *Methanoseaeta* y *Methanoregula* [67].

Las comunidades metanotrofas corresponden a un grupo diverso de bacterias y arqueas [68-71] que oxidan el CH₄ a través de distintas vías metabólicas. Además del oxígeno las metanotrofas pueden usar diferentes aceptores de electrones, como por ejemplo el nitrato [72], el sulfato [73] y metales como el hierro (Fe) y el manganeso (Mn) [74, 75]. Las bacterias metanotrofas oxidan el CH₄ a través de la enzima metano monooxigenasa [76], mientras que las arqueas utilizan la metil-coenzima reductasa M [70]. Las comunidades metanotrofas son clasificadas dentro de tres grupos, es decir: las de Tipo I que incluyen a los géneros *Methylobacter*, *Methylomonas*, *Methylomicrobium* y *Methylophaera*; las de Tipo II donde se encuentran los géneros *Methylocystis*, *Methylosinus* y *Methylocella* y un tercer grupo las de Tipo X que cuenta con el género *Methylococcus* [77, 78].

Todos estos procesos biogeoquímicos asociados a la producción y consumo de GEI en humedales costeros son altamente afectados por cambios de la salinidad. Por ejemplo, menores tasas de nitrificación y desnitrificación fueron observadas en zonas con mayor salinidad en el estuario de la bahía de San Francisco (USA) [59] y el estuario Bretón Sound [79], respectivamente. Adicional a esto en el sedimento del estuario Schelde (Holanda), se observó que en zonas con mayor salinidad ($16,2 \text{ g L}^{-1}$ de Cl^-) dominaron las especies *Nitrosomonas ureae* y *N. oligotropha*, a diferencia de las zonas con menor salinidad ($0,7 \text{ g L}^{-1}$ de Cl^-), donde dominó una nueva especie de *Nitrosomonas sp.*, con una contribución relativa de *N. marina* y *N. aestuarii* [80]. En contraste con estos resultados en un estudio desarrollado en el estuario “Ythan” (Escocia), se observó una mayor dominancia del género *Nitrosospira* en zonas más salinas (25-30‰), en comparación con las zonas más influenciadas por el agua dulce (0-5‰), donde predominaron las *Nitrosomonas* [81]. Por otro lado, en el estuario del río Colne (Escocia, UK), en zonas con menor salinidad dominó el género *Methanosaeta*, a diferencia de las zonas con mayor salinidad, donde dominaron los géneros *Methanosarcina* y *Methanococcoides* [82]. Contrario a esto los géneros *Methanosaeta*, *Methanobacterium*, *Methanocella*, junto con el género metanotrófico *Methylocystis* fueron tolerantes a zonas de mayor salinidad [83].

1.4 Vulnerabilidad de los humedales costeros, eutrofización y su potencial efecto en la emisión de GEI

Las zonas costeras cubren un 4% del área total terrestre del planeta, sin embargo, estas áreas albergan a casi un tercio de la población [84]. Entre los ecosistemas costeros más impactados por la elevada densidad poblacional y la actividad antrópica se encuentran los humedales costeros que en las últimas décadas son afectados por el aumento en el nivel del mar, entre otras consecuencias del calentamiento global [1, 85-87]. En efecto, los humedales son los ecosistemas costeros más vulnerables a nivel mundial [88], por ejemplo, más del 50% de las áreas de turberas, estuarios, zonas ribereñas, zonas de lagos litorales y llanuras inundadas de América del Norte, Europa y Australia, se han

degradado [84]. Con respecto al continente americano, se realizó un estudio en 18 humedales costeros distribuidos dentro de una gradiente latitudinal de 500 kilómetros en una zona semiárida de Chile central. Desde sus resultados y mediante el uso de un índice de perturbación antrópica (IDI), se observó un aumento significativo del IDI desde el norte hacia el sur de la zona semiárida de Chile central [89], lo cual se explicaría por el significativo aumento de la población y del turismo en la región de Valparaíso [90]. Entre las principales actividades antrópicas que pueden afectar a los humedales costeros se destacan: la descarga de aguas residuales y residuos provenientes desde actividades antrópicas por ejemplo, enriquecimiento orgánico y de nutrientes fertilizantes y pesticidas utilizados en la agricultura, metales pesados, entre otros contaminantes emergentes, además de la extracción del agua, entre otros usos de estos sistemas [91].

La eutrofización es un proceso que se entiende como la acumulación de residuos orgánicos en ecosistemas acuáticos, como estuarios, lagos, lagunas, embalses, entre otros, que posteriormente induce la proliferación excesiva de productores primarios. En los humedales costeros, la eutrofización ocurre de manera natural y también es inducida por el enriquecimiento del agua con nutrientes (nitrógeno y fósforo) estimulando el crecimiento de plantas, algas y microalgas [92]. Este florecimiento algal causa turbiedad en la columna de agua y constituye un aporte de MO que al ser descompuesta induce una disminución en la concentración de oxígeno y un aumento en la producción de compuestos reducidos (NH_4^+ , H_2S , CH_4) que pueden ser tóxicos para otros organismos acuáticos. De esta forma, las variaciones de factores químicos del agua por efecto de la eutrofización, inducen cambios significativos sobre la biodiversidad del ecosistema [92]. Un ejemplo de esto se observa en el hecho que, la mayoría de los estuarios en los Estados Unidos, están categorizados como al menos moderadamente eutróficos [93], sugiriendo que la eutrofización en los humedales costeros es un problema ambiental significativo en países desarrollados. Los procesos de eutrofización pueden modificar las emisiones de CO_2 y CH_4 en los humedales [94], por ejemplo, la fertilización con nutrientes en marismas de la

costa “New Brunswick” (Canadá), por un periodo de un año, generó cambios en los flujos de N_2O emitidos desde el sedimento, pasando desde una condición de “sumidero” (pre fertilización), a una condición de fuente, (post fertilización) [95]. Por otra parte, zonas cercanas a puntos de descarga de aguas residuales presentan concentraciones de CH_4 y N_2O significativamente mayores en comparación con las zonas alejadas al punto de descarga en el río Guadalete (España) [96].

1.5 Zona Semiárida de Chile-central (ZSACHC) y humedal costero “El Sauce” como caso de estudio por contaminación de aguas residuales

América del Sur es el continente con mayor área cubierta por ecosistemas acuáticos (ríos, lagos, humedales, etc.) en el planeta, representando más del 20 % de su superficie total [97]. Chile central cuenta con un total de 412 humedales costeros que cubren un área total de 38.167 hectáreas [98]. La zona semiárida de Chile-central (ZSACHC) se extiende desde las regiones Metropolitana y de Valparaíso hasta la región de Coquimbo ($33^{\circ}77'S$ - $29^{\circ}82'S$) y se caracteriza por presentar un clima mediterráneo que es principalmente influenciado por procesos oceánicos como la Corriente de Humboldt y el Anticiclón del Pacífico Sur Oriental [99]. Desde su extremo sur hacia su extremo norte, sus precipitaciones son cada vez menores y a causa de esto es posible observar una sucesión de bioclimas que van desde zonas más influenciadas por las lluvias a zonas más secas y desérticas [100]. Como consecuencia de esto, los caudales de los principales ríos (es decir: Maipo, Aconcagua, Limarí y Elqui) disminuyen de sur a norte (**Figura I-1**). Dentro de toda la franja costera de la ZSACHC, la región de Valparaíso cuenta con 82 humedales costeros y la región de Coquimbo con 40. Todos estos humedales costeros están clasificados dentro de la categoría micro-mareal [98] y, por lo general, presentan una barra de arena que se puede encontrar cerrada, abierta, o bien, semi-abierta. La transición desde una barra abierta a cerrada resulta en cambios fisicoquímicos (temperatura, pH, salinidad, conductividad y oxígeno disuelto), que pueden influir directamente sobre el funcionamiento ecosistémico de los humedales costeros. La conductividad es un factor que varía ampliamente en

los humedales costeros de la ZSACHC, donde es posible observar humedales costeros con rangos de salinidad subsalinos (1500-5000 $\mu\text{S}/\text{cm}$), hiposalinas (5000-35000 $\mu\text{S}/\text{cm}$) y mesosalinos (35000-70000 $\mu\text{S}/\text{cm}$). Esta variación posiblemente se deba a diversos factores como por ejemplo: el tamaño, la tasa de evaporación, la influencia marina y continental, entre otros factores climáticos que potencialmente se relacionan con la disponibilidad de agua de la cuenca en una gradiente latitudinal de norte a sur en la zona de estudio.

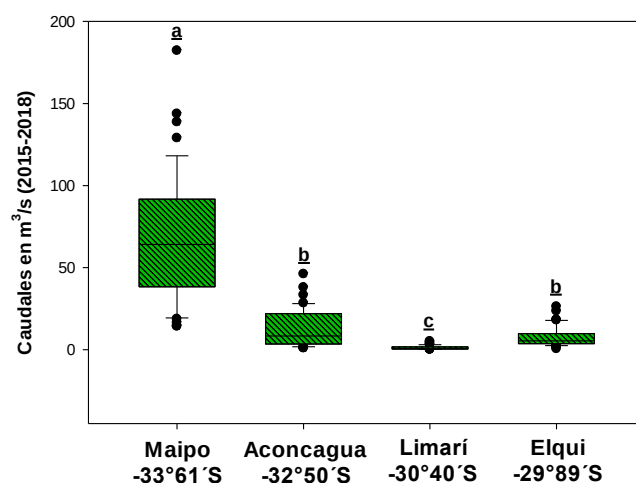


Figura I-1: Caudales de las principales cuencas de la ZSACHC entre los años 2015 y 2018. Los caudales disminuyen significativamente de sur a norte, sin embargo, el caudal del Aconcagua no es significativamente mayor que el del Elqui. Los datos corresponden al promedio mensual de cada mes desde el año 2015 hasta el año 2018 para cada cuenca. Todos los datos fueron descargados desde la página de la DGA (MOP). <http://snia.dga.cl/BNAConsultas/reportes>

De manera similar al escenario mundial, a nivel local los humedales costeros de la ZSACHC también son vulnerables y se han visto amenazados por diversos factores asociados a la actividad antrópica. En un documento hecho por el Ministerio del Medio Ambiente (MMA) que se titula “Estrategia Nacional de Biodiversidad 2017-2030”, se da cuenta de que la mayoría de los sistemas acuáticos costeros de Chile-central se encuentran en estado eutrófico [101]. Por otro lado, en un estudio desarrollado por Figueroa et al. (2009) en 20 humedales costeros pertenecientes a la ZSACHC, se informó que sólo un 35% de los humedales se encontraron con estado de conservación de muy buena o buena calidad [102]. Estos autores señalaron que el resto de los humedales se

encuentran en categoría de media, mala y muy mala calidad, destacándose en esta última categoría el humedal costero “El Sauce” ubicado en la localidad rural de Laguna Verde perteneciente a la región de Valparaíso [102].

El humedal costero “El Sauce”, se conforma principalmente por la contribución de aguas provenientes del estero “Las Cenizas” y el embalse la Luz, además de la escorrentía natural. Su cuenca, se encuentra crónicamente afectada por la contaminación de diversas fuentes, como por ejemplo las aguas residuales provenientes desde la PTAS ESVAL, ubicada en la localidad de Placilla. Además, el humedal costero “El Sauce” recibe lixiviados de un vertedero municipal y en su desembocadura por fuentes no puntuales de aguas domésticas originadas por la comunidad de “Laguna Verde”. Estudios desarrollados en la Universidad de Playa Ancha han podido constatar que tanto el estero “Las Cenizas”, como también el humedal costero “El Sauce” dentro de toda su extensión, presentan aguas de mala calidad según la norma chilena 1333 (NCh1333) [103, 104]. El humedal costero “El Sauce”, no cumplió con 10 de los 13 parámetros que exige esta normativa, entre los cuales, el DBO₅, la contaminación fecal, los nutrientes, cloruros, entre otros factores, superaron los valores máximos permitidos por la norma para el uso de riego [104]. Por lo tanto, el humedal costero “El Sauce” debido a su elevado grado de intervención durante un largo periodo de tiempo funciona como un ecosistema modelo para estudiar en detalle el impacto antrópico sobre las comunidades microbianas y su relación con las condiciones fisicoquímicas del agua, incluyendo variables de calidad del agua y GEI.

De esta manera asumiendo que la ZSACHC cuenta con una gran variedad de humedales costeros que presentan una gradiente en aumento de su conductividad y descenso en su impacto antrópico hacia el norte de la zona se planteó la siguiente pregunta de investigación:

¿Cómo influye la salinidad con otros factores ambientales y el impacto antrópico sobre las concentraciones de GEI y estructura del microbioma bentónico, incluyendo grupos funcionales asociados al reciclaje de GEI en humedales costeros de una zona semiárida de Chile central?

CAPÍTULO II. HIPÓTESIS Y OBJETIVOS

HIPÓTESIS

Desde los antecedentes recién mencionados, se pretende poner a prueba las siguientes hipótesis:

Hipótesis 1

El aumento en la salinidad disminuye la abundancia relativa de grupos funcionales nitrificantes, metanogénicas y metanotróficos en humedales costeros de la ZSACHC, disminuyendo las concentraciones de GEI.

Hipótesis 2

La descarga desde una PTAS aumenta la abundancia de comunidades metanogénicas y disminuyen la abundancia de comunidades nitrificantes y metanotrofas en el estero “El Sauce” (Laguna Verde, Valparaíso), incrementando las concentraciones de GEI.

OBJETIVOS

Objetivo general

Determinar la influencia de la conductividad y el impacto antrópico sobre la estructura del microbioma bentónico y las concentraciones de GEI en humedales costeros.

Objetivos específicos

- 1)** Identificar la relación entre la conductividad y la distribución de GEI (CO_2 , CH_4 , N_2O) de humedales costeros ubicados en un gradiente latitudinal de disponibilidad de agua e impacto antrópico en la zona semiárida de Chile central.
- 2)** Identificar la relación entre las comunidades microbianas bentónicas representativas de humedales costeros con la distribución latitudinal de la concentración de GEI y cambios físico-químicos del agua en la zona semiárida de Chile central.
- 3)** Determinar la influencia del efecto antrópico asociado a efluentes de plantas de tratamiento de aguas residuales sobre la estructura del microbioma bentónico y de grupos funcionales nitrificantes, metanogénicas y metanotróficos del estero El Sauce (Laguna Verde).

**CAPÍTULO III. DISSOLVED GREENHOUSE GASES AND BENTHIC
MICROBIAL COMMUNITIES IN COASTAL WETLANDS OF THE CHILEAN
COAST SEMIARID REGIÓN**

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DISSOLVED GREENHOUSE GASES AND BENTHIC MICROBIAL COMMUNITIES IN COASTAL WETLANDS OF THE CHILEAN COAST SEMIARID REGION

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ABSTRACT

Coastal wetlands are ecosystems associated with intense carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) recycling, modulated by salinity and other environmental factors that influence the microbial community involved in greenhouse gases production and consumption. In this study, we evaluated the influence of environmental factors on GHG concentration and benthic microbial community composition in coastal wetlands along the coast of the semiarid region. Wetlands were situated in landscapes along a south-north gradient of higher aridity and lower anthropogenic impact. Our results indicate that wetlands have a latitudinal variability associated with higher organic matter content at the north, especially in summer, and higher nutrient concentration at the south, predominantly in winter. During our sampling, wetlands were characterized by positive CO_2 μM and CH_4 nM excess, and a shift of N_2O nM excess from negative to positive values from the north to the south. Benthic microbial communities were taxonomically diverse with > 60 phyla, especially in low frequency taxa. Highly abundant bacterial phyla were classified into *Gammaproteobacteria* (*Betaproteobacteria* order), *Alphaproteobacteria* and *Deltaproteobacteria*, including key functional groups such as nitrifying and methanotrophic bacteria. Generalized additive model (GAM) indicated that conductivity accounted for the larger variability of CH_4 and CO_2 , but the predictions of CH_4 and CO_2 concentration were improved when latitude and pH concentration were included. Nitrate and latitude were the best predictors to account for the changes in the dissolved N_2O distribution. Structural equation modeling (SEM), illustrated how the environment significantly influences functional microbial groups (nitrifiers and methane oxidizers) and their resulting effect on GHG distribution. Our results highlight the combined role of salinity and substrates of key functional microbial groups with metabolisms associated with both carbon and nitrogen, influencing dissolved GHG and their potential exchange in natural and anthropogenically impacted coastal wetlands.

Keywords: Salinity, Estuary, Nutrients, 16S rRNA gene sequencing, Wetland microbiome.

1. Introduction

Wetlands are ecosystems that provide critical ecological functions that include organic matter decomposition and nitrogen and carbon recycling, among others. Globally, wetlands act as a source of CH₄ and N₂O [1], two of the most relevant greenhouse gasses (GHG). Compared with other aquatic natural sources, wetlands are the ecosystems with the greatest contributions to global CH₄ emissions outweighing the contribution of lakes, ponds and other water bodies [2].

Dissolved nutrients and GHG wetland inventories are driven by microbial metabolic processes, showing a temporary (daily, seasonal, monthly) and spatial variability along environmental changes, including solar radiation, temperature, salinity and redox potential, as well as microbial community composition dynamics [3–5]. Microbial processes, such as methanogenesis, are carried out by strict anaerobic archaea that convert acetate, H₂, CO₂, as well as methylated compounds into CH₄, producing up to 90% of total CH₄ available on the planet [6]. A diverse and metabolic versatile microbial community composed by methanotrophic bacteria and archaea, oxidizes CH₄ [7–9]. Besides oxygen, they can use different electron acceptors, including nitrate [10], sulfate [11], and metals, such as Fe and Mn [12, 13]. On the other hand, N₂O is aerobically produced during the first step of nitrification by ammonia-oxidizing bacteria and archaea or as an intermediate product of denitrification, the anaerobic reduction of NO₃⁻ to nitrogen gas [14]. The functional microbial communities involved in GHG are often found residing in wetlands sediments where they are subjected to a wide range of environmental fluctuations from natural disturbances to anthropogenic perturbations [3, 15].

The 6,500 km Chilean coastline harbors a great diversity of wetlands, which are widely distributed across different climatic regions influenced by oceanographic processes associated with the Humboldt Current System dynamics [16].

Geophysical processes, such as earthquakes and tsunami events also have a great impact on the coastline, resulting in coastal wetlands with distinctive environmental and biological characteristics, including a high biodiversity and bearing an exceptional endemism [17–19]. Due to this, several of those wetlands have been categorized as Ramsar sites [20]. Wetlands studies are mostly focused in Patagonia fluvial areas and peatlands recently reviewed by León et al. [21]. Based on GHG and microbiological characterization, these ecosystems have been classified as sensitive areas for global warming due to the impact on CH₄ emissions mediated by H₂/CO₂-based methanogens stimulation [22], highlighting the role of temperature as a driving factor of CH₄ emissions globally [23]. Additional studies have provided insights for desert coastal wetlands in Atacama mainly associated with drainage catchment areas and river mouths [24, 25] and hypersaline pools [26], where salinity and substrate availability control methanogenesis/sulfate reduction competition. To our knowledge, there are no studies exploring the relation between GHG distribution and microbial communities in coastal wetlands of the semiarid region of central-northern area, as a transition between the hyper arid and humid areas.

The Semiarid Zone of central-northern Chile (SAZCCh) holds permanent estuary coastal wetlands characterized by the presence of sand bars and water variable salinity, in general, saltier and warmer at north depending on the water sources [27]. This region has a desert-oceanic Mediterranean bioclimate, with precipitation concentrated in the winter season [28], however, experiencing a mega-drought for the last 30 years [29]. In addition, the wetlands along the Chilean coastline are subjected to variable sources of anthropogenic perturbations, such as, agriculture, mining, tourism, at arid northern area [24] and higher urbanization, litter, industrial and sewage disposal in the central north area [27]. In general, these anthropogenic activities have been shown to considerably affect water quality and quantity [24] and potentially also GHG dissolved concentration in the coastal wetlands semiarid region as reported globally. For instance, the extraction of water and changes of land-use as well as the discharges of agricultural fertilizers have been reported to induce freshwater wetlands salinization [30], triggering

wetlands eutrophication, and significantly increasing CH₄ and N₂O emissions [3]. The wide range of fluctuating environmental conditions and stressors present in the coastal wetlands belonging to SAZCCh provide a natural platform to explore how the environmental and anthropogenic factors contribute to shaping microbial communities composition and activity as well as the underlying biogeochemical processes related to GHG cycling.

In this study, the distribution of dissolved GHG and the benthic microbial community structure was explored for the first time in a selected group of estuarine coastal wetlands situated in landscapes along a south-north gradient of higher aridity and lower anthropogenic impact in central-northern Chile. Based on these aridity and anthropogenic influence gradient, we addressed the following objectives: 1) To determine physical and chemical variability including dissolved nutrients and GHG, 2) To identify the combination of salinity and other factors accounting for the variability of dissolved GHG, and 3) their role on benthic microbial community composition, including known functional groups. We hypothesize that salinity and latitudinal changes associated with water availability and nutrients enrichments, related with more anthropogenically influenced wetlands (polluted or urban), will increase dissolved GHG inventories by altering the contribution of nitrifiers, methanogens and methanotrophs in the benthic microbial communities. These results will provide for the first time a baseline related to GHG distribution along central-northern Chilean coastal wetlands, highlighting those environmental factors that predominantly affect their dynamic and the responsible key benthic microbial functional groups, serving as guidance for future studies in both natural and impacted coastal wetlands.

2. Material and methods

2.1 Study area and wetlands of the semiarid zone of central-northern Chile

The group of 24 estuarine type coastal wetlands were selected within the semiarid zone of central-northern Chile (SAZCCh) extending from latitude 29°82' to 33°77', considering their environmental surrounding conditions associated with

aridity and differential anthropogenic impact (**Figure III-1, Table S III-1**). The water source of these wetlands drain mainly from four larger and small catchment areas, Maipo, Aconcagua, Elqui and Limari (**Figure III-1**). Maipo and Aconcagua basins are considered low aridity basins within our latitudinal gradient since they received an annual rainfall average of 20.0 mm and a river discharge of 0.98–46.01 and 14.18–182.16 in Maipo and Aconcagua rivers, respectively (data obtained from the national water monitoring agency, <https://snia.mop.gob.cl/BNAConsultas/reportes>). In contrast, Elqui and Limari', as high aridity basins, are characterized by low annual rainfall (average of 0.4 mm) and where their main rivers (Elqui, Grande and Choapa rivers) registered an average annual flow of 3.5 m³ s⁻¹ [31]. Moreover, Valparaíso region encompasses more than one hundred glaciers, which represents more than tenfold the glaciers of Coquimbo region [31].

2.1a Wetlands from high aridity basins (HAB)

The wetlands sampled from HAB are located within the Coquimbo geopolitical region and are subjected to a variable anthropogenic perturbation. For instance, Punta Teatinos, the mouth of Elqui River and El Culebrón associated with the Coquimbo bay (**Figure III-1, Table S III-1**) are impacted by the fast urbanization and expansion of Coquimbo and La Serena cities (**Figure S III-1**). Whereas, another set of wetlands located further south, such as, Lagunillas, Estero Tongoy, Salinas Chica, Salinas Grande and Pachingo, are characterized by high biological diversity, some listed in RAMSAR category (see links to Ramsar sites in **Table S III-1**). In general, the vegetation of these coastal wetlands is mainly dominated by *Sarcocornia fruticosa* (Salinas) and *Typha angustifolia* (Tifales) [32].

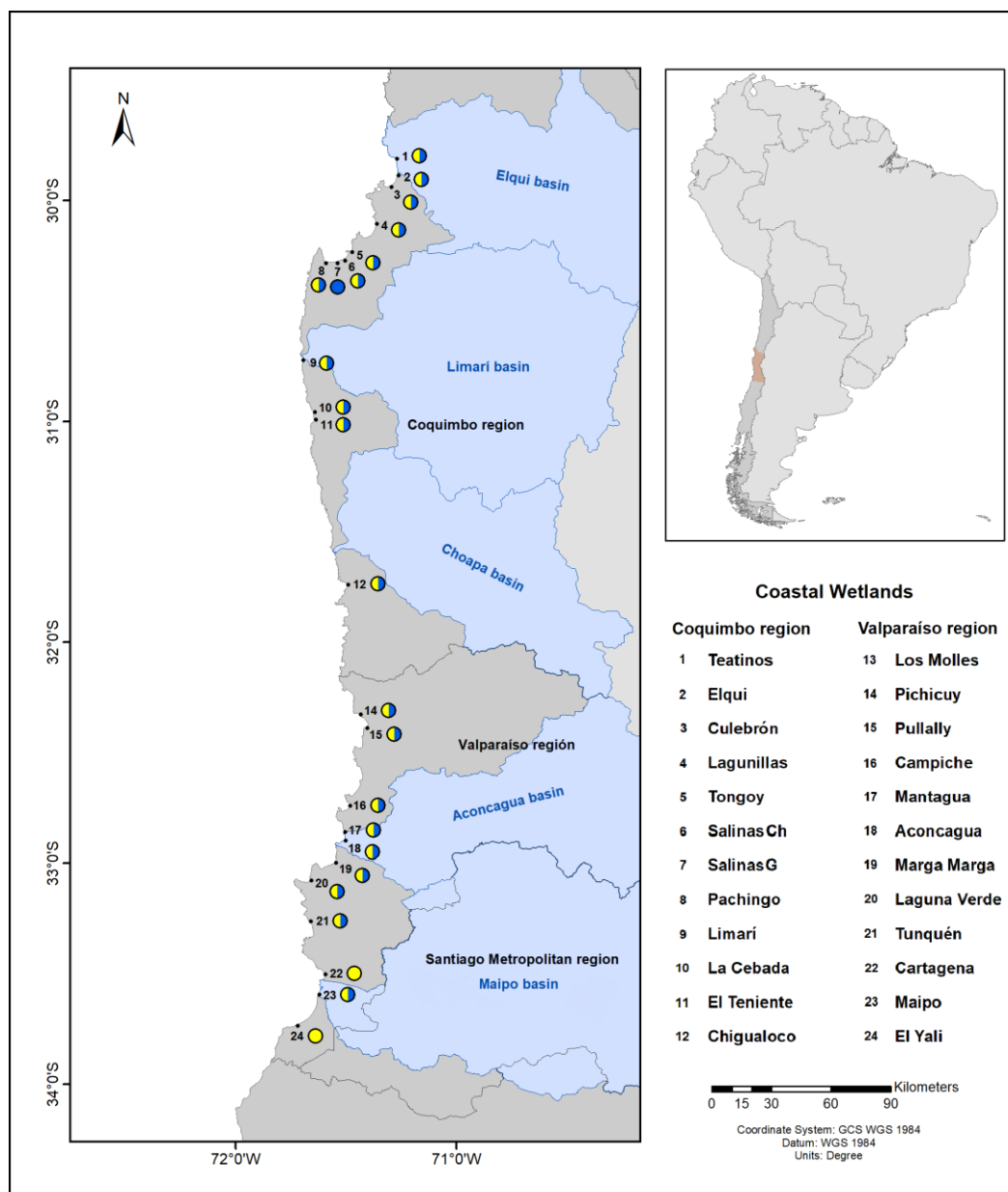


Figure III-1. Study area map showing the coastal wetlands sampled through latitudinal gradient in winter and summer (blue/yellow circles). Cartagena and Yali wetlands were only sampled in summer (yellow circles) and Salinas G wetland was only sampled in winter (blue circles). Map was created using cartography Shapefiles publically available, i.e., “Basin Layer” from the Water Resources Directorate (Dirección General de Aguas, DGA) and “Administrative layer” the Subsecretary of Regional Development (Subsecretaría de Desarrollo Regional y Administrativo (SUBDERE)). The wetland geographical position was mapped using QGIS, open code version 2.18 version software (<https://qgis.org/downloads/>).

2.1b Wetlands from low aridity basins (LAB)

The wetlands sampled from LAB are located within the Valparaíso geopolitical region (**Figure III-1, Table S III-1**). Wetlands belonging to LAB are characterized by higher anthropogenic impact, including agricultural, industrial, sewage and different types of contamination in the main rivers Aconcagua and Maipo, and other estuaries such as Los Molles, Campiche, Laguna Verde and Cartagena [33]. Moreover, this area concentrates a larger population and coastline causing a great impact on the coastal wetlands [17]. Only Tunquén and El Yali were classified as class II, attractive sites with high landscape value and natural reserve, also listed in RAMSAR [20] (S2 Fig).

2.2 Field sampling campaigns in the semiarid zone of central-northern Chile

Winter field campaigns in the Valparaíso Region (southern basins) were conducted on July 28, 29 and 30, and in the Coquimbo Region (northern basins) on August 25, 26 and 27, 2017. In summer, samples were collected in the Valparaíso Region on December 27, 28 and 29, 2017 and in the Coquimbo Region on January 3, 4 and 5, 2018. A summary of the wetland geographic positions, sampling dates and all the data available for our study for winter and summer seasons is included in the S1 Table.

All samples were taken from the estuary mouth in a single site at a low downstream flow area. All estuaries sampled are freely accessible through their mouths that adjoin public beaches. To visit natural protected areas with protection such as Yali permits were given for the Universidad de Playa Ancha by the Corporación Natural Forestal (CONAF) between the years 2014 and 2019 considering the policy of the national regulation of natural protected zones (Sistema Nacional de Áreas Silvestres Protegidas del Estado, SNASPE). The sampling was carried out wearing waders, using a standard procedure in the different wetlands, consisting in standing against the downstream flow to avoid sediment resuspension and sample contamination at a comparable depth of approximately 40–60 cm, without the influence of seawater intrusion or tides.

Water surface temperature, pH, dissolved oxygen (DO) and conductivity were determined in-situ using a multiparameter (probe YSI and ProDSS model) at a single point. Water samples for nutrient determination, i.e., nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{3-}) and silicic acid (SiO_4^{4-}), were collected in triplicate directly from the surface using a syringe and immediately filtered ($0.45\ \mu\text{m}$ GFF) in “Nalgene” bottles (60 mL). These samples were transported at 4°C during field campaigns and then frozen until analyzed at the laboratory. To determine GHG (CO_2 , N_2O and CH_4) concentrations, water samples were collected in triplicate using 20 mL gas tight vials, avoiding bubbles, immediately fixed with 50 μL mercury chloride (saturated) and hermetically sealed. The vials were stored at room temperature in the dark until performing the analyses at the laboratory. Samples for GHG analyses were collected in summer in all regions, and in winter only in the Valparaíso Region. Fine grain wet-sediment samples (occupying an approximate volume of 300–500 μL) were collected at a single point in cryotubes (1.5 mL) filled with RNA-later (Ambion, Life Technologies, USA), a buffer used to preserve both RNA and DNA (Gray et al., 2013) [34]. These samples were transported at 4°C to the laboratory and stored at -80°C until subsequent DNA extraction. Sediment sampled in the winter campaign from Salinas G, El Teniente and Chigualoco (Coquimbo Region) and Pichicuy, Laguna Verde Cartagena, Tunquén and El Yali (Valparaíso Region) and sediment sampled in the summer campaign from El Teniente and Chigualoco (Coquimbo Region) and Pichicuy, Marga Marga, Laguna Verde and Tunquén, were excluded from nucleic acid extraction because they contained high levels of very coarse grain, which could bias further analysis.

2.3 Chemical analyses determined in the water and sediments of the wetland sampled

Nutrient concentration in the water was determined using spectrophotometric methods. NO_2^- concentration was analyzed as indicated by Strickland and Parsons (1972), whereas NO_3^- , PO_4^{3-} and SiO_4^{4-} were measured using a nutrient autoanalyzer [35].

Dissolved GHG (CO₂, CH₄ and N₂O) concentrations were determined by gas chromatography using the headspace technique [36], in a gas chromatograph (Greenhouse GC-2014, Shimadzu). The chromatograph was equipped with an electron capture detector (ECD) for N₂O and a flame ionization detector (FID) for CO₂ and CH₄ determinations, attached to a methanizer for the conversion of CO₂ to CH₄.

GHG contribution ($\Delta[A]$) was estimated based on discrete GHG determination, following Sarmiento and Gruber (2004) [37]:

$$\Delta[A] = [A]_w - [A]_{equilibrium}$$

were, $[A]_w$ is the gas concentration in the water and $[A]_{equilibrium}$ is the equilibrium with the atmosphere gas concentration. The last one is calculated with the following formula:

$$[A]_{equilibrium} = S_A \times p^A$$

were, S_A is the solubility of the gas in the water A and p^A is the partial pressure of the gas A in the atmosphere. This calculation allowed us to classify each wetland as a potential source or sink for atmospheric GHG. Therefore, when $\Delta[A] > 0$, the wetland is a source of GHG, and if $\Delta[A] < 0$, the wetland is a sink of GHG.

Surface sediment samples were collected in sealable polyethylene bags at a single point in each wetland and transported at 4°C to the laboratory and stored at -20°C to the laboratory until analysis within a month. Organic matter was determined by the loss on ignition (LOI) method in a Muffle furnace. For granulometry determination, the samples were dried at 40°C and then analyzed using the standard method for mesh analysis to separate different sizes of sand, gravel, and silt.

2.4 Benthic microbial community composition characterization in the wetlands using 16S rRNA gene

Nucleic acid extraction and sequencing of 16S rRNA gene were used for benthic microbial community characterization. After RNA-later removal, 250 mg of wet sediment were weighted and then DNA was extracted using the DNeasy Power Soil DNA Isolation kit (MoBio Laboratories), following the manufacturer's instructions. DNA extracts were quantified with the dsDNA BR (Broad Range) Qubit 2.0 fluorometer (Thermo Fisher Scientific) and the DNA quality (260/290) was measured by spectrophotometry using Cytation Take3 nanodrop (BioTek). To verify 16S rDNA amplification, a PCR was carried out using the following primers: 27F (5'-AGRGTTYGATYMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3'). PCR conditions were the following: 95°C for 5 min followed by 35 cycles of 95°C for 45 s, 56°C for 45 s, and 72°C for 45 s, and a final elongation step of 5 min at 72°C using GoTaq1 DNA Polymerase (Promega Corporation). PCR products were checked using electrophoresis for 45 min at 70 volts in an agarose gel (1,5%). DNA extracts were sequenced using Illumina Miseq sequencing platform at Mr. DNA laboratory (Texas, USA), using in-house primers 515F (GTGYCAGCMGCCGCGGTAA) [38] and 806R (GGAATACNVGGGTWTCTAAT) [39] for the V4 region of the 16S rDNA gene. Sequences were deposited in the European Nucleotide Archive (ENA) under the project accession ID PRJEB44346 and the primary accession samples (ERR5725941- ERR5725957).

Sequencing data -processing analyses- were carried out using QIIME2 (v.2019.7) for sequences curation and taxonomic classification into ASVs (Amplicon Sequence Variant) [40]. First, sequences were imported to QIIME2 using Casava. The demultiplexed, poor quality and short sequences were extracted (S2 Table), leaving c.a. 210 bp length sequences, using qiime dada2 denoised-paired plugin [41]. The taxonomic classification was performed using the SILVA132 database (release 132–99% OTUs, 515–806 region) with the feature-classifier classify-consensus-vsearch plugin [42]. Then, readings on abundance of

known functional groups taxa associated with ammonia and methane recycling mainly related with oxidation metabolic pathways, known to produce N₂O or consume CH₄ respectively, were selected for their subsequent modeling with the environmental factors.

Alpha diversity analysis of the microbial community was estimated using ASVs (diversity, richness, and evenness). Bray-Curtis similarity analyses were performed at different taxonomy levels (phyla and ASVs) using squared-root transformed data. Principal Coordinates Analysis (PCO) was generated to assess changes in the microbial community's structure and its relationship with the environmental factors using Primer6 software [43]. Similarity Percentage (SIMPER) was estimated to identify the phyla and ASVs contribution to similarity/dissimilarity between seasons (winter and summer), saline category (hyposaline and mesosaline wetlands) and arid category.

2.5 Statistical analysis to identify environmental factors influence on GHG and benthic microbial community

To determine spatial and seasonal changes of the environmental factors, nutrients and OM a two-way ANOVA was used. Spearman multiple correlation analyses were carried out between conductivity and other environmental factors, with the GHG and functional groups, since most of the variables did not present a normal distribution (Shapiro-Wilk normality test, $p < 0.05$). A Non-metric multidimensional scaling (NMDS) was conducted to determine the influence of the sampled seasons (winter versus summer) as a factor, based on the stress level of NMDS fit, which shows the relationship between ordination distance and observed dissimilarity. It provides a flexible framework for modeling the relationship between individual predictors and dependent variables. Then, GAM was used to determine the contribution of conductivity with other environmental factors on GHG and functional groups variability. The "mgcv" package [44] and the R statistical software [45] were utilized.

GHG response was evaluated using the following linear predictor:

GHG response was evaluated using the following linear predictor:

$$GHG_i = \mu + s(C) + s(lon) + s(lat) + s(t) + s(pH) + s(d_oxy) + s(nitrate) + s(p) + s(s) + s(nitrite)$$

~Negative Binomial

were GHG_i is gas concentration; μ is the intercept; $s()$ is the spline softener; C is the conductivity effect on gas; i , lat is the latitude effect on gas; i , lon is the longitude effect on gas; i , t is the water temperature effects on gas; i , pH is the pH effect on gas; i , d_oxy is the dissolved oxygen effects on gas; i , nitrate is the nitrate effect on gas; i , p is the phosphate effect on gas; i , s is the silicic acid effect on gas; i , nitrite is the nitrite effect on gas i . For the dependent variable, a negative binomial error distribution with a link type “log” was used. GHG models were built on N = 32.

The functional group response was evaluated using the following linear predictor:

$$taxa_i = \mu + s(C) + s(lon) + s(lat) + s(t) + s(pH) + s(d_oxy) + s(nitrate) + s(p) + s(s) + s(nitrite)$$

~Negative Binomial

were taxa_i is taxa i , μ the intercept; $s()$, the spline softener; C is the conductivity effect on taxa; i , lat is the latitude effect on taxa; i , lon is the longitude effect on taxa; i , t is the water temperature effects on taxa; i , pH is the pH effect on taxa; i , d_oxy is the dissolved oxygen effects on taxa; i , nitrate is the nitrate effect on taxa; i , p is the phosphate effect on taxa; i , s is the silicic acid effect on taxa; i , nitrite is the nitrite effect on taxa i . For the dependent variable, a negative binomial error distribution with a link type “log” was used. Taxa models were built on N = 34, except the models that included nitrite, since they had N = 24.

Structural equation modeling (SEM) was used to determine multivariate causal relationships by considering key factors previously identified by GAM as significant to explain either the variability of GHG and specific functional groups. Those factors include latitude, pH, conductivity, nitrate and organic matter. The resulting latent variables were “environmental variables, ENV”, “functional groups,

FG” and “GHG”, and the structural relationship between them was explored considering as a theoretical causal model that ENV influences FG community structure resulting in changes in GHG distribution. SEM model was run by means of lavaan project (ugent be) in R studio, using diagonally weighted least squares (DWLS) estimation to fit the weight covariance matrix using only the diagonal of this matrix and the NLMINB optimization method. Chi-square ($p > 0.05$) test and root mean square error of approximation (RMSEA), comparative fit index (CFI) and Tucker-Lewis Index (TLI) were included to evaluate the model fit.

3. Results

3.1 Coastal wetlands physical and chemical conditions in the water and sediments of the latitudinal gradient of the semiarid zone of central northern Chile.

The physical and chemical conditions in the water and sediments varied depending on the wetland geographic localization and the season sampled. The average temperature ranged between 11 and 30.5°C, reaching significantly higher values ($p < 0.001$) in the high aridity basins (HAB) compared with the low aridity basins (LAB) and temperature was significantly greater in summer ($p < 0.01$) unlike winter (**Figure III-2A; Table III-1**). Other variables, such as pH ranges (7.18 –10.38 pH units) registered significantly higher values ($p < 0.001$) in summer compared to winter (**Figure III-2B; Table III-1**). Conductivity was the environmental factor that showed the highest variability, ranging from 0.550 to 42.528 and from 1.162 to 63.519 mS/cm in winter and summer, respectively, with significantly higher values ($p < 0.05$) in the HAB versus LAB (**Figure III-2C, Table III-1**). In addition, DO ranged from 4.27 to 19.34 mg/L in winter and from 6.9 to 17.18 mg/L in summer (**Figure III-2D**). Differences among dissolved nutrients across seasons were not observed, however, nitrate and silicic acid showed significantly greater values in LAB compared to HAB (**Figures III-2E and H, Table III-1**). Phosphate and nitrite registered low variability (**Figures III-2F and G**) and phosphate did not show

significant spatial and seasonal changes (**Table III-1**), but reached maximum concentration in polluted wetlands, in winter and summer. Indeed, waters from Maipo, Laguna Verde, Aconcagua, Marga Marga and Elqui, all estuaries located close to urban or industrial areas, registered higher concentrations of phosphate (20-35 μM), nitrate (100-500 μM) and/or nitrite (10-70 μM) than the other wetlands (**Table S III-1**).

The wetland sediment granulometry was associated with different grain sizes ranging from silt to very coarse sand types (**Figure S III-3**). The high level of silt and very fine sand sediments were characteristic of wetlands from HAB, and only a few from LAB, mainly in summer (**Figure S III-3B**). Besides, HAB wetlands showed significantly higher organic matter content ($> 4 \text{ g}$), in addition organic matter content was significantly greater in summer compared to winter (**Figure S III-3 and Table 1**).

Dissolved GHG concentration in the wetlands revealed a broad range: CH_4 , 4.4–1206 nM, CO_2 , 0.6–507.2 μM and N_2O , 3.7–83.3 nM (90–1,941 ppb) (**Table S III-1**). CO_2 presented significantly higher concentrations in winter than in summer (One-Way ANOVA $p < 0.001$) in LAB wetlands. In general, most wetlands are CO_2 sources, except for six wetlands sampled in summer, i.e., Chigualoco, Los Molles, Pichicuy, Campiche and Yali, (**Figure III-3A**). All wetlands registered an excess of CH_4 , except Limarí, which showed dissolved concentration in equilibrium with the atmosphere (**Figure III-3B**). N_2O was the only GHG showing a marked gas excess shifts across latitude from positive to negative values, suggesting that LAB wetlands act as a potential N_2O source, whereas HAB wetlands, except for the Elqui, act as an N_2O sink (**Figure III-3C**). Interestingly, Laguna Verde (in summer) and Maipo (in winter), two of the wetlands most severely impacted by pollution, registered the highest magnitude of N_2O excess (**Figure III-3C**).

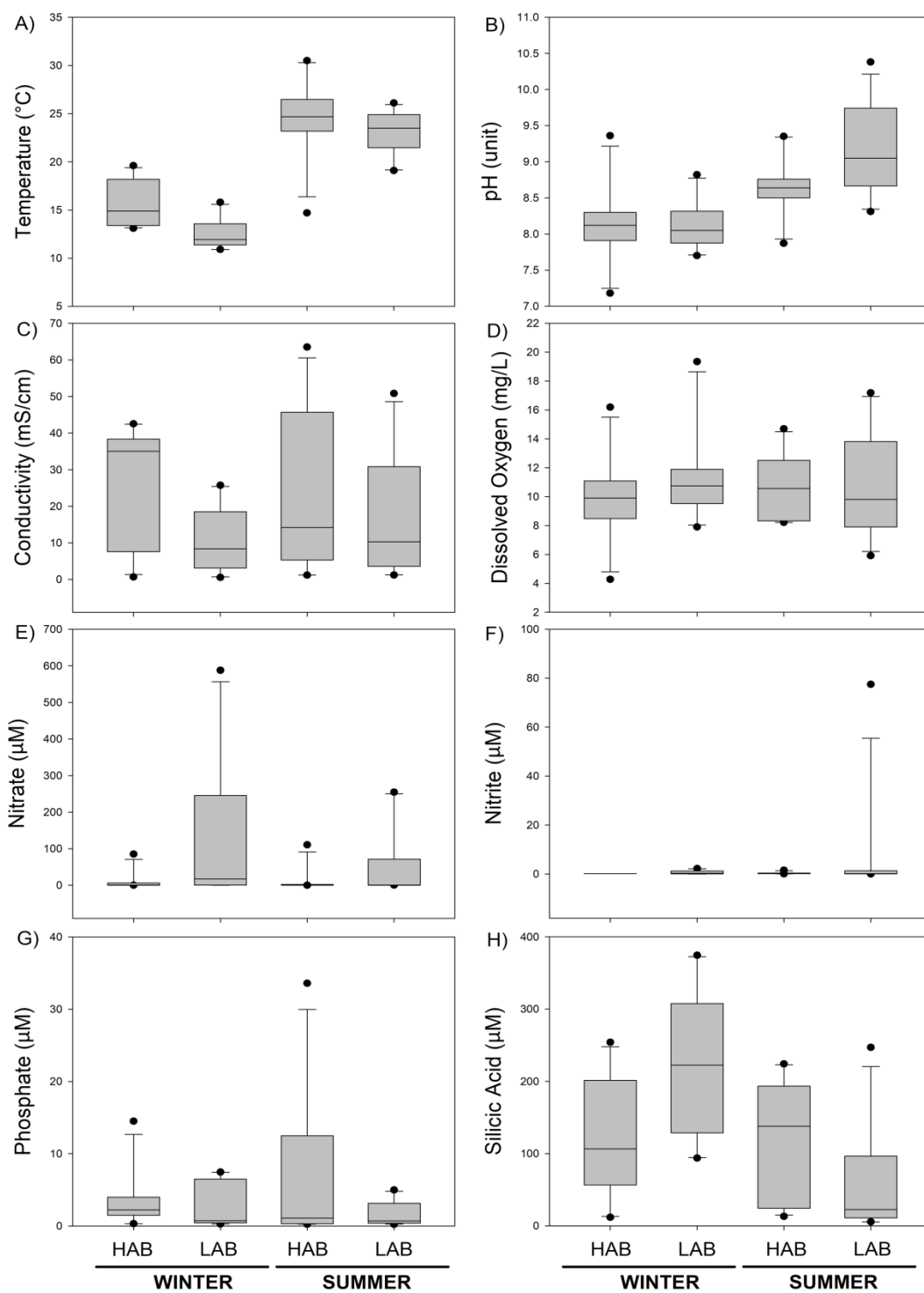


Figure III-2. Physical and chemical conditions and dissolved nutrients determined in high versus low aridity basins wetlands water during summer and winter. Outliers are indicated as black dots. The detailed table containing data for each wetland can be found in S2 Table.

Table 1. Two-way ANOVA comparing physical and chemical conditions, dissolved nutrients and organic matter determined in high versus low aridity basins wetlands water during summer and winter. Significant values ($p < 0.05$) are highlighted in grey.

Variable	Season	HAB vs LAB	Shapiro-Residuals	Levene
Temperature	4.86.E-03	1.38.E-14	9.35.E-03	3.98.E-01
pH	5.08.E-06	1.15.E-01	1.51.E-01	6.28.E-01
Conductivity	4.38.E-01	4.51.E-02	6.71.E-02	1.44.E-01
Dissolved Oxygen	8.34.E-01	4.25.E-01	3.97.E-02	8.12.E-01
Nitrate	2.37.E-01	2.25.E-02	3.58.E-07	4.34.E-02
Phosphate	7.28.E-01	8.31.E-02	8.24.E-09	3.75.E-01
Silicic Acid	1.60.E-01	1.42.E-02	5.46.E-02	5.86.E-02
Nitrite	—	—	—	—
OM	4.717.E-03	4.097.E-06	4.554.E-02	3.06.E-03

3.2 Relationship between environmental factors, nutrients and their modulating role on GHG distribution

Spearman multiple correlation analysis indicates that conductivity presented a high number of significant correlations with GHG concentration, including silicic acid ($r = -0.502$, $p < 0.001$), nitrate ($r = -0.488$, $p < 0.001$), CO₂ ($r = -0.624$, $p < 0.001$), CH₄ ($r = -0.400$, $p < 0.05$) and N₂O ($r = -0.495$, $p < 0.01$) (**Table S III-3**). Water temperature was significantly correlated with N₂O ($r = -0.632$, $p < 0.001$) and pH ($r = 0.59$, $p < 0.001$) and pH was correlated with CO₂ ($r = -0.704$, $p < 0.001$) and dissolved oxygen ($r = 0.424$, $p < 0.01$) (**Table S III-3**). In addition, the sediment organic matter (OM) was significantly correlated with temperature ($r = 0.463$; $p < 0.001$) and very fine sand ($r = 0.439$; $p < 0.01$) and very coarse sand ($r = -0.396$; $p < 0.05$) (**Table S III-3**).

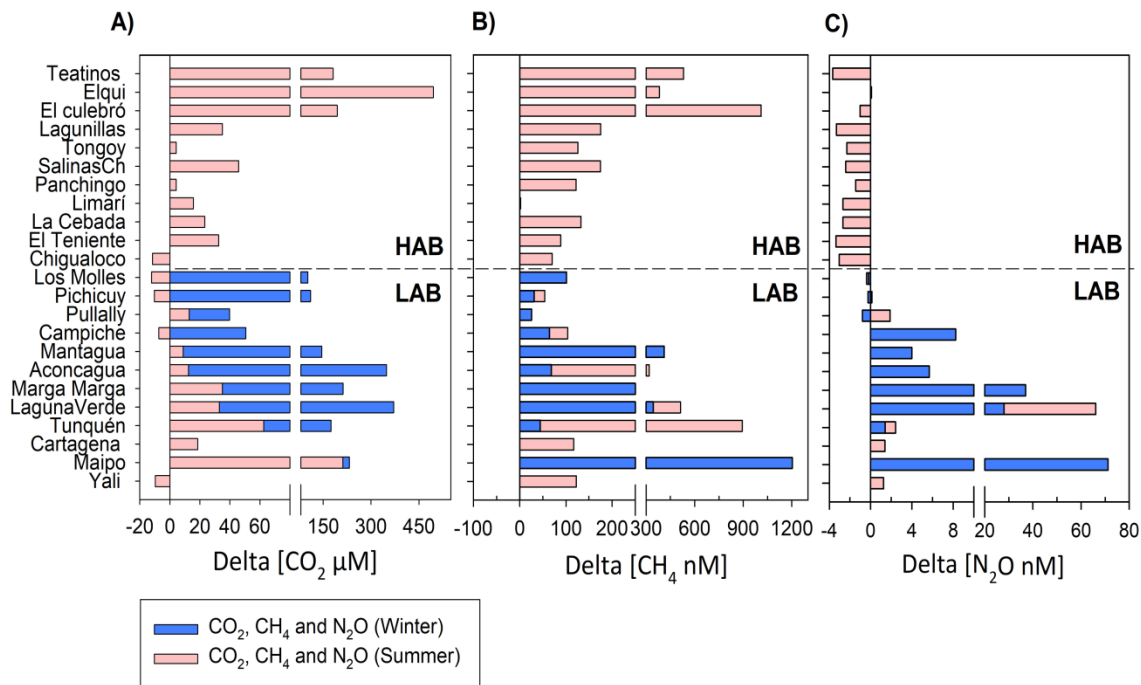


Figure III-3. CO₂ (A), CH₄ (B) and N₂O (C) greenhouse gases “excess” that were estimated from original concentration in the water in winter (pink bars, only for LAB wetlands) and summer (blue bars, for HAB wetlands).

Generalized additive models (GAM) indicate that among all the variables analyzed, conductivity and latitude were significant predictors of CH₄ distribution in the wetlands, accounting for 60.7% and 415.6 of deviance and AIC, respectively (**Figure III-4A and B; Table III-2**). In the case of CO₂, conductivity and pH were explanatory variables with 81.4% and 330.7 of explained deviance and AIC, respectively (**Figure III-4C and D; Table III-2**). Regarding N₂O, conductivity was not a good predictor to account for changes in its distribution across wetlands. The two most significant predictors to account for changes in N₂O concentration were latitude and nitrate, representing 89.4% of the deviance and 191.5 of AIC (**Figure III-4E and F; Table III-2**).

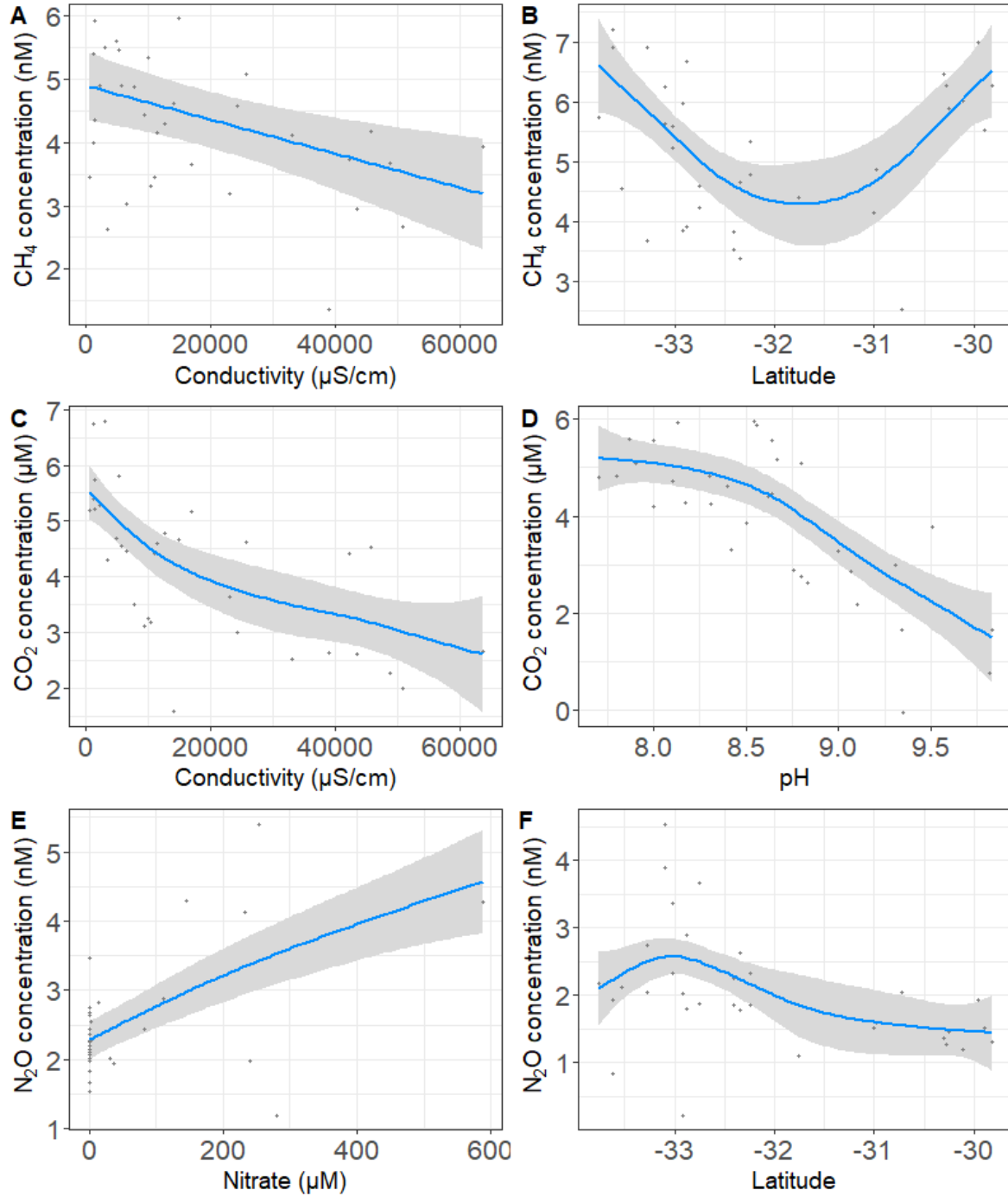


Figure III-4. GAM models showing the relationships between the greenhouse gas distribution and different environmental factors: CH₄ with conductivity (A), Latitude (B), CO₂ with conductivity (C) and pH (D) and N₂O with nitrate (E), and latitude (F). Axes “Y” correspond to the effect of the different predictor variables.

Table III-2. GAM models significant results using environmental factors with nutrients as predictor variables and greenhouse gasses as response variables (n=33).

GHG	Models	AIC	ED	p-value
CH ₄	~ s(C) +s(Lat)	415.6	60.7	C = 1.65e-3; Lat = 4.53e-6
	~ s(C)	430.9	34.2	C = 4.33e-4
CO ₂	~ s(C) +s(pH)	330.7	81.4	C = 2e-16; pH = 2e-16
	~ s(C)	364.6	31.4	C = 1.06e-5
N ₂ O	~ s(Nitra) +s(Lat)	191.5	89.4	Nitra= <2e-16; Lat = 5.66e-5
	~ s(C) +s(Nitra)	213.2	74.7	C = 0.105; Nitra = <2e-16
	~ s(Nitra)	213.3	73.2	Nitra = <2e-16
	~ s(Nitri)	225.4	62.5	Nitri = <2e-16
	~ s(C) +s(Nitri)	225.7	61.9	C = 1.29e-02; Nitri = 7.18e-5
	~ s(C) +s(Lat)	226.0	61.9	C = 2.24e-2; Lat = 2.25e-5
	~ s(C) +s(MO)	224.1	55.3	C = 3.968e-03; MO = 1.71e-4
	~ s(C) +s(Temp)	231.4	55.8	C = 9.6e-4; Temp= 4.1e-4
	~ s(C) +s(Lon)	234.2	49.5	C = 1.11e-4; Lon = 6.646e-3
	~ s(C)	239.8	33.1	C = 2.59e-4
Analyses combination used: s(C); s(C) +s(Lat); s(C) +s(Lon); s(C) +s(Temp); s(C) +s(pH); s(C) +s(DO); s(C) +s(Nitra); s(C) +s(P);s(C) +s(Nitri); s(C) +s(MO); s(Nitra); s(Nitri). Acronyms: s() = smoothness, C = conductivity, Lat = latitude, P = phosphate, S = silicic acid, OM = Organic Matter, Nitri = nitrite Temp = temperature, DO = dissolved oxygen.				

3.3 Microbial community structure and composition characteristics in the coastal wetlands

In general, alpha diversity estimators indicate that there was a higher variability in winter than in summer, i.e., richness (201–675 versus 416–593 ASV), diversity (3.784–5.237 versus 4.236–5.193 H') and evenness (0.62–0.80 versus 0.66–0.81), (**Table S III-4**). A discrete set of wetlands, including Limarí, La Cebada, Mantagua, Aconcagua and Maipo, presented contrasting seasonal changes, characterized by the highest richness (> 550 ASVs) in winter than in summer (**Table S III-4**). Principal Coordinate Analysis (PCoA) allowed us to visualize the variability of microbial community structure at the ASV level, for which PC1 and PC2 accounted for c.a. 32.7% of the total variability (**Figure S III-4**). The overlaid correlated environmental variables visualized as vectors highlighted that the microbiome structure was mostly influenced by conductivity (Spearman correlation $r = 0.644$ with PC1) categorized according to their salinity i.e., mesosaline versus hyposaline (> 20 mS/cm vs < 20 mS/cm). Nitrate was also significantly correlated with PC1 ($r = -0.312$), likewise, temperature ($r = 0.619$), organic matter ($r = 0.522$) and silt ($r = -0.370$) correlated well with PC2.

The microbial composition analysis of wetland sediments showed that bacteria (2,821,908 total reads) predominate over archaea (70,397 total reads), representing 97.53% and 2.47%, respectively. The bacterial community was associated with 64 phyla, among which 49 of them contributed less than 1% of the total retrieved sequences (**Figure S III-5**). The most abundant bacterial phylum was Proteobacteria, showing a winter versus summer variability; in the summer, these changes include a slight decrease in the average relative abundance of Gammaproteobacteria and Alphaproteobacteria from 16.26 to 13.04% and from 10.79 to 9.33%, respectively. In contrast, Deltaproteobacteria increased smoothly in summer compared with winter (8.76 versus. 11.41%) (**Table S III-5**). Other abundant phyla in the wetlands were Bacteroidetes and Cyanobacteria, but only the latter showed higher abundance in summer (11.17%) compared with winter (7.82%). When considering wetlands salinity, Gammaproteobacteria class was the most abundant taxa contributing to 15.55% in hyposaline wetlands and 13.82% in mesosaline wetlands (**Table S III-5**). On the other hand, archaeal community was composed of 9 phyla mainly affiliated to Thaumarchaeota, Nanoarchaeota and Euryarchaeota (**Figure S III-6**). SIMPER analysis indicated that in winter Thaumarchaeota and Nanoarchaeota account for 68.1 and 18.14% of the total archaeal sequences, while in summer their contribution was 24.76 and 66.09%, respectively. SIMPER analysis using salinity predicted slightly higher percentages of contribution to the archaeal sequences in hyposaline compared with mesosaline wetlands for Thaumarchaeota (49.37 vs. 45.73%) and Euryarchaeota (12.35 vs. 11.76%). In contrast, the opposite was found for Nanoarchaeota, which showed an increase from 37.65% to 41.9% (**Figure S III-6 and Table S III-5**).

3.4 Relationship between environmental factors, nutrients, and factors reshaping microbial functional groups

The microbial communities associated with wetlands were characterized by many functional groups, including archaea and bacteria ammonia oxidizers, methanogens and methanotrophs, which significantly correlates with different environmental factors sampled (**Table S III-6**). *Nitrosomonas* sp. ($r = 0.67$) and

other genera affiliated with nitrifying bacteria including AOB and *Nitrospira* sp. ($r \geq 0.55$) presented a significantly high correlation with N_2O (**Table S III-6**). Ammonia and methane oxidizing bacteria belonging to Betaproteobacteria (*Nitrosomonas* sp. and *Methylotenera* sp., respectively) and the nitrite oxidizing bacteria (*Nitrospira* sp.), were the only genera significantly correlated with conductivity and frequent enough to allow modeling with environmental factors including conductivity (**Table III-3, Table S III-1**). Moreover, summer and winter data were combined considering that non-metric multidimensional scaling analysis indicated that season (summer versus winter) was not an influencing factor of the structure of the mentioned functional groups (**Figures S III-7A and B**). GAM results generated with a selected microbial functional groups considering their representation ≥ 28 wetlands (S1 Table) indicated that potential factors accounting for Betaproteobacteria order distributions were conductivity and OM and nitrate (**Figures III-5A and B**), based on the deviance and AIC obtained (**Table III-3**).

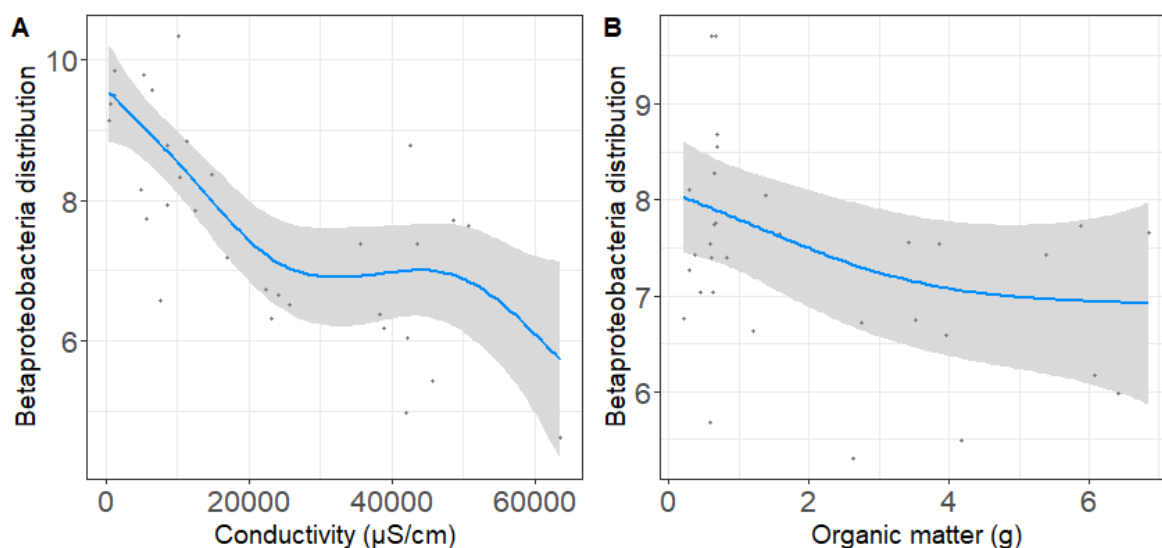


Figure III-5. GAM models showing the relationship between the Betaproteobacteria changes with conductivity (A) and organic matter (B) as predictors. Axes “Y” correspond to the effect of the different predictor variables.

In addition to that, SEM model analysis was performed using conductivity, pH, latitude, season, nitrate and organic matter to determine potential multivariate causal relationship on the variability of a selected microbial communities known for their contribution on ammonia and methane oxidation named as functional groups (FG) and then identify the FG influence on GHG using a path diagram presented in **Figure III-6**. The result showed that latent variables (ENV, Environment; FG, Functional Group; GHG, Greenhouse gases) were differentially and significantly affected by the observed factors (**Figure III-6**). A relationship between the latent variables Env—FG and FG—GHG presented significant ($p < 0.001$) linear correlation $r^2 > 0.75$ (**Figure III-6, Table S III-7**). The SEM model was supported by a good prediction, based on Chi-square (X^2 , $p = 0.301$) and the model fit indexes obtained (i.e., RMSEA = 0.058; TLI = 0.965 and CFI = 0.971 (R output is summarized in **Table S III-7**).

Table III-3. GAM models significant results using environmental factors with nutrients as predictor variables and functional groups as response variables (n=34).

GHG	Models	AIC	ED	p-value
<i>Betaproteobacteria</i>	~ s(C) +s(OM) ~ s(C) +s(P) ~ s(C) + s(Lat) ~ s(C)	563.4 596.2 599.0 601.1	67.2 63.8 60.0 55.7	C = $p < 2e-16$; MO = 2.56e-2 C = $p < 2e-16$; P = 4.55e- 3 C = $p < 2e-16$; Lat = 0.0444 C = $p < 2e-16$
<i>Methylothera</i> sp	~ s(C) +s(Nitri) ~ s(C)	229.3 343.1	52.6 20.0	C = 1.56e-4; Nitri = 8.061e-3 C = 1.08e-3
<i>Nitrosomonas</i> sp	~ s(C)	394.8	28.5	C = 6.73e-5
<i>Nitrospira</i> sp	~ s(C) +s(Temp) ~ s(C) +s(P) ~ s(C) +s(DO) ~ s(C)	486.4 487.5 487.4 488.0	39.1 29.8 24.4 19.2	C = 9.82e-3; Temp = 3.53e-3 C = 3.86e-3; P = 7.512e- 2 C = 1.42e-4; DO = 5.0427e-2 C = 1.74e-3
Analyses combination used: s(C); s(C) +s(Lat); s(C) +s(P); s(C) +s(MO); s(C) +s(Nitri); s(C) +s(Temp); s(C) +s(DO). Acronyms: s() = smoothness , C = conductivity, Lat = latitude, P = phosphate, S = silicic acid, OM = Organic Matter, Nitri = nitrite Temp = temperature, DO = dissolved oxygen.				

4. Discussion

Coastal wetlands in the semiarid region of central-northern Chile are characterized by heterogeneous physical and chemical conditions in waters and sediments across latitude and season. Indeed, our study revealed that hyposaline waters and coarse-gravel sediments are more frequently observed in the southern basins, belonging to the low aridity basins (LAB). In contrast, mesosaline water conditions and very fine-sand sediments with higher organic matter content were mostly found in the northern basin's wetlands, belonging to the high aridity basins (HAB) (**Figure III-2A and B, Figure S III-3A and B**). These results are consistent with the geographic location of the wetlands and landscapes scales, associated with aridity shifts from north to south basins. Our study found that LAB wetlands belonging to southern basins were characterized by high nutrient enrichments (**Figure III-2, Table S III-1**), which could be potentially associated with a greater anthropogenic pressure from urban areas and greater utilization of water for industry and agriculture in the southern respect to their northern counterpart. This is in agreement with previous reports that showed the significant impact of irrigated agriculture on N-content in the Aconcagua watershed [46]. In addition, various pollution sources, besides domestic water, were reported in Laguna Verde, a wetland categorized for having the worst state of conservation compared with other wetlands from central-northern Chile [32], including water discharge from sewage treatment plants and percolation of municipal landfill [47]. Moreover, the central-northern area of Chile is experimenting a strong climate change scenario including a megadrought [29], that influences the water availability and quality in the basins [48], and potential variable salinization mechanisms, besides seawater intrusion, as observed in other coastal wetlands [30].

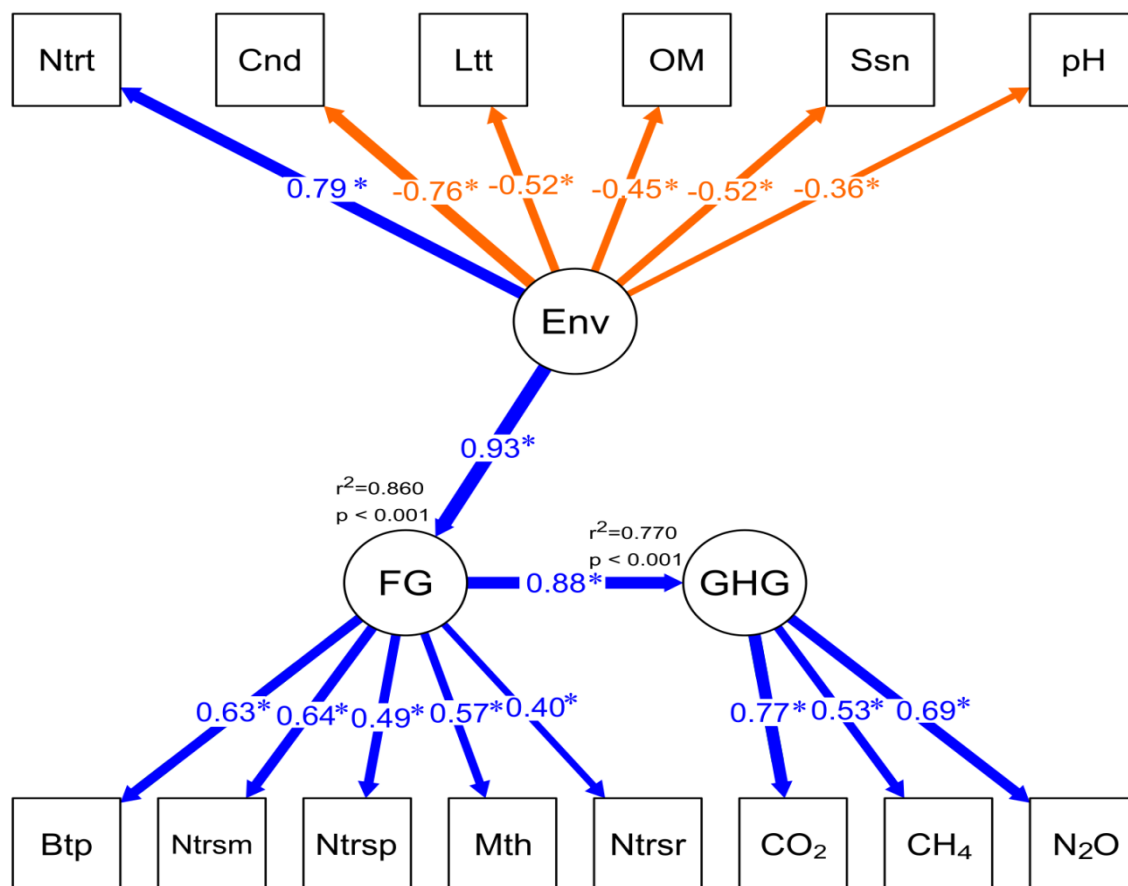


Figure III-6. Structural equation model (SEM) showing the influences of observed factors on latent variables and the identification of the effect of the environment (ENV) on functional microbial groups (FG) and the FG influence on Greenhouse gases (GHG). Arrows represent the relationships between observed factors and latent. Significance was signed with an asterisk (<0.05) and path coefficients values were included for latent potential causality effects.

4.1 Dissolved greenhouse gases and potential regulation by environmental factors associated with latitude changes HAB to LAB

The results of dissolved greenhouse gasses (GHG) distribution indicated that the coastal wetlands situated at LAB presented higher concentration compared with their counterparts from HAB, resulting in a greater GHG excess estimation, which indicates the potential of wateratmosphere exchange. As in other estuarine wetlands a positive CH₄ excess was registered, whereas CO₂ was more variable, showing a high excess magnitude in winter at the LAB area (**Figure III-3**). On the other hand, N₂O excess indicated a clear latitudinal trend, as HAB wetlands were potential N₂O sinks, while LAB wetlands were potential sources of N₂O to the

atmosphere in summer. We are aware that the results of analyses related to GHG excess can be considered as a potential trend, considering that only a single point of each estuarine wetland was studied, and samples were exclusively associated with water. Moreover, a great diel variability is expected in the water and vegetation of coastal wetlands, as reported in other wetlands associated with *P. australis*, one of the most prevalent aquatic plants inhabiting estuaries around the world [5]. In our study sites, a greater influence of vegetation is expected in the wetlands located in LAB (see pictures **Figures S III-1 and 2**). Future studies should consider flux estimation approaches that include sediments, water and surrounding biomes to identify net GHG contribution of the wetlands in central-northern Chile and corroborate our baseline result.

Our GAM models indicate that conductivity was a common factor predicting CO₂ and CH₄ variability (**Figure III-4, Table III-2**). However, the variability of the prediction for CH₄ and CO₂ increased when considering latitude and pH, respectively (**Figure III-4A and D; Table III-2**). On the other hand, latitude and nitrate were the two best predictors accounting for the distribution of N₂O concentrations and not conductivity. Our results are consistent with previous studies in temperate areas considering the role of salinity combined with the effect of nutrients in some wetlands. For example, a decrease in CO₂ and CH₄ effluxes in the Yellow River Delta wetland [49] and the Yangtze Estuary [50], associated with higher salinity, water depth and tide cycles [50–52].

On the other hand, experimental studies combining the effect of salinity and nutrients enrichment in freshwater wetlands have reported that independent of salinity, N₂O increment and reduction/suppression of CH₄ production were associated with nitrate inputs [53]. Therefore, nutrients play an important role in the dynamics of GHG in estuaries and wetlands. In the study area, potential latitudinal changes in the role of microbial processes involved in nitrogen recycling, such as nitrification and denitrification are expected. Supporting this idea, we found that nitrate as a product of nitrification was significantly correlated with the N₂O in the coastal wetlands (**Figure III-4E and F; Table S III-3**), as reported elsewhere for

constructed wetlands [54]. Moreover, our correlations suggest a greater potential role of nitrification in the N_2O accumulation in LAB wetlands compared with HAB. Likewise, reads assigned to ammonia oxidizing bacteria and archaea were correlated with N_2O and NO_3^- (e.g., *Nitrosomonas* and *Nitrosoarchaeum*, and other genera S6 Table). In addition, the predominant N_2O sink estimated in the HAB wetlands potentially indicates an active consumption that could be associated with anaerobic processes, such as denitrification.

4.2 Microbial community structure and key functional groups potentially associated with GHG regulation

The benthic microbial community associated with SAZCCh coastal wetlands was found to be diverse and exhibited changes associated with environmental factors including nutrients, temperature and conductivity variability. For example, PCoA analysis revealed that at ASV level, the microbial community structure was predominantly grouped according to hyposaline and mesosaline conditions, with slight changes in their diversity (**Figures S III-4A and B**). Salinity is a well-known environmental factor modulating microbial community structure at global [55] and local [56] scales, such as estuaries [57]. In these transition areas, the microbial community structure changes along salinity gradients were associated with a greater stochastic input of microbes from marine habitats during the tidal regime [58] and by survival and adaptation of those microorganisms to freshwater environments [59].

Functional groups associated with GHG recycling were also retrieved in our 16S rRNA gene survey, including ammonia oxidizing archaea and bacteria, methanogens and methanotrophs, showing significant correlations with environmental variables including GHG and negative correlations with conductivity (**Table S III-6**). GAM analyses support environmental factors such as conductivity, latitude and OM as predictor factors in the distribution of Betaproteobacteria (**Table III-3**).

The high number of negative correlations with conductivity agrees with previous reports focused in functional groups. For instance, *Nitrosomonas* dominated the freshwater areas compared to *Nitrospira* which was more abundant in brackish water of the Ythan estuary (Scotland) [60]. Previous research has documented that the abundance of other methanotrophic species, such as *Methylobacillus* *oxifera* and *Methylocystis* sp., decreased in areas with higher salinity of coastal wetlands [61, 62].

Structural equation modeling (SEM) supports the importance of the environment (ENV) considering environmental factors, such as, conductivity, pH, OM, nutrients, geographical and temporality conditions sampled (latitude and season) as significant predictors for the selected functional microbial group (FG) conformed by five grouped selected taxa (Betaproteobacteria, *Nitrosomonas*, *Nitrospira*, *Methylobacillus* and *Nitrosoarchaeum*), which are further related with GHG as latent variable.

The ammonia oxidizer *Nitrosomonas* sp. presented a significant contribution to the variability within the FG using SEM modeling and also individually based in GAM associated with conductivity. This result supports reports associated with *Nitrosomonas* sp. as a predominant ammonia oxidizer in estuaries including anthropogenically perturbed wetlands [63]. Therefore, possibly responsible for the higher N₂O concentration found in the LAB wetlands resulting in positive excess.

The potential role of functional groups associated with both methanotrophic, nitrification and denitrification pathways should be further studied to evaluate their role in N₂O production. More adequate methods, such as specific quantification by RT-PCR, together with a higher spatiotemporal replicability, could help to generate more integrative analyses and model the contribution of key microbial groups in nutrients GHG dynamics, including potential latitudinal trends associated with a higher accumulation of N₂O in the water of hyposaline wetlands situated at the southern basins.

5. Conclusion

Coastal wetlands in the semiarid zone of central-northern Chile are heterogeneous and very distinctive ecosystems characterized by a latitudinal variability of their physicochemical conditions and their benthic microbial community. The findings of this study indicate that salinity combined with different environmental factors depending on the specific GHG studied accounted for the dissolved greenhouse gas variability. The results revealed a latitudinal shift in the dissolved N₂O distribution likely associated with the input of allochthonous nutrients probably associated with higher anthropogenic influence on these wetlands located in the south. Salinity and nutrients were factors associated with the composition of key functional groups of microorganisms, including methane-oxidizing, such as *Methylobacter* sp. and *Methylothermobacter* sp. Our study highlights how a set of highly diverse wetland microbiomes respond to the combined effect of salinity and nutrient input, contributing directly or indirectly to the regulation of the biogeochemistry of the wetlands. The dynamic of these coastal water bodies is becoming especially important to get a better understanding of the potential greenhouse gases emissions, where a greater effort is needed to integrate spatiotemporal variability of nutrients, GHG and microbial communities.

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8. Supplementary material

Supplementary figures



Figure S III-1. Coastal wetlands pictures sampled in the Coquimbo region.



Figure S III-2. Coastal wetlands pictures sampled in the Valparaíso region.

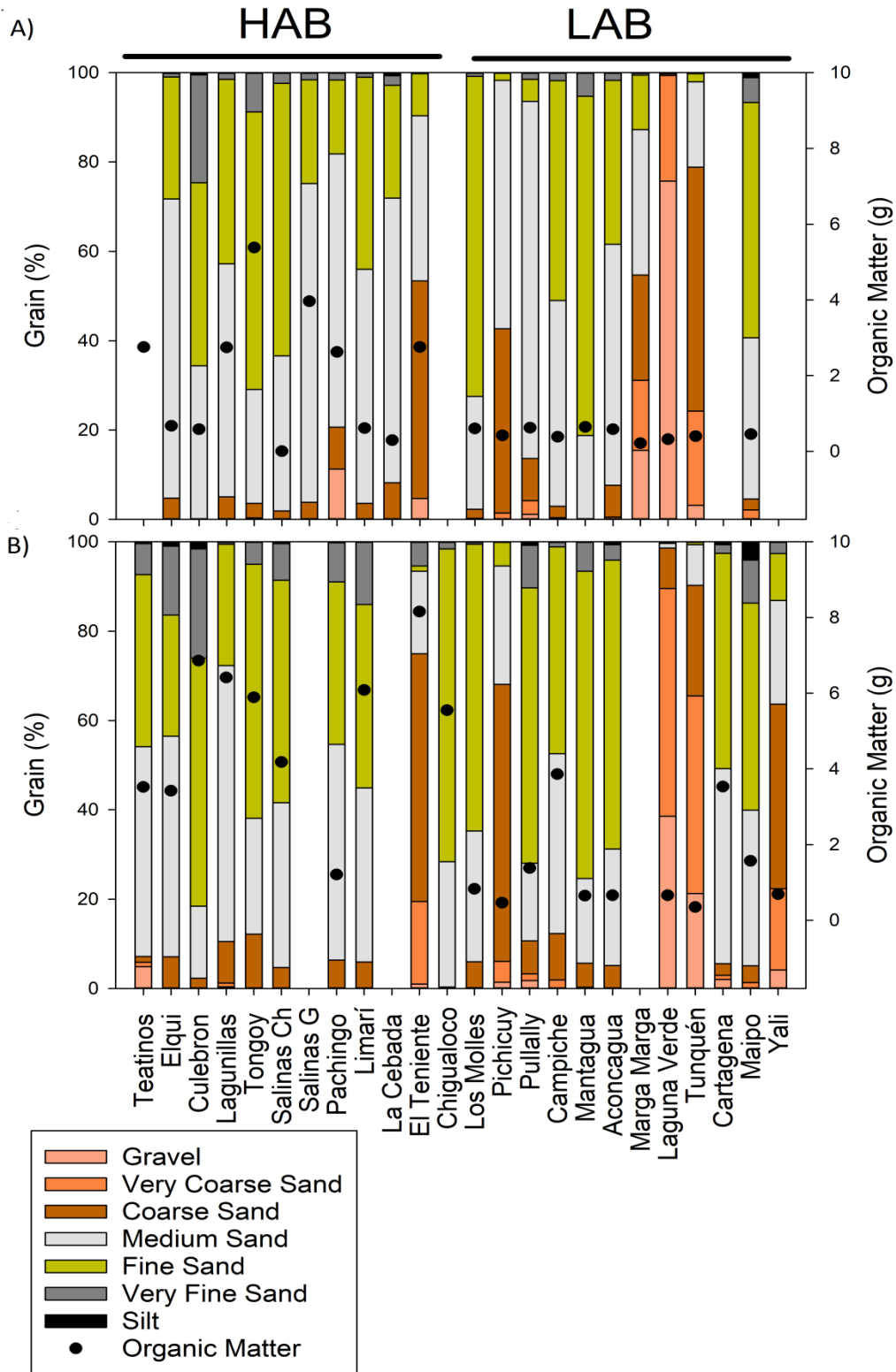


Figure S III-3. Granulometry and organic matter in the sediments of the sampled wetlands in winter (A) and summer (B). Teatinos, Chigualoco, Cartagena and Yali wetlands were only measured in summer and Salinas G, La Cebada and Marga Marga wetlands were only measured in winter.

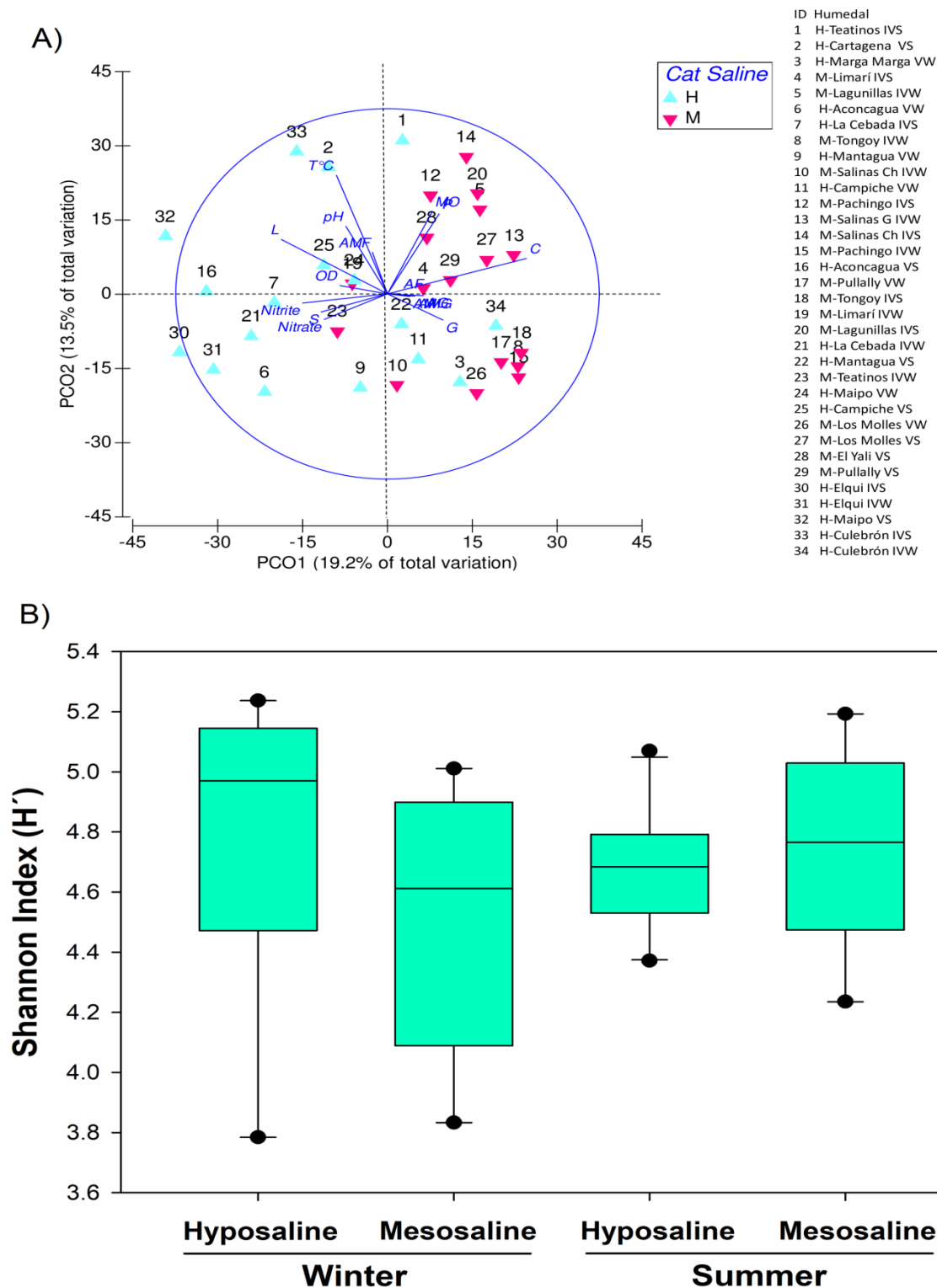


Figure S III-4. A) Principal Coordinate Analysis in hyposaline and mesosaline wetlands. Acronyms in the wetland names are H= hyposaline, M= mesosaline, IV= Coquimbo Region, V= Valparaíso Region, W= Winter, V= Summer. B) Boxplot (median, quartiles, and outliers) showing grouped richness values considering the saline categories.

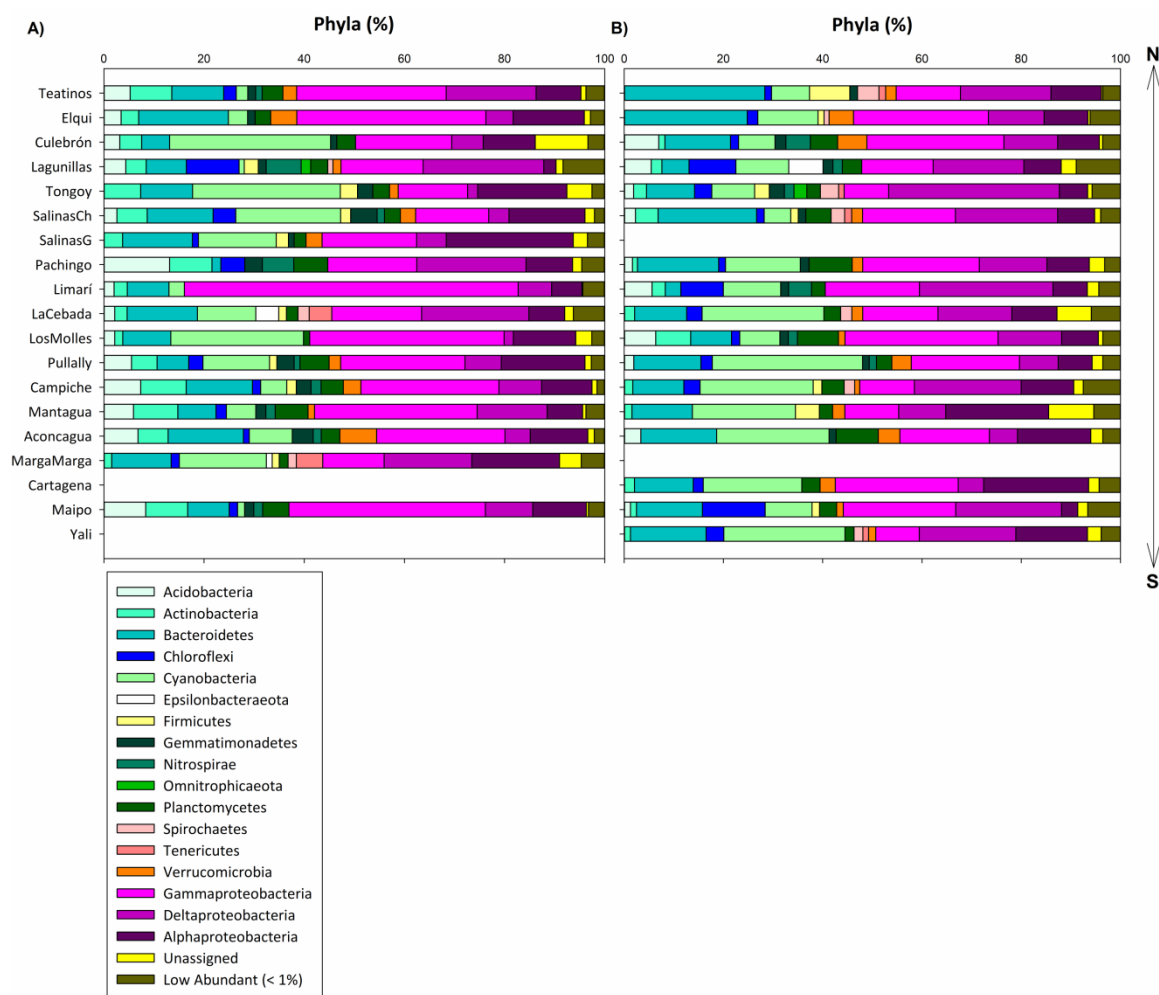


Figure S III-5. Benthic bacterial phyla observed in winter (A) and summer (B). The Proteobacteria phylum was separated in the Gamma, Delta and Alphaproteobacteria classes. Low abundant Phyla (<0.1%, Acetothermia, Aegiribacteria, Anck6, Armatimonadetes, Atribacteria, BHI80-139, BRC1, CK-2C2-2, Caldiserica, Calditrichaeota, Chlamydiae, Cloacimonetes, Coprothermobacteraeota, Dadabacteria, Deferribacteres, Deinococcus-Thermus, Dependientiae, Elusimicrobia, Entotheonellaeota, Epsilonbacteraeota, FBP, FCPU426, Fibrobacteres, Fusobacteria, GAL15, Halanaerobiaeota, Hydrogenedentes, Kiritimatiellaeota, LCP-89, Latescibacteria, Lentisphaerae, MAT-CR-M4-B07, Margulisbacteria, Marinimicrobia (SAR406 clade), Modulibacteria, Nitrospirae, Omnitrophicaeota, PAUC34f, Patescibacteria, Poribacteria, Rokubacteria, Schekmanbacteria, Synergistetes, TA06, Thermotogae, WOR-1, WPS-2, WS1, WS2, WS4, Zixibacteria.

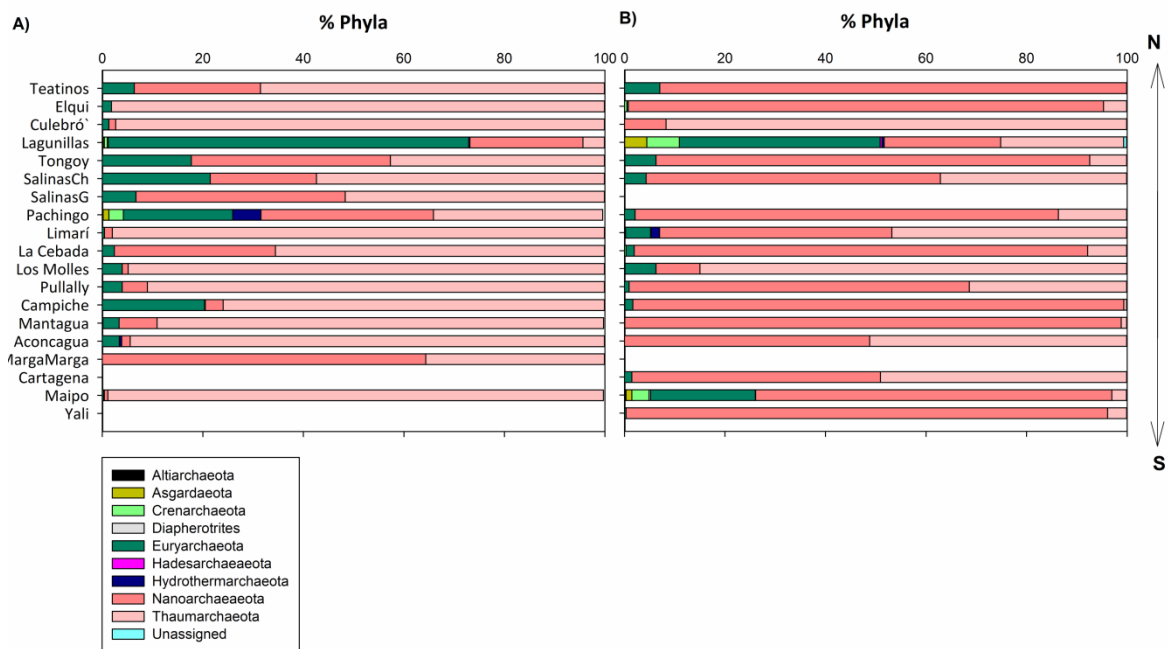


Figure S III-6. Benthic archaea phyla retrieved in winter (A) and summer (B).

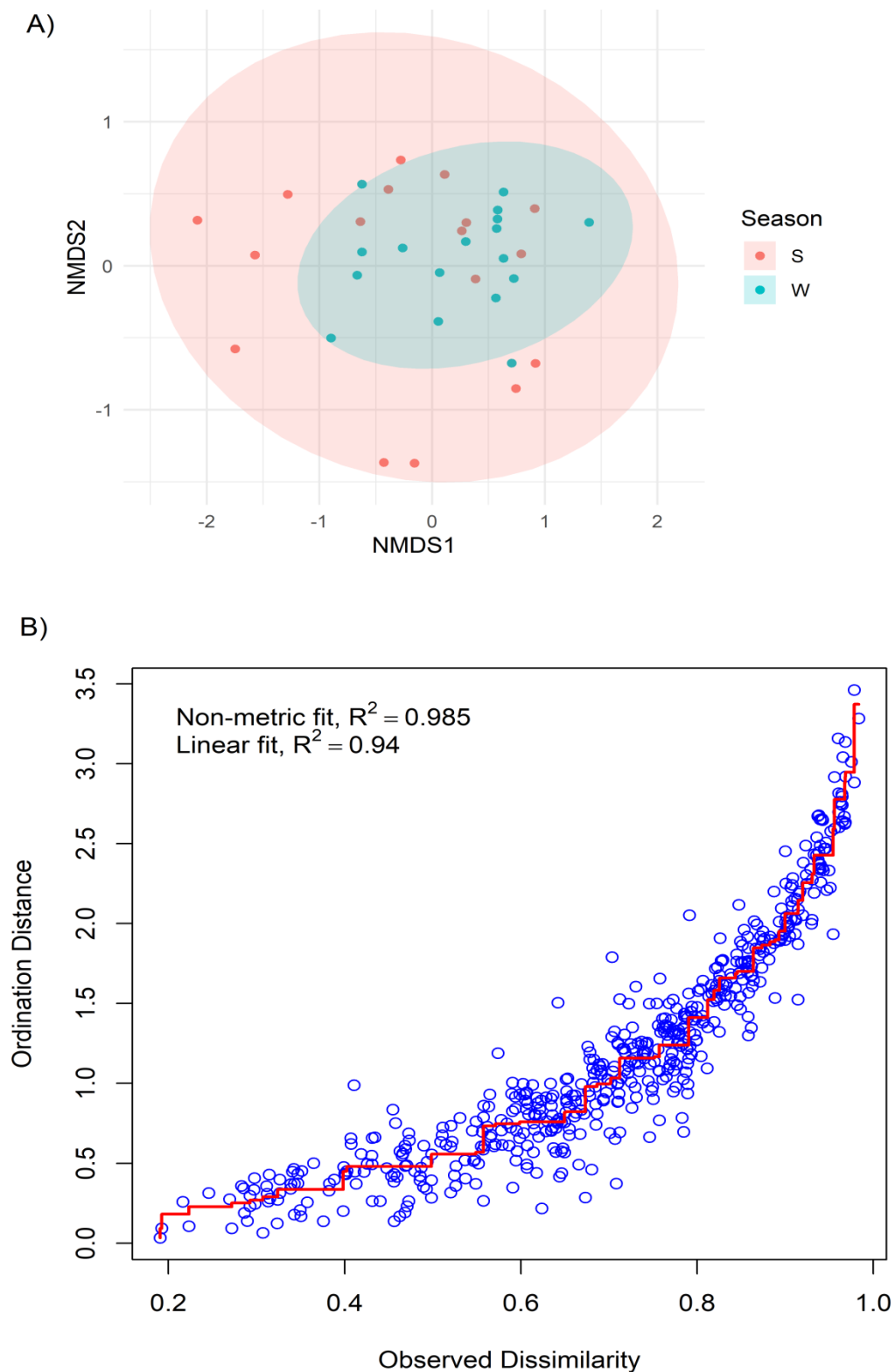


Figure S III-7. A) Non-metric multidimensional scaling (NMDS) analysis for selected functional groups (methane oxidizing bacteria, ammonia oxidizing bacteria and archaea, and methanogens). B) Linear fit associated with the NMDS analysis.

Supplementary tables (bigger tables were not able to be attached to the text, therefore, they were referred to their DOI)

Table S III-1. Physical, chemical and biological (functional groups and phyla) data determined during our study from a selected group of estuarine coastal wetlands of the central-northern Chile. <https://doi.org/10.1371/journal.pone.0271208.s001>

Table S III-2. Sequencing reads count information before and after denoise and links for repository download. CBD = Count Before Denoise and CAD = Count After Denoise.

Season	Region	Estuary	CBD	CAD	Sequence Link
Winter	Coquimbo	Teatinos	69104	62947	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	Elqui	78326	68154	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	Culebrón	52344	44440	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	Lagunillas	54780	48737	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	Tongoy	80195	69202	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	Salinas Chica	52950	45351	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	Salinas Grande	90974	79018	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	Pachingo	15604	12627	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	Limarí	177637	145232	ENA Browser (ebi.ac.uk)
Winter	Coquimbo	La Cebada	163255	146744	ENA Browser (ebi.ac.uk)
Winter	Valparaíso	Los Molles	92307	81854	ENA Browser (ebi.ac.uk)
Winter	Valparaíso	Pullally	84504	73595	ENA Browser (ebi.ac.uk)
Winter	Valparaíso	Campiche	97631	87581	ENA Browser (ebi.ac.uk)
Winter	Valparaíso	Mantagua	101926	90313	ENA Browser (ebi.ac.uk)
Winter	Valparaíso	Aconcagua	125666	112354	ENA Browser (ebi.ac.uk)
Winter	Valparaíso	Marga Marga	123793	107338	ENA Browser (ebi.ac.uk)
Winter	Valparaíso	Maipo	90968	81114	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	Teatinos	113280	101730	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	Elqui	64787	55676	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	Culebrón	93151	84681	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	Lagunillas	114372	104334	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	Tongoy	112201	98327	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	Salinas Chica	109738	95343	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	Pachingo	156152	141968	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	Limarí	99088	88849	ENA Browser (ebi.ac.uk)
Summer	Coquimbo	La Cebada	101123	91694	ENA Browser (ebi.ac.uk)
Summer	Valparaíso	Los Molles	69370	60434	ENA Browser (ebi.ac.uk)
Summer	Valparaíso	Pullally	89026	75201	ENA Browser (ebi.ac.uk)
Summer	Valparaíso	Campiche	64986	59073	ENA Browser (ebi.ac.uk)
Summer	Valparaíso	Mantagua	114609	104334	ENA Browser (ebi.ac.uk)
Summer	Valparaíso	Aconcagua	69370	60434	ENA Browser (ebi.ac.uk)
Summer	Valparaíso	Cartagena	113917	101987	ENA Browser (ebi.ac.uk)
Summer	Valparaíso	Maipo	73221	59761	ENA Browser (ebi.ac.uk)
Summer	Valparaíso	Yali	139496	127349	ENA Browser (ebi.ac.uk)

Table S III-3. Spearman multiple correlation analyses between physical and chemical water conditions, nutrients and greenhouse gases distribution, sediment organic matter content and granulometry. The values show the correlation coefficient (r) and the three colors the significance level of p-value.
<https://doi.org/10.1371/journal.pone.0271208.s003>

Table S III-4. Alpha -diversity indexes (richness, Shannon index and evenness) of the benthic microbial community from the coastal wetlands in winter and summer.

	Winter			Summer		
Wetlands	Richness (ES)	Shannon Index (H')	Evenness	Richness (ES)	Shannon Index (H')	Evenness
Teatinos	438,6	4,14	0,6686	442,1	4.402	0,7012
Elqui	504,9	5.052	0,7984	504,1	5,07	0,8028
Culebrón	418,2	4.805	0,7873	430,2	4.573	0,7363
Lagunillas	386,5	4.437	0,7367	463,8	4.453	0,7065
Tongoy	425,6	4.778	0,7757	455	4.785	0,7623
SalinasCh	473,4	5.011	0,8045	510,4	4.762	0,744
SalinasG	502,7	4,94	0,778	ND	ND	ND
Pachingo	201	3.974	0,7494	469,7	4.236	0,6645
Limarí	570,9	4,68	0,7127	444,7	4,54	0,7284
La Cebada	674,3	4,97	0,7421	450,5	4.586	0,7349
Los Molles	374,1	3.833	0,6317	511,5	5.111	0,8075
Pullally	430,8	4.545	0,7283	593,3	5.193	0,7975
Campiche	378,8	3.784	0,6197	416	4.758	0,7776
Mantagua	603,3	5.237	0,7999	539,2	4.626	0,7127
Aconcagua	634,3	5,23	0,7859	526,2	4.372	0,6738
Marga Marga	520,3	4.872	0,7591	ND	ND	ND
Cartagena	ND	ND	ND	555,7	4.743	0,7273
Maipo	611,3	5,06	0,7701	468,1	4.862	0,7774
El Yali	ND	ND	ND	544,4	4.769	0,7322

Table S III-5. SIMPER analyses associated with the comparison of microbial communities contributing >5% associated with the different categories analyzed, i.e., conductivity, latitude and season sampled.

Domain	Taxa	% Hypos aline	% Mesosal ine	% HAB	% LAB	% Winte r	% Summ er
Bacteria	Gammaproteobacteria	15,55	13,82	14,27	15,1	16,26	13,04
	Alphaproteobacteria	9,85	10,46	11,38	8,93	10,79	9,33
	Deltaproteobacteria	9,65	10,39	9,73	10,5	8,76	11,41
	Bacteroidetes	11,67	10,12	11,53	9,37	10,23	10,8
	Cyanobacteria	8,8	10,12	10,69	8,04	7,82	11,17
	Planctomycetes	5,33	5,94	5,55	5,71	5,82	5,36
Archaea	Thaumarchaeota	49,37	45,73	48,05	45,6	68,1	24,76
	Nanoarchaeota	37,65	41,9	42,76	35,1	18,14	66,9
	Euryarchaeota	12,35	11,76	ND	16,5	13,33	ND

Table S III-6. Spearman multiple correlation analyses between physical-chemical variables (environmental factors and nutrients) and the functional group. The green taxa were selected for the GAM analyses. The values show the correlation coefficient (r) and the three colors the significance level of p-value.

<https://doi.org/10.1371/journal.pone.0271208.s006>

Table S III-7. Structural equation model (SEM) statistical summary results

<https://doi.org/10.1371/journal.pone.0271208.s007>

CAPÍTULO IV. MICROBIAL AND BIOGEOCHEMICAL SHIFTS IN A HIGHLY ANTHROPOGENICALLY IMPACTED ESTUARY (“EL SAUCE” VALPARAÍSO)

Capítulo en elaboración:

Francisco Pozo-Solar, Marcela Cornejo-D’Ottone, Roberto Orellana, Carla Acuña, Cecilia Rivera, Polette Aguilar-Muñoz, Céline Lavergne, Verónica Molina. Para ser publicado en la revista “Water” de la MDPI.

MICROBIAL AND BIOGEOCHEMICAL SHIFTS IN A HIGHLY ANTHROPOGENICALLY IMPACTED ESTUARY (“EL SAUCE” VALPARAÍSO)

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ABSTRACT

Coastal zones are sensitive ecosystems to climate change and anthropogenic pollution, resulting in a potential loss of biodiversity and ecosystem services through eutrophication, nutrient imbalances, among others. The coastal El Sauce catchment, Central Chile, is under multiple anthropogenic pressures including a wastewater treatment plant (WWTP) discharge, which its broad effect remains underexplored. In order to assess the impact of the WWTP on the El Sauce stream, the benthic microbial communities and key functional groups variability (i.e., nitrifiers, methanogens and methanotrophs) were determined by 16S rDNA high throughput sequencing and by functional genes quantification, respectively, during two contrasted seasons in three catchment areas (pre-, WWTP- and post-discharge). Microbial communities' structure profiles were associated with the water quality, nutrients, greenhouse gas (GHG) distribution and the organic matter isotopic signatures in the sediments for the first time in this ecosystem. Results showed that organic matter isotopic signatures using nitrogen and carbon ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$), and the physicochemical conditions in the El Sauce Estuary changed from pre- and WWTP discharge (i.e., a pH decrease of 0.5 unit and an increase of 4-6°C the water temperature). The WWTP discharge area was characterized by low nutrient concentration and significantly higher GHG (> 600 μM CO_2 , > 30000 nM CH_4 and > 3000 nM N_2O). In addition, benthic microbial communities structure shifted spatially and seasonally, including specific phyla known as sewage bioindicators, such as Firmicutes (Clostridiales order) and Bacteroidetes. Besides, other taxa were enriched or only retrieved in the sediments of the WWTP influenced area, e.g., Tenericutes, Lentisphaerae, Synergistetes and LCP-89. Methanogens were enriched near the WWTP discharge compared to the predischage site in both seasons, while methanotrophs and ammonia oxidizers were unfavored only during winter. Our results indicate that the WWTP discharge impacts biogeochemical conditions in the El Sauce catchment modifying the benthic microbial communities including a decrease in key functional groups able to mitigate CH_4 and regulate nutrients recycling in these aquatic ecosystems.

Keywords: Wetland, Greenhouse gases, organic matter, benthic microbial community, qPCR, 16S rRNA genes.

1. Introduction

Organic matter (OM) is a central factor regulating nutrient recycling in several aquatic ecosystems. Processes involved in controlling the transformation of OM are especially relevant in coastal environments, such as estuaries and wetlands, since they contribute to global carbon mitigation [1]. Estuarine OM is composed of a diverse mixture of allochthonous and autochthonous sources [2]. While autochthonous OM enriched by plant-derived polymers is locally originated from the high primary productivity associated with phytoplankton and aquatic vegetation, allochthonous OM is often advected from rivers, tributaries, groundwater, or allocated sources mainly from terrestrial origin. The seasonal distribution of OM sources, its quantity, quality and environmental physical and chemical characteristics, are key factors governing biogeochemical processes in estuaries. Therefore, it affects the net flux of nutrients to coastal marine ecosystems. Several of those elements are currently threatened by climate change and anthropogenic activities [3–5]. Indeed, many estuaries receive industrial, domestic, agricultural and wastewater discharges, which often are highly enriched by fractions of sewage and fossil fuels, and may include complex OM mixtures [6,7] and other pollutants [8,9] resulting in eutrophication, biodiversity loss [10] and affecting aquatic ecosystem functions [11].

Among other environmental factors, the origin, composition and distribution of OM play a relevant role in reshaping the structure and composition of natural microbial communities of estuaries [12–14].. Anthropogenic OM sources and composition were reported to influence water quality and microbial community taxonomic composition and structure in aquatic ecosystems [15]. Wastewater treatment plants (WWTP) are facilities in which several processes aim to remove solids as well as transform liquid wastes into an acceptable final effluent. Partial degradation of OM and nutrients mineralization occur through a series of stages, such as activated sludge, aeration tanks, bioreactors, and disinfection processes before water is discharged to the environment. However, multiple studies have

reported that WWTPs are important sources of several contaminants that may negatively impact aquatic ecosystems, including those of emerging concern [16–18]. Besides persistent chemicals residual, OM and microorganisms remain in treated waters influencing both bio-physical and ecological conditions, especially when the receiving water body is a semi-closed aquatic transition ecosystem, such as estuaries with higher water residence times [19–22]. Several lines of evidence suggest that wastewater discharge in estuaries increases greenhouse gas (GHG) emissions to the atmosphere [16,23,24], as well as other freshwater ecosystems, like river mouths [25] and lakes [26]. WWTP discharges carry over a significant proportion of remaining microbes associated with the human gut microbiome, including pathogens and other microbial communities besides Coliform bacteria. These microbes are typical indicators of wastewater that can shape the natural community structure and substantially affect their biogeochemical activity [27]. In addition, WWTP discharge changed the water quality by increasing its temperature, altering nutrient concentration and stoichiometry, as well as, adding antibiotics and other emerging pollutants that affects the microbial community structure [28]. Moreover, WWTP could cause shifts in functional microbial groups, for instance, methanogenic communities are favored, while methanotrophs and phototrophs are disfavored in WWTP impacted zones [29,30].

El Sauce catchment, located in Laguna Verde, Valparaíso, has been considered an eutrophic system for several years mainly due to runoff and loading of various sources of pollution from the constant water discharge of a WWTP, as well as inputs from leachates from a municipal landfill and domestic wastewater [31]. Nevertheless, it is unknown how these disturbances influence GHG and the native microbiome including functional groups associated with GHG recycling. This study aimed to determine the influence of WWTP discharge on the overall biogeochemical conditions along the El Sauce Estuary in winter and summer, including the changes on dissolved nutrient and GHG concentration (CO_2 , CH_4 and N_2O), OM quality and benthic microbial community structure.

2. Material and Methods

2.1 Study area and sampling procedures

El Sauce Estuary is located in Valparaíso (Central Chile; 33°6'S—71°38'O) and its main water flow from Las Cenizas basin area is connected with “La Luz” reservoir. The studied stream is situated within the Peñuelas subwatershed representing a total of 320.9 km² (DGA, 2014). [32] As a part of the coastal watershed complex called “Aconcagua/Maipo Coast”, the water flow is mainly dependent on rainfall and anthropogenic activities. It is important to note that a sandbar avoiding superficial water exchanges between the stream and the ocean is almost constantly present at the estuary and was present during both sampling periods. Field work to collect physicochemical data water and sediments samples were conducted on August 26, 2019 (austral winter) and January 07, 2020 (austral summer). Five sites were sampled along the main tributary and estuary mouth of the Las Cenizas basin following a previous report (Rivera-Castro et al., 2020) [31]. Two sampling sites from the upper area of the main tributary were located 30 m upstream (F2) and 30 m downstream the WWTP discharge (F3). A third site (E7) was located at an intermediate tributary area characterized by a pond. The last two sampling sites were situated near the estuary (E10) and in the mouth (E11) (Figure VI-1). During winter and summer, the tributary upper and intermediate portions were shallow 10–20 cm water-depth, whereas at the estuary mouth the water depth was approximately 50–60 cm.

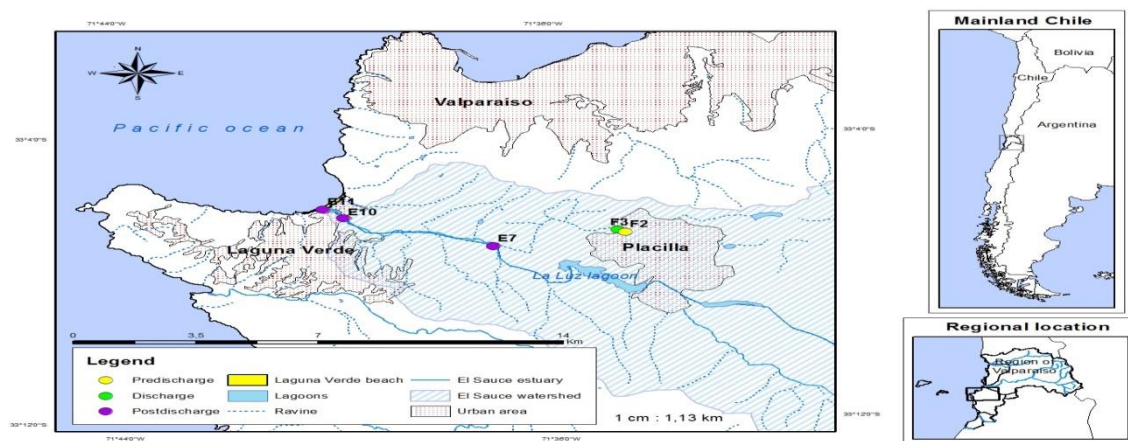


Figure IV-1: Sampled sites of El Sauce Estuary considering five stations.

Physicochemical parameters such as temperature, pH, dissolved oxygen (DO), and conductivity were recorded in the surface water in triplicate (except for DO) using a multiparameter probe (YSI and ProDSS model). Triplicate water samples were collected from the water surface for nutrients including nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{3-}) and silicic acid (SiO_4^{4-}) determinations. The water was filtered through a 0.45 μm GFF filter and collected into 60 mL “Nalgene” bottles (Nalgene, USA). These bottles were kept cool for less than 3 h and frozen at $-20\text{ }^\circ\text{C}$ until further analysis. Also, water samples were collected to determine the concentration of dissolved GHG (CO_2 , N_2O and CH_4) using 20 mL gas-tight vials, in triplicate. These vials were filled under water to avoid bubbles, then were immediately fixed with 50 μL saturated HgCl_2 solution and stored at room temperature in the dark until their analysis in the laboratory. Water samples for standard water quality parameters according to Chilean regulation NCh409/84, were also collected in triplicate and transported to the laboratory following procedures established by the Standard Methods Book [33]. For the determination of Chlorophyll-a concentration, in triplicate discrete water samples (500 mL) were filtered onto GF/F filters (Whatman® glass microfiber filters, USA) and then stored and frozen until laboratory analyses.

Surface sediment samples were sampled in sealable bags for organic matter and granulometry determinations and were kept at $4\text{ }^\circ\text{C}$ for further laboratory analyses. Fine-grained sediment samples were collected to determine carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotopes and stored in cryotubes (2 mL, in duplicate). Also, fine-grained sediments were sampled for molecular microbial community composition analysis in cryotubes (2 mL, in duplicate) filled with RNA-later (Ambion, Life Technologies, USA) to preserve nucleic acids [34]. All the sediment samples were transported under cool conditions using gel packs (less than 3 h). Sediments for isotopic and microbial community analyses were stored frozen at $-80\text{ }^\circ\text{C}$ until analyses.

2.2 Laboratory procedures for biochemical parameters

Nutrients concentrations were determined through spectrophotometric methods. Nitrate and nitrite concentration were determined following Strickland et al. (1972) [35] and PO_4^{3-} and SiO_4^{4-} were measured following Atlas et al. (1971) [36] using a nutrient autoanalyzer. Chlorophyll-a, was determined in discrete seawater samples according to the method described by Holm-Hansen et al. 1965 [37]. Dissolved GHG concentrations in water were determined by gas chromatography through the headspace technique McAuliffe, 1971 [38] using a Greenhouse GC-2014 Shimadzu chromatograph equipped with an electron capture detector (ECD) for N_2O and a flame ionization detector (FID) for CO_2 and CH_4 , attached to a methanizer for the conversion of CO_2 to CH_4 . Calibration was carried out using three calibration points using helium, air and a standard of 600, 5 and 1 for CO_2 , CH_4 and N_2O mixture for absolute quantification (Scotty gas mixture; Air Liquid Co., Paris, France). Standard water quality parameters such as transparency, turbidity, color, dissolved total solids (DTS), sulfates, chlorides, fecal coliforms and BOD_5 (biochemical oxygen demand) according to Chilean regulation NCh1333/78, were measured following the Standard Methods Book [33]. All chemicals used for the above analyses were analytical grade.

2.3 Sediment granulometry, organic matter and isotopic analyses

Sediment granulometry of samples was assessed using dry sediment (40 °C during 24h) and analyzed by standard mesh analysis method to sort sand, gravel and silt size fractions (Friedman and Sanders 1978) [39]. Total organic matter was determined using the Loss-On-Ignition (LOI) method in a muffle furnace [40]. Carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotopes were analyzed using mass spectrometry (Thermo Scientific Delta V Advantage IRMS with EA-2000 Flash Elemental analyzer), according to Pee Dee Belemnite (VPDB) and air standard at the Laboratory of Biogeochemistry and Applied Stable Isotopes (LABASI, PUC, Chile).

2.4 Benthic microbial community characterization using molecular approaches

DNA was extracted using the DNAeasy PowerSoil DNA Isolation Kit (Qiagen, USA). Briefly, after thawing the RNA-later buffer was removed from the sample, then 250 mg of wet sediment were weighted and distributed into the PowerBead tubes provided by the manufacturer. DNA extracts were quantified (1.3–40 ng/μL) with the dsDNA BR (Broad Range) Qubit 2.0 fluorometer (Thermo Fisher Scientific) and DNA quality (260/280) was determined by spectrophotometry using Cytation 5 (BioTek) and template amplification was tested using conventional 16S rDNA PCR using 27F and 519R primers. DNA extracts (n = 10) were sent to the Illumina Miseq sequencing platform at Mr. DNA laboratory (Texas, USA) to be sequenced using 515F (GTGYCAGCMGCCGCGGTAA) [41] and 806R (GGACTACNVGGGTWTCTAAT) primers [42] for the V4 region of the 16S rDNA gene. Sequences were deposited in the European Nucleotide Archive (ENA) under project accession ID PRJEB44347 and primary accession samples (ERR9814522-ERR9814533). The stations were separated into three categories, e.g., predischage (F2), discharge (F3) and postdischarge (E10 and E11). Samples from station E7 were not included in microbial communities' analysis. The same criteria was used in the qPCR analyses.

2.5 Quantitative PCR analyses

Ammonia oxidizing bacteria from the Betaproteobacteria (βAOB), comammox *Nitrospira* sp, Methanotrophic bacteria associated with *Methylobacter*-like and Methanogenic archaea functional groups were quantified using the following functional genes *amoA*, *comaA*, *pmoA* and *mcrA*, respectively, with primer references and qPCR conditions shown in the **Table IV-1**. The qPCR reactions used approximately 10 ng of DNA as template and 20 μL reactions using the Power SYBR Green Master Mix (Applied Biosystems) with the QuantStudio 3 thermocycler (Applied Biosystems, USA).

Table IV-1. qPCR table indicating its gene, group, organism (sequence ID), primers, primers concentration, annealing temperature, efficiency, R² and reference.

Gene	Group	Primers	Primer Concentration (μM)	Annealing Temperature (°C)	Efficiency (%)	R ²	Reference
Ammonia monooxygenase subunit A (<i>amoA</i>)	Betaproteobacteria AOB	amoA1F amoA2R	0.4 0.4	56	92.55%	0.998	[43]
Ammonia monooxygenase subunit A (<i>comaA</i>)	COMAMMOX Nitrospira Clade A	comaA-244F coma-659R	0.4 0.4	52	83.10%	0.999	[44]
Methane monooxygenase subunit A (<i>pmoA</i>)	Methanotrophs	A189F Mb661R	0.4 0.4	55	90.34%	0.999	[45] [46]
Methyl coenzyme M reductase subunit A (<i>mcrA</i>)	Methanogens	mlas mcrA-rev	1 1	55	90.59%	0.999	[47]

2. 6 Sequencing data curation and taxonomic classification processing

QIIME2 (v.2019.7) was used for sequence curation and taxonomic classification into Amplicon Sequence Variant (ASVs) [50]. First, sequences were imported to QIIME2 using Casava. Then, sequences were demultiplexed and poor-quality or short sequences (<210 bp length) were removed using qiime dada2 denoised-paired plugin [51]. Summer samples from E7 contained few sequences, and thus were removed for further analyses. Taxonomic classification was performed using the SILVA132 database , V4 subunit of the 16S rRNA gene region) with the feature-classifier classify-consensus-vsearch plugin [52].

2.7 Statistical analyses

All statistical analyses were carried out using R software [53]. Because normality assumption cannot be verified with our data, Spearman correlations were conducted to evaluate relationships between physical and chemical conditions in water and sediments using the `rcorr` function with the 'Hmisc' package [54]. Graphical visualization, including significance, were generated using the `corrplot` package [55]. Taxa bar plots for phylum and functional groups related with GHG recycling spatial and winter/summer changes were generated using the 'phyloseq' package [56]. In addition, to determine potential changes associated with the influence of WWTP discharge in the benthic microbial community structure and their association with environmental conditions, samples were grouped as: predischage (F2), discharge (F3) and post-discharge stations (E10 and E11) and plotted using the `amp_heatmap` function of the 'ampvis2' package [57]. Redundancy analysis (RDA) was constructed with the `ordinate` function of the 'phyloseq' package to determine the relation between microbial community (Phylum level) shifts with environmental factors using the `envfit` function of `vegan` package [58] to identify the significance of the correlation. Differential analysis of ASVs was performed using the 'DESeq2' package, specifically the `DESeq` function. The data used for the comparison are the ASV of each crude sample, without normalizing. The results were visualized in a Volcano Plot [59]. SIMPER (Similarity Percentage) analysis was conducted using PRIMER6 software [60] to identify phylum contribution between predischage, discharge, and post-discharge stations.

3. Results

3.1 Environmental factors, nutrients and water quality variables

Surface water temperature of El Sauce Estuary was characterized by lower values in winter (12.1–17.6 °C) compared to summer (17.63–26.1 °C), and the maxima were found associated with the WWTP discharge station F3 (**Figure IV-2A and B**). Water pH was lower at the upper stations, particularly in F3 station, which was characterized by pH values <6.86 in winter and summer, compared to the post-discharge stations where pH >7.4 was detected (**Figure IV-2A and B**). Water conductivity was found to increase from the upper portion towards the mouth, ranging between 766 to 1,304 $\mu\text{S cm}^{-1}$ and 828 to 4,150 $\mu\text{S cm}^{-1}$ in winter and summer, respectively. In general, DO followed the spatial conductivity shifts, but with a higher variability in winter compared to summer (**Figure IV-2C and D**).

Nutrients (i.e., nitrite, nitrate, silicate, and phosphate) at F2 and F3 stations were characterized by extreme changes in nitrate and nitrite, which reached the lowest values at the WWTP discharge station (F3 station) <14 μM for nitrate and < 3 μM for nitrite in winter and summer, respectively. The highest values of nitrate and nitrite concentrations were registered in the upper tributary and intermediate pond stations (F2 and E7) of >200 and μM > 100 μM values in summer (**Figure IV-2 E and F**). Estuary stations E10 and E11 were characterized by similar nitrate and nitrite concentrations, and slightly higher concentration towards the mouth (from E10 towards E11) during summer (**Figure IV-2 E and F**). Phosphate concentrations were higher in the upper tributary station (F2) and unlike other nutrients, greater concentrations were found in winter (> 15 μM) than summer (< 5 μM). F3 and E11 stations exhibited lower concentration (< 1 μM) in both seasons (**Figure IV-2G and H**). Except for the upper tributary (Station F2) in summer, silicate presented lower concentrations during winter (320–100 μM) versus summer (100–250 μM) from F3 station towards the estuary mouth (**Figure IV-2 G and H**).

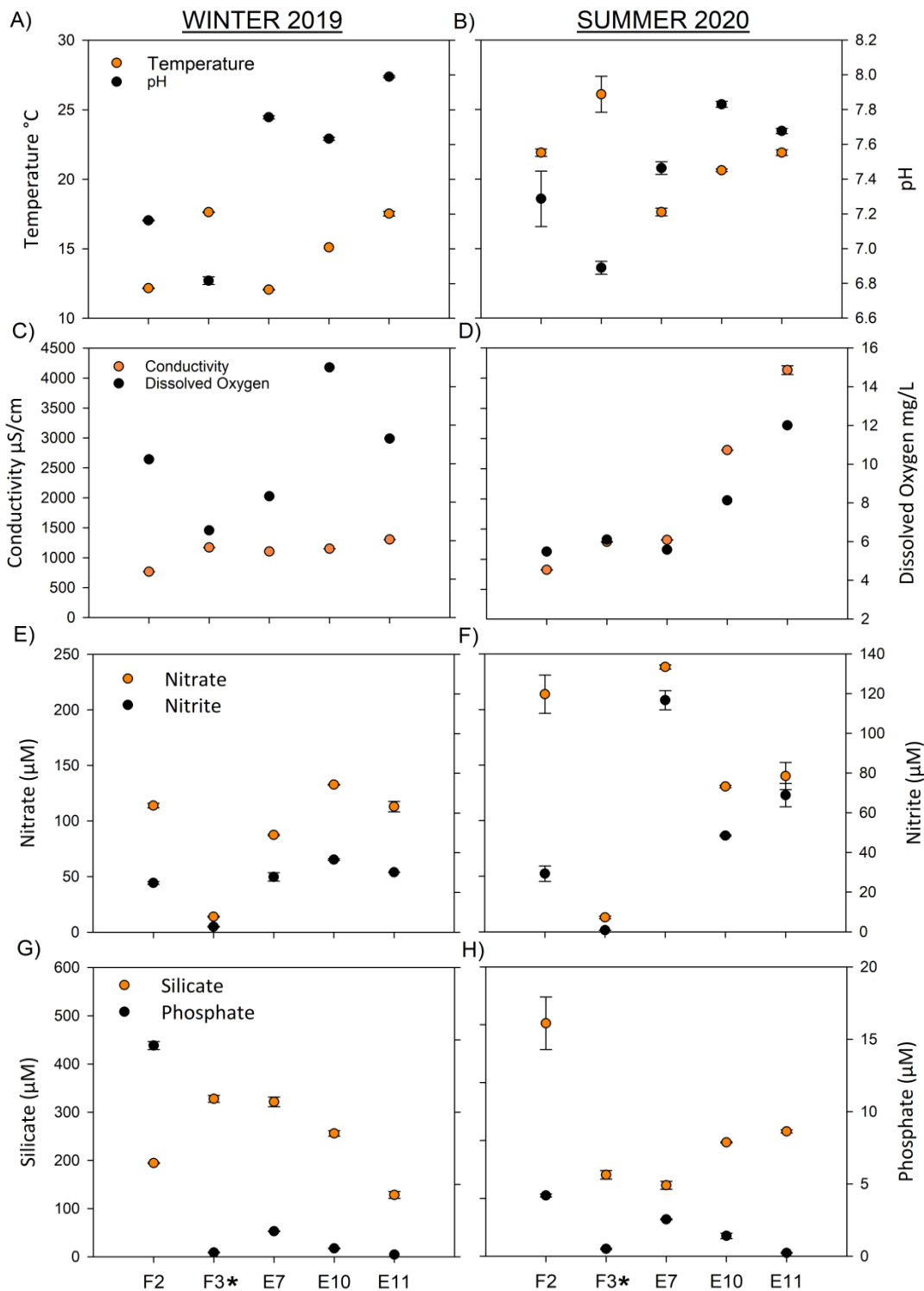


Figure IV-2. Physicochemical and nutrients conditions in all sampled sites during winter (left panels) and summer (right panel) of El Sauce Estuary.

Water quality conditions based on additional physicochemical variables, such as transparency, color and dissolved total solid (DTS) exceeded maximum permitted values for agricultural irrigation and animal consumption according to the environmental Chilean regulation NCh1333/78 (**Table IV-2**). Besides, fecal coliforms also exceeded allowed values (>1,000 CFU/mL) in the upper tributary (F2) and in the mouth (E11) in both summer and winter seasons.

IV-Table 2. Water quality factors in all sampled stations during winter and summer. All black numbers are values that exceed the maximum allowed limit (MAL). The values in brackets are the results previously reported by Rivera et al (2020).

	WINTER					SUMMER					
FACTOR	F2	F3	E7	E10	E11	F2	F3	E7	E10	E11	AML
DTS (mg/L)	381	587	543	563	647	413	645	660	1406	>2000	< 500
Transparency (cm)	18	40	12	60	84	ND	ND	ND	60	4	≥ 120
Turbidity (NTU)	5.54	18.53	15.60	10.02	14.27	1.93	32.83	55.40	4.91	10.16	50
Color (Pt/C)	139.00	316.00	299.67	183.00	278.67	254.00	624.00	715.00	239.	218.00	< 100
Sulfate (mg/L)	85.33	244.00	141.33	133.33	136.00	124.85	159.15	153.66	205.8	270.28	250
Chlorides (mg/L)	75.63	146.53	146.53	184.34	255.24	0.70	1.43	1.57	5.30	8.73	200
Total Coliforms (NMP)	9200	<1.8	<1.8	ND	1100	≥ 16000	0	110	500	2200	1000
Fecal Coliforms (NMP)	140	<1.8	<1.8	ND	490	260	0	80	300	140	1000
BOD5 (mg/L)	7.30	0.00	4.35	4.51	11.98	11.34	65.79	66.41	17.71	20.31	20
Chlorophyll a (mg/L)	6.52	0.6	0.19	20.02	41.14	31.81	0.39	39.28	22.74	32.92	10

3.2 GHG distribution along the Laguna Verde estuary during winter and summer

The WWTP discharge has a high impact on the dissolved concentration of GHG in the Laguna Verde estuary, that presented a maximum at station F3 (WWTP discharge site) reaching up to 1,200 μM CO_2 in summer, and 55,301 nM CH_4 and 3,930 nM N_2O in winter (**Figure IV-3**). These dissolved CH_4 concentration

values were up to 60 fold higher than the upper tributary predischarge water (Station F2). While the WWTP discharge increases the concentration of methane to a greater extent than the concentrations of N₂O and CO₂, those gases registered a continuous decrease from the intermediate pond (Station E7) towards the estuary mouth (Figure IV-3).

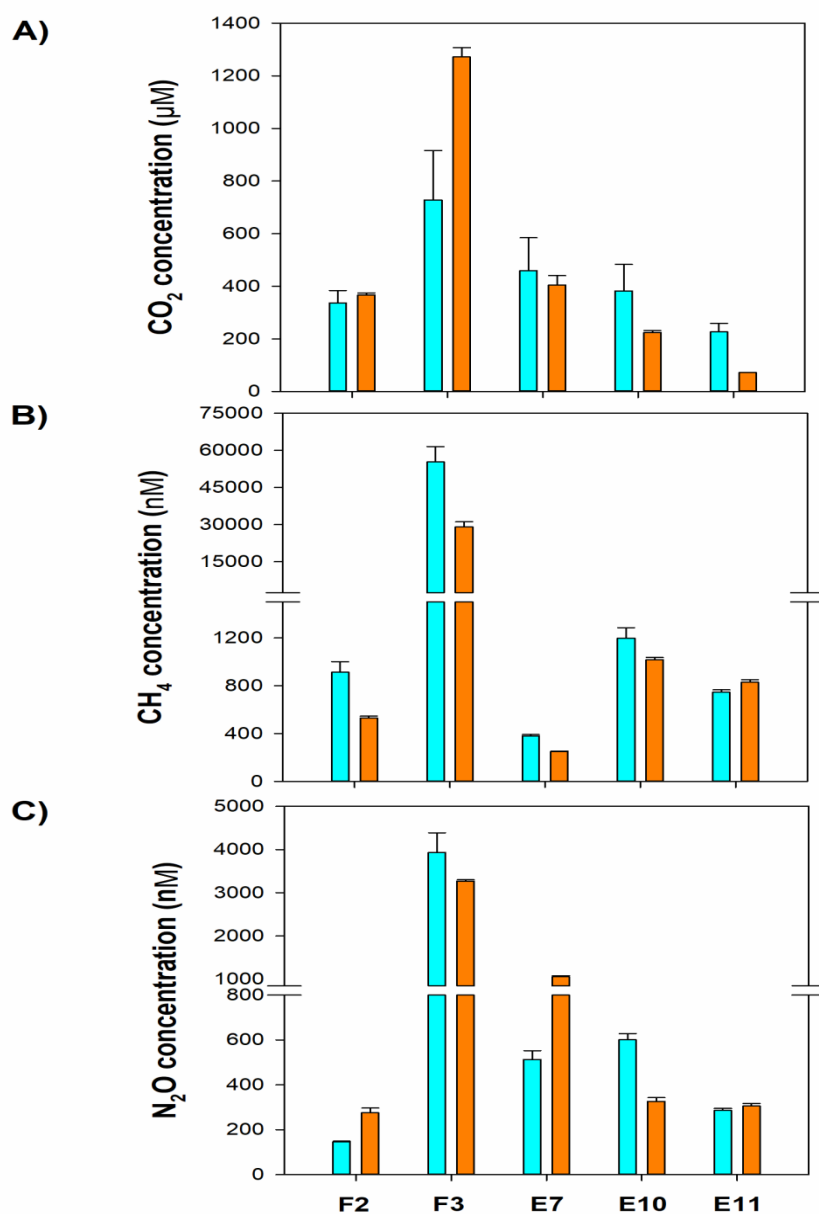


Figure IV-3. Greenhouse gas concentration (CO₂, CH₄ and N₂O) dissolved in water in winter (cyan bars) and summer (orange bars) of El Sauce Estuary.

3.3 Sedimentary conditions and potential organic matter quality

Sediments were characterized by the predominance of larger particle sizes (i.e., medium-coarse and very coarse) in the upper tributary, whereas lower particle sizes (i.e., silt and very fine sand) were found in the sediments of the mouth especially during winter compared to summer sampling (**Figure IV-4**). Total organic matter (TOM) increased by >100 % (<4 to >8 g) from the upper tributary (F2 and F3) and intermediate pond (E7) post-discharge WWTP towards the mouth stations (E10 and E11) (**Figure IV-4**). Isotopic organic matter signatures coincided with TOM shifts characterized by a marked difference in $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ isotopes ratios predominantly ~ 29 $\delta^{13}\text{C}$, ~ 6 $\delta^{15}\text{N}$ in the upper tributary compared to the mouth estuary sediment ~ 26 $\delta^{13}\text{C}$, ~ 12 $\delta^{15}\text{N}$ (**Figure IV-5A**), showing higher $\delta^{15}\text{N}$ ratios in summer compared to winter. In addition, higher C/N ratios were found in the WWTP discharge during summer and the intermediate pond water during winter (C/N >10), whereas lower ratios were determined towards the mouth Station (E10 and E11) and the intermediate pond water during summer reaching C/N <8 (**Figure IV-5B**).

3.4. Spearman Correlation Analyses Associated with Water Quality Parameters and GHG

Among the physicochemical parameters, pH and conductivity were significantly linked with water quality parameters, GHG concentrations, nutrients and sediments properties (**Figure IV-6**). For instance, pH was significantly negatively correlated with GHG in the water and granulometry in sediments (p-value <0.05), while positively correlated with TOM in sediments (p-value <0.05) (**Figure IV-6**). Conductivity was significantly positively correlated with TDS, sulfate and sedimentary TOM (p-value <0.05). Water quality variables as the turbidity was significantly correlated with color (p-value <0.05) and both variables with BOD₅, TDS and sulfate (p-value <0.05) in the water and with TOM (p-value <0.05) in the sediments. In addition, Chlorophyll-a was significantly correlated with fecal coliforms, CO₂, nitrate, nitrite and $\delta^{15}\text{N}$ (p-value <0.05). Interestingly, GHG was significantly correlated among them (p-value <0.05) and also with nitrate (p-value

<0.05) (Figure 6). Regarding sediment granulometry, medium sand was significantly correlated (p-value <0.05) within the different categories and with other sediment variables (**Figure IV-6**), for example with TOM, $\delta^{15}\text{N}$ and several water physico-chemical variables, including Chlorophyll-a, GHG, nitrite and nitrate. Coarse sand percentage was also significantly correlated with pH, Chlorophyll-a, CH_4 , nitrite and nitrate (p-value <0.05) (**Figure IV-6**).

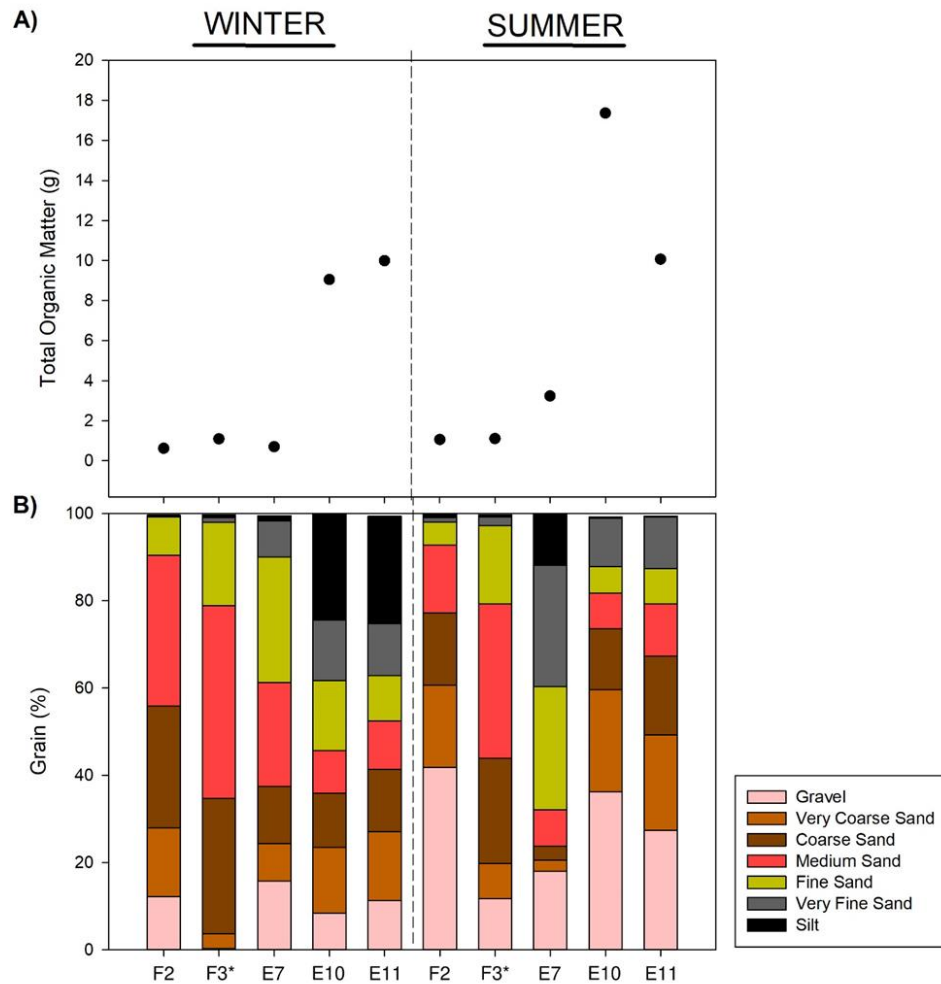


Figure IV-4. A) Total organic matter percentage and B) sediment granulometry distribution.

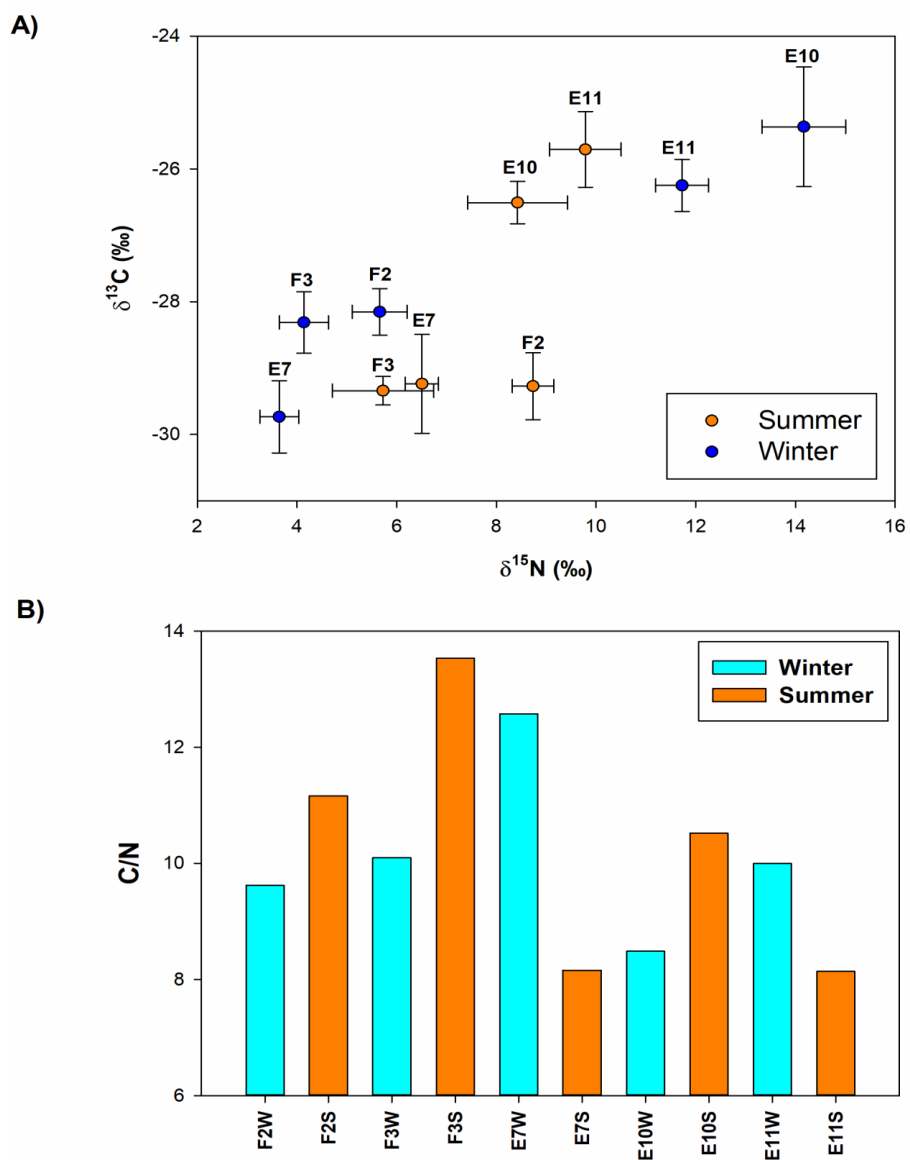


Figure IV-5: Isotopic organic matter signal (A) and C/N rates (B) in all sampled sites during winter and summer of El Sauce Estuary.

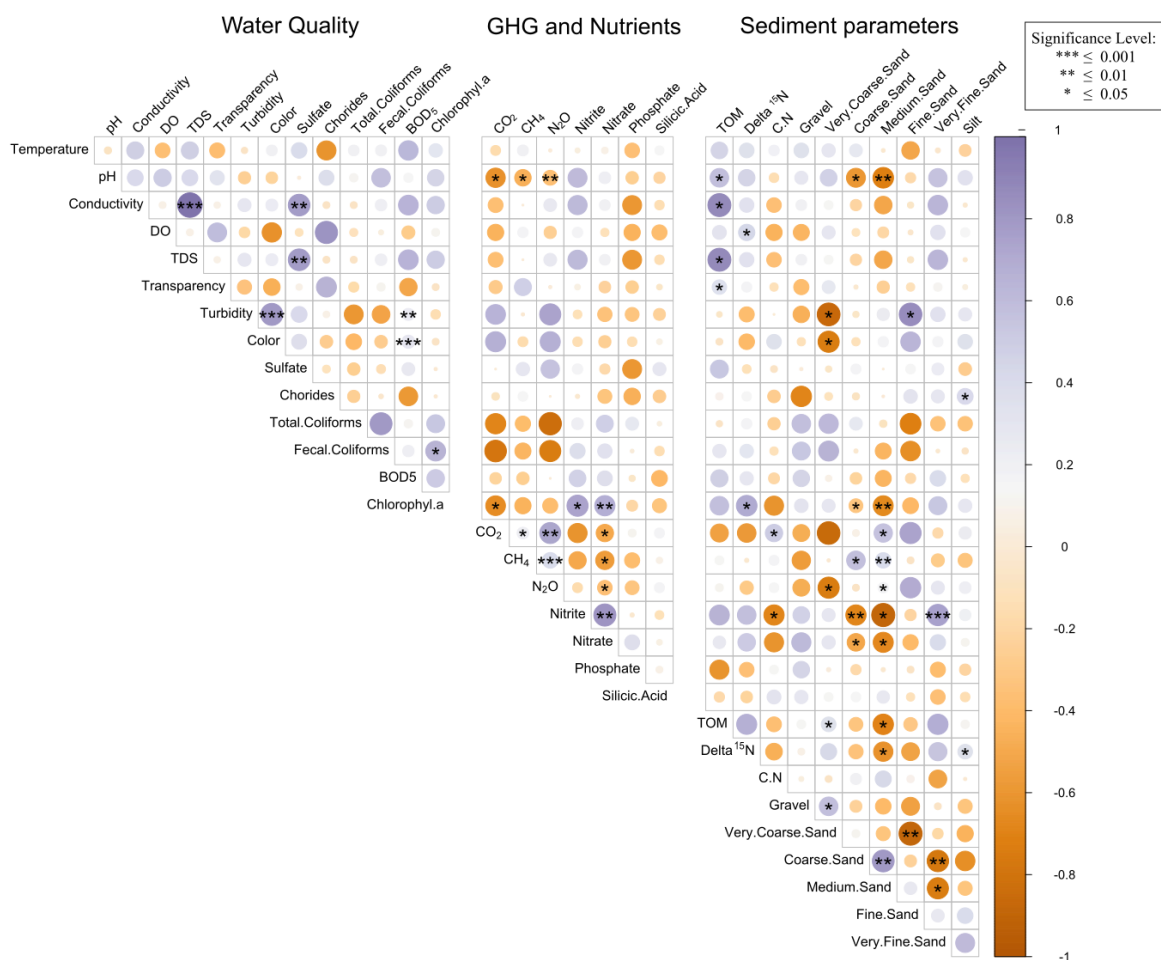


Figure IV-6: Spearman correlation analysis between water quality factors, GHG, nutrients and sediment factors.

3.5 Benthic microbial community composition

The benthic microbial communities of El Sauce Estuary were characterized by the presence of 56 bacterial and 8 archaea Phylum (**Supplementary Table 1**). The microbial communities were dominated by Proteobacteria affiliated to Gammaproteobacteria class mainly represented by the Betaproteobacteriales order, followed by Bacteroidetes and Chloroflexi. Both spatial and temporal variations were observed, including changes in Chloroflexi that was predominantly detected towards the mouth of the estuary stations (E10 and E11), whereas Firmicutes and Verrucomicrobia had a higher relative contribution to the total microbial community towards the upper tributary (Stations F2 and F3), also showing slight changes in the winter versus summer (**Figure IV-7**).

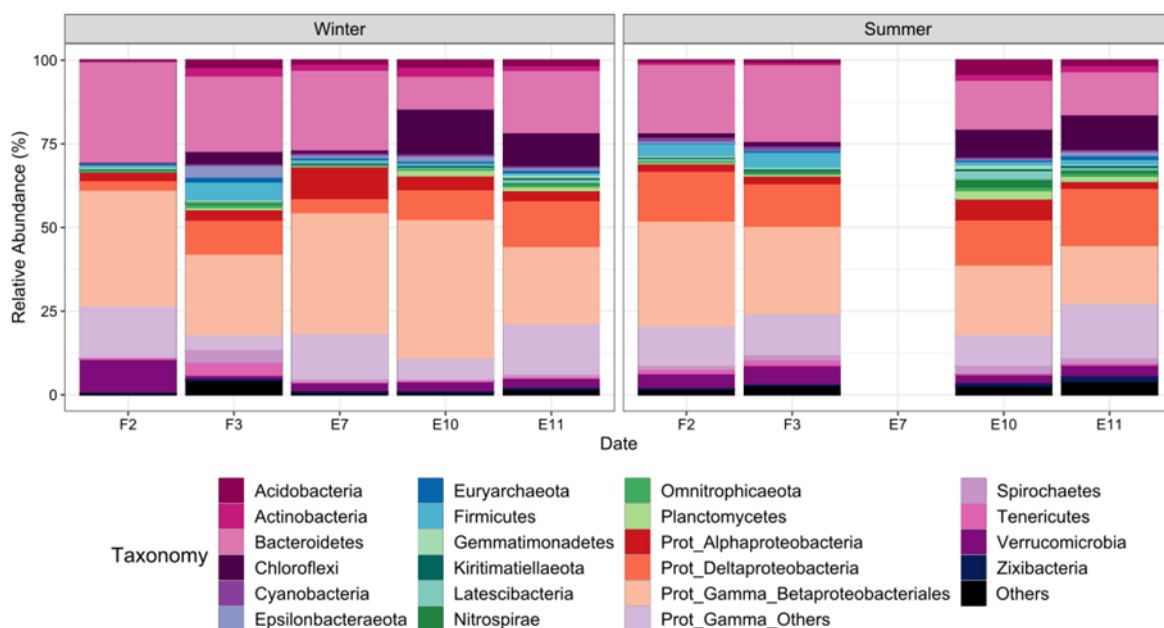


Figure IV-7: Taxa bar plot showed microbioma distribution at the phylum level during winter and summer in all sampled sites.

SIMPER analysis considering the spatial grouping, excluding Station E7, indicated that the core benthic microbial community i.e., Proteobacteria, Bacteroidetes and Verrucomicrobia were consistently present at the predischage site compared to the discharge and post-discharge areas of the estuary (**Figure IV-1S**). Discharge benthic microbial communities were differentiated by a greater contribution of Firmicutes and Spirochaetes and by the exclusive presence of Tenericutes, Lentisphaerae, Synergistetes and LCP-89. In addition, Hydrogenedentes, Nitrospirae, Latescibacteria, Gemmatimonadetes and Zixibacteria were exclusively found in the post-discharge area, whereas Actinobacteria, Acidobacteria, Planctomycetes and Cloroflexi had a greater contribution (**Figure IV-1S**).

At the pre-discharge area, Xhantomonadales, Sphingobacteriales, Cytophagales, Verrucomicrobiales, Flavobacteriales and Pseudomonadales were among the twenty most abundant orders, contributing the most in terms of abundance during winter (**Figure IV-8**). At the discharge zone, Bacteroidales and Clostridiales were highly prevalent during winter and summer, whereas Desulfuromonadales, Syntrobacterales, Chitinophagales, Campylobacteriales and Spirochaetales had a greater contribution during summer. At the post-discharge

zone, Desulfobacterales, Anaerolineales and Myxococcales were abundant during winter and summer, while Steroidobacterales exhibited a greater contribution during summer (**Figure IV-8**).



Figure IV-8. Heatmap showed the most dominant orders in the predischarge, discharge and postdischarge stations during winter and summer.

Differential analysis based on the comparison of microbial communities at ASVs level showed a greater number of ASVs present in the discharge compared to predischarge waters (**Figure IV-9**). Eighty-one ASVs, predominantly affiliated to the orders Betaproteobacteriales, Bacteroidales, Syntrophobacteriales, Campylobacteriales and Chitinophagales were more abundant in the discharge compared to predischarge site, followed by a less proportion of ASVs belonging to Spirochaetales, DTU014 (Clostridium), Izimaplasmatales and Methanofastidiosales. In contrast, only eight ASVs affiliated with the orders Verrucomicrobiales and Flavobacteriales were enriched at predischarge compared to discharged water.

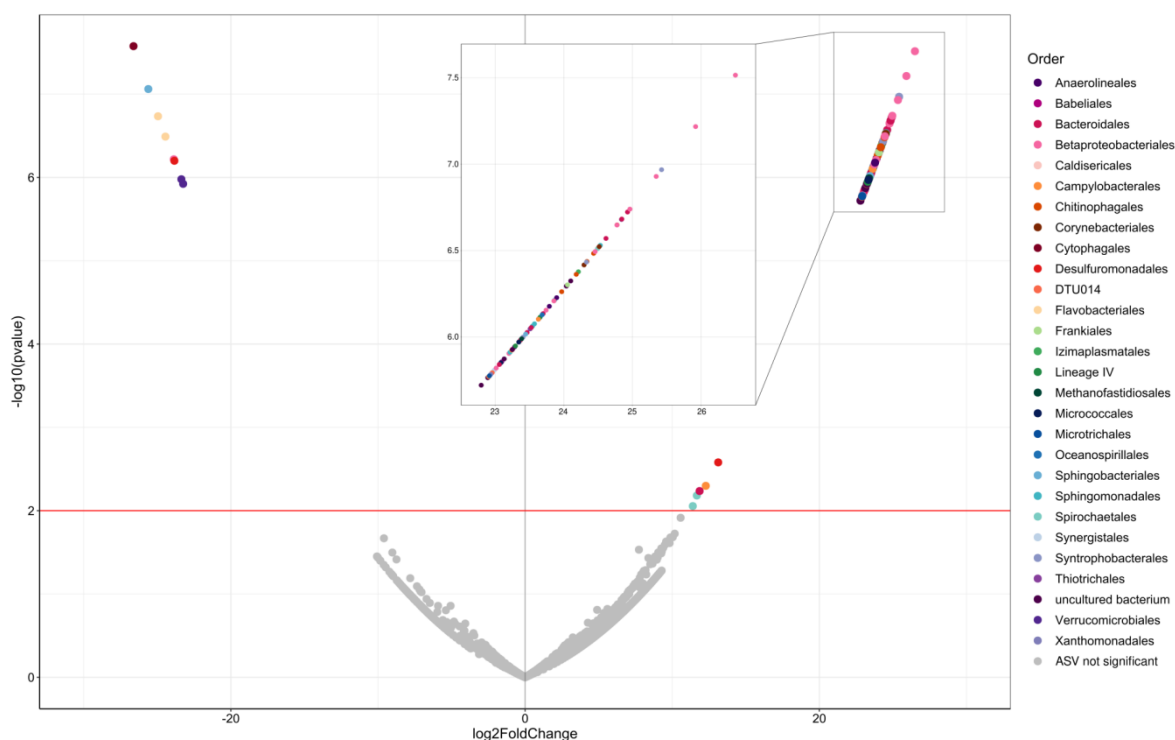


Figure IV-9. Volcano plot of differentially abundant ASVs identified between Discharge and PredischARGE. A positive log₂ Fold Change in relative abundance indicates the feature predominated among Discharge, and vice versa for PredischARGE. The gray dots denote the ASVs without marked differences (p-value > 0.01, red line).

Redundancy analysis (RDA) confirmed the spatial variability of the microbial community structure at the phyla taxonomic level, revealing a clear separation between discharge station (F3) and the mouth stations (E10 and E11) (**Figure IV-10**). Both first and second axes accounted for 75.6% of the total variation of the benthic microbial community structure. Among the environmental variables accounting for the microbial community variability at discharge waters, values of N₂O, CH₄ and medium and coarse sands were significant, , whereas very fine sand, TOM, pH and δ¹⁵N were significantly higher at the post-discharge stations E10 and E11 (**Figure IV-10**). Many water column quality parameters were associated with the benthic microbial community structure but were not significant at a p value <0.05.

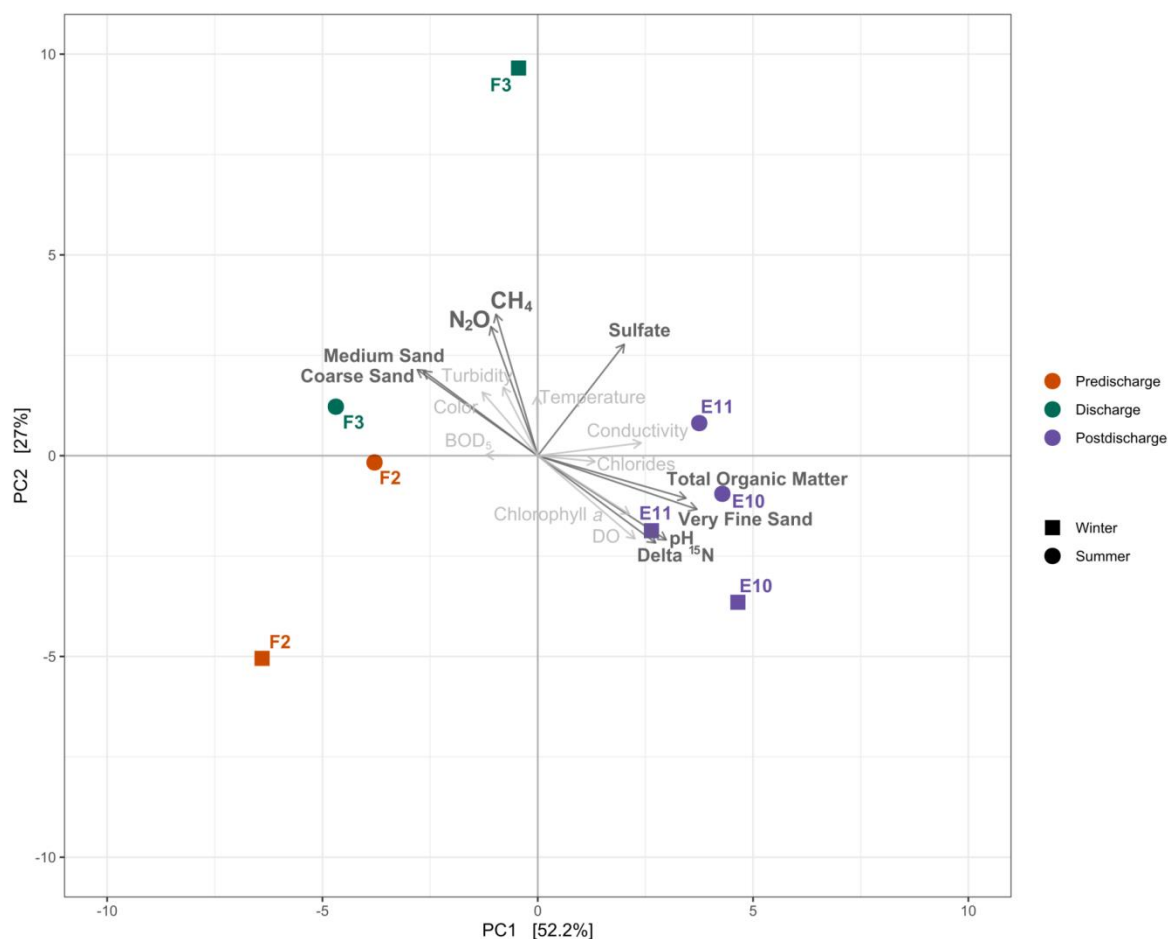


Figure IV-10. Bray-Curtis based redundancy analysis comparing benthic microbiomes dissimilarities between predischage, discharge and post-discharge stations and its relationship with physicochemical parameters. Parameters significantly correlated with the ordination identified through envfit function p -value < 0.05 were depicted in black.

3.6 Functional microbial groups spatial variability during winter and summer in the El Sauce estuary

Nitrifying communities' contribution based on 16S rRNA gene sequencing from the studied sediment presented a distinct spatial pattern between winter and summer (**Figure IV-11A**). For instance, the Nitrosomonadaceae family was ubiquitous all along the stream during winter and summer, whereas Nitrospiraceae and ammonia oxidizing archaea were detected mainly at the Estuary mouth stations (**Figure IV-11A**). A higher richness of methanotrophs/methylotrophs were detected during winter compared to summer (**Figure IV-11B**), with the detection of Methylococcaceae at the discharge station. The El Sauce benthic microbial communities were characterized by a rich methanogenic community particularly

abundant at the discharge station (F3) in winter (**Figure IV-11C**). During winter, Methanobacteriaceae and Methanosarcinaceae were the predominant methanogens in the mouth stations (**Figure IV-11C**).



Figure IV-11. Taxa bar plot showed functional group abundance at the family level during winter and summer in all sampled sites. Note that no sequencing data were available for E7 in summer.

The abundance of the functional genes (*amoA*, *comA*, *pmoA* and *mcrA*) evaluated through qPCR, showed a greater contribution of methanogens, ammonia and methane oxidizers during summer compared to winter especially at the Estuary mouth (**Figure IV-12A**). In winter, the discharge station exhibited a lower abundance of ammonia and methane oxidizers compared to the pre and postdischarge sites (**Figure IV-12A and C**). During summer, Betaproteobacteria ammonia oxidizers (β AOB) decreased in abundance towards the estuary mouth (**Figure IV-12A**), unlike comammox bacteria that presented a similarly high contribution at all sites with a slightly greater contribution at the discharge station (**Figure IV-12B**). On the other hand, during winter and summer, methanogens

abundance was higher at the discharge and postdischarge zones of the Estuary (Figure IV-12D).

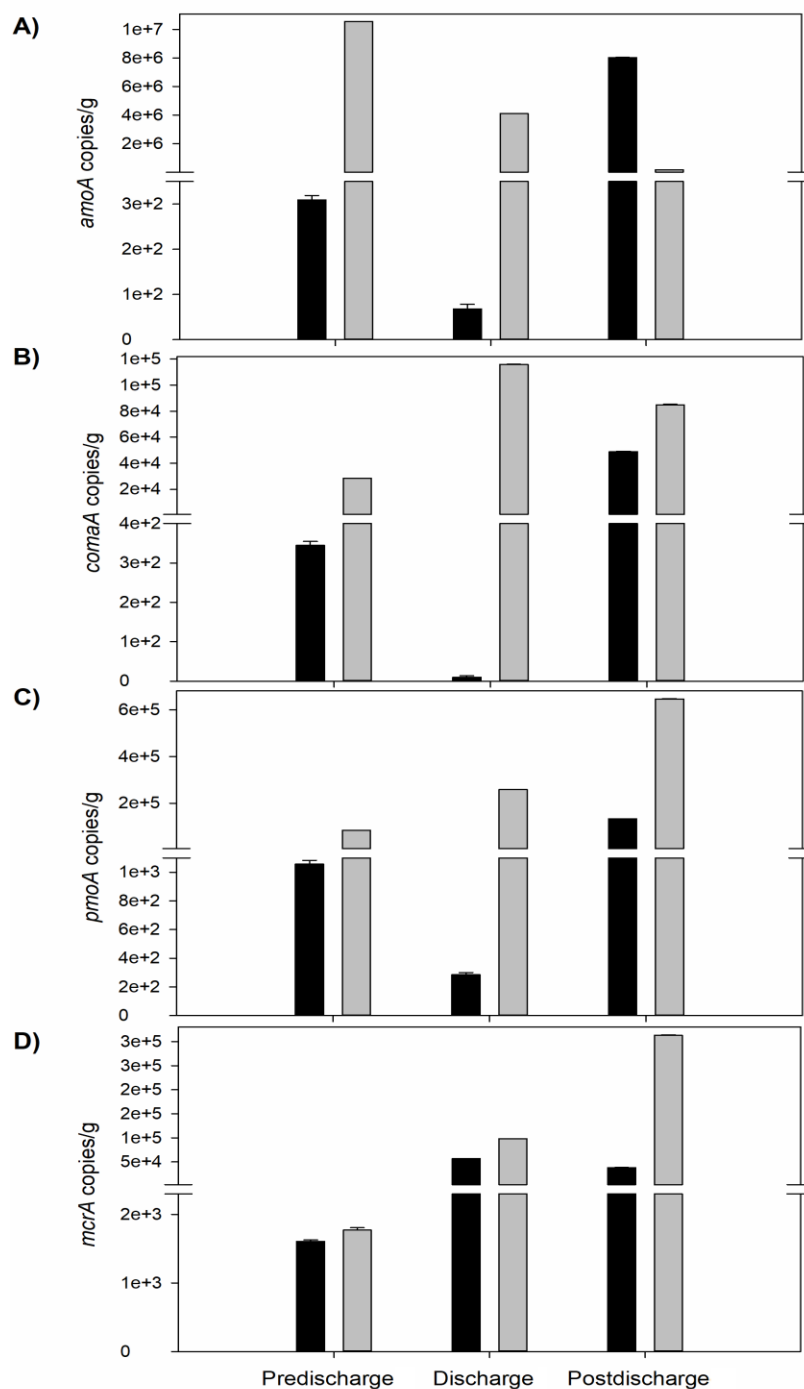


Figure IV-12. Abundance of all targeted functional genes (*amoA*, *commaA*, *pmoA* and *mcrA*) in the predischarge, discharge and postdischarge stations during winter (black bars) and summer (grey bars). The abundance is expressed in copies per gram of sediment dry weight (DW).

Spearman multiple correlation analysis was carried out to evaluate relationships between the functional groups abundance based on qPCR and the contribution of functional microbial groups composition by 16S rDNA sequencing. The *mcrA* gene abundance (i.e., in copies/g sediment DW) showed a positive correlation between several taxa of methanogens, including counts belonging to the Methanomassilicoccaceae, Methanosaetaceae, Methanoregulaceae correlated with Methanospirillaceae, Methanofastidiosaceae and total methanogens. On the other hand, methanotrophic communities quantification using *pmoA* gene copies/g sediment DW was positively correlated with the Methylacidiphilaceae family, whereas Methylococcaceae and Methylophilaceae families retrieved from 16S rRNA gene sequencing correlated positively with total MOB (**Figure IV-13**). Neither β AOB and commamox *amoA* gene copies/g sediment DW were correlated with total nitrifiers communities recovered from 16S rRNA gene sequencing. However, counts belonging to several families of nitrifiers positively correlated with each other. This was the case of Nitrosopumilaceae and uncultured Crenarchaeota with Nitrosomonadaceae, Nitrosotaleaceae and Nitropiraceae, whereas Nitrosomonadaceae and Nitrosotaleaceae correlated with Nitropiraceae (**Figure IV-13**). Moreover, functional microbial groups abundance based on gene quantification qPCR were correlated with environmental factors (**Table IV-S2**). Except for methanogens, all functional groups were significantly correlated with $\delta^{15}\text{N}$ was (p-value < 0.05). Also, comammox and methanotrophs were significantly correlated with temperature, BOD₅ and TOM (p-value < 0.05). The commamox was significantly correlated (p-value < 0.05) with conductivity and DTS, and methanogens with phosphate (Table S2).

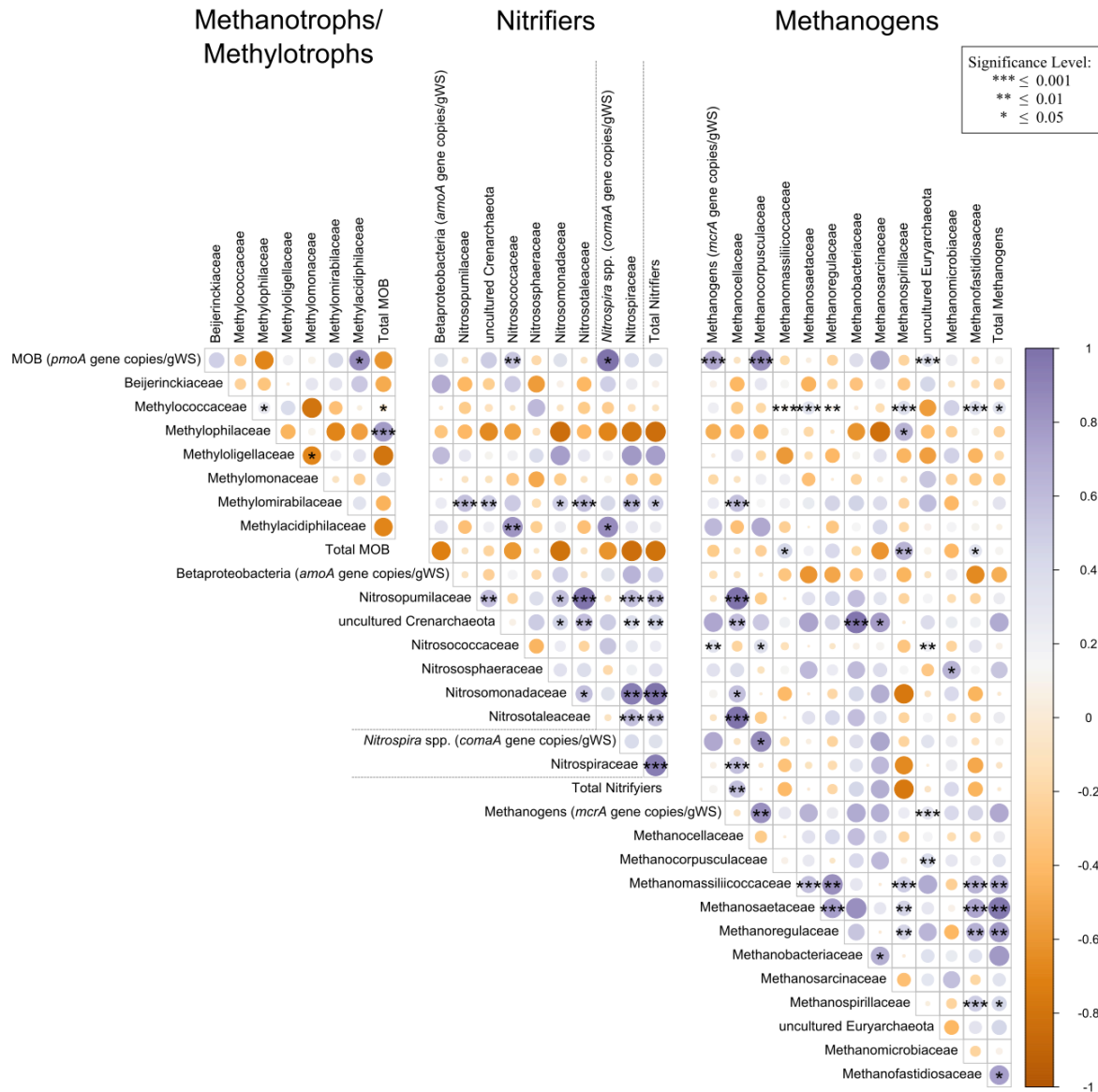


Figure IV-13. Spearman correlation analysis between functional microbial groups correlations based on qPCR genes abundances correlation with reads.

4. Discussion

El Sauce Estuary is a fresh surface water body located in the Valparaíso region whose main water flow comes from Las Cenizas basin. Previous studies have reported that El Sauce is under anthropogenic pressure, categorizing the estuary as a permanent hypertrophic estuarine environment mainly impacted by WWTP discharges, as well as percolated liquids from a nearby landfill, and sewage discharges from houses not included in the sewage system (Rivera-Castro et al.,

2020) [31]. These inputs are often composed of a mixture of partially degraded OM, nutrients, microorganisms, many of them pathogens, and emerging contaminants that have the potential of reshaping the natural community structure and biogeochemical activity, especially in semi-closed environments, such as, El Sauce. Our study reports the first evidence of the WWTP discharge impact on water quality, benthic microbial communities and dissolved greenhouse gases of El Sauce Estuary, providing new insights into how WWTP discharges influence on the biogeochemistry of receiving water bodies, especially in complex and fragile environments such as polluted coastal ecosystem of Valparaíso Region.

4.1 The WWTP altered physicochemical properties of El Sauce stream

The physicochemical conditions of the watershed, such as color, transparency, turbidity, DTS and total coliforms suggested its poor water quality (**Table IV-2**). Moreover, the WWTP discharge (F3 station) considerably affected the physicochemistry of the estuary, decreasing pH and the concentration of nitrate, nitrite and phosphate compared to the upper tributary water in both winter and summer (**Figure IV-2A and B**). DO decreased exclusively in summer, whereas water temperature increased in both seasons. The discharge comes from Esval-Placilla wastewater treatment plant (WWTP) that includes an aerated lagoon and chloride gas bubbling as secondary treatments which explain the changes of pH, temperature and nutrient concentrations during our study. Our results agreed with previous reports showing that El Sauce Estuary is significantly affected by WWTP water discharge besides other sources of pollution which have categorized the estuary as a eutrophic system for many years [31]. Moreover, in our study the WWTP discharge station (F3), exhibited twenty times more dissolved CH₄ concentration compared to the other stations, including the stations situated at the mouth of El Sauce catchment (**Figure IV-3**). Similar results have been found in sewage draining rivers and in the Guadalete Estuary (e.g., Hu et al. 2018, Burgos et al. 2015) [61,62]. The CH₄ accumulation observed in the WWTP discharge station may be explained either by a stimulation of the methanogenesis or by the inhibition of CH₄ oxidation [63]. Also, the aerated lagoon may stimulate nitrification

and denitrification processes contributing to nitrogen loss, by means of NO, N₂O and N₂ production [64,65].

The Isotopic composition of OM exhibited a marked spatial variability between upstream stations (predischarge and discharge) and the mouth stations (post-discharge) (**Figure IV-5A**). The values of sedimentary isotopic signatures indicated that OM in the upstream station was associated with a terrestrial OM origin ($\delta^{13}\text{C} = -28$ to -29.5 and $\delta^{15}\text{N} = 4$ to 8.7) unlike the mouth of the estuary ($\delta^{13}\text{C} = -25.3$ to -26.5 and $\delta^{15}\text{N} = 8.4$ to 14.2) that evidenced a marine OM origin (**Figure IV-5A**). OM isotopic $\delta^{13}\text{C}$ magnitudes were within values previously reported in estuaries of temperate areas, i.e., Godavari (-24.6), Loire (-26.8), Ems (-24.2) and Sado (-25.6) mouth estuary [66,67]. Conversely, the OM isotopic $\delta^{15}\text{N}$ values determined in our study could be associated with various origins, including phytoplankton or, residual anthropogenic nitrogen by rivers and diffuse runoff, i.e., $\delta^{15}\text{N} = 7.3 \pm 2.1 \%$ [68, 69].

4.2. Benthic Microbial Communities and Functional Groups of the El Sauce Estuary

The El Sauce Estuary holds a diverse benthic microbial community with the predominance of Proteobacteria, mainly Betaproteobacteriales and Bacteroidetes phyla (**Figure IV-7**). These results agreed with microbial community composition reported from coastal wetlands of the semi-arid zone of central Chile [70] and with other tidal estuarine sediment systems [71]. Regarding to the water physicochemical properties, the benthic microbial community structure along El Sauce Estuary was enriched by Firmicutes (Clostridiales order) and Bacteroidetes (Bacteroidales order) mainly in the WWTP discharge waters (Figure 7). These bacterial phyla are often dominant in wastewater treatment processes [72–74]. Moreover, in our study, Tenericutes, Lentisphaerae, Synergistetes and LCP-89 phyla were exclusively detected in the WWTP discharge station (F3) compared to the other sampling areas (e.g., **Figure IV-1S**). Besides a significant number of ASVs changed at the discharge station compared to predischarge (**Figure IV-9**). Firmicutes have been reported to be prevalent in the human stool microbiome and

therefore, enriched in WWTP discharge [75,76]. In particular, the orders Clostridiales and Bacteroidales have been proposed as good bioindicators to estimate the degree of influence of sewage and fecal source in water catchment impacted by WWTP discharge [73,77,78]. However, its integration to alternative tools to identify and model the influence of WWTP discharge on the microbial communities in aquatic environments remained to be explored in depth.

In general, microbial groups belonging to Proteobacteria, Verrucomicrobia and Actinobacteria were dominant phyla in upstream waters of the El Sauce Estuary, as well as other wetlands of the semiarid region of central Chile [70]. These groups were found to significantly decrease in the WWTP discharge station; however, they recover again in the mouth of the estuary. Similar studies associated with WWTP discharge in urban and sub-urban rivers also found shifts in relevant phyla characterized by an increment of Verrucomicrobia and Actinobacteria, while a decrease of Deltaproteobacteria [77].

Moreover, phylum associated with functional groups, such as, Cyanobacteria showed a low abundance in all sampled sites (**Figure IV-7**), which is probably attributed to the higher water turbidity which decreases solar radiation penetration towards the sediment, thus inhibiting benthic photosynthetic processes [79]. In addition, other known functional taxa associated with nutrient recycling, such as, Nitrospirae and Chloroflexi phylum were rare or undetected at the WWTP discharge station mainly compared to the Estuary mouth (**Figure IV-7**). These phyla increased their relative sequence abundance towards the mouth (E10 and E11 stations) of the estuary (**Figure IV-7**), where higher dissolved nutrient concentrations were detected in the water. Nutrient availability and other compounds in the WWTP effluent could affect sensitive microbes such as Chloroflexi as previously suggested [77].

The quantification of specific functional microbial groups by qPCR associated with nutrients and GHG recycling were influenced by WWTP discharge and by the sampling season. Nitrifying (β AOB and comammox) and methanotrophic communities abundances decreased in the WWTP discharge station (F3) during winter (**Figure IV-12A and C**) and increased towards the

mouth, which is important since they can still co-oxidize a variety of remaining xenobiotic compounds [80]. These results are in agreement with the decrease of the relative abundance of Betaproteobacteriales order in the WWTP discharge station (**Figure IV-9**). Similarly, β AOB gene abundances were low in activated sludge from WWTP compared to coastal sediments ($5.68 \times 10^{-6} - 4.79 \times 10^{-5}$ versus $5.4 \times 10^{-1} - 34.4 \times 10^{-1}$ copies/g sample) [81] and between sewage effluents in eutrophic lakes sediments unlike adjacent areas [82]. Further research is required to elucidate if the decrease of Betaproteobacteriales is due to an outcompetition with ammonia-oxidizing archaea and anaerobic ammonium-oxidizing bacteria or other specific environmental fluctuation.

In addition, all the targeted functional groups (β AOB, comammox and methanotrophic) were enriched in the discharge area during summer unlike winter and in both seasons downstream at the estuary mouth (**Figure IV-12A, B and C**). In particular, a higher abundance of methanogenic communities was observed in areas affected by the WWTP discharge (**Figure IV-12D**), as in other estuaries impacted by WWTP effluents [29]. Besides, a rich methanogenic community was observed in winter at the discharge station and downstream based on 16S rRNA sequencing accounting for the relevance of methanogenesis in wetlands.

The BOD₅ in the water, as well as total OM and $\delta^{15}\text{N}$ in the sediments were significantly correlated with comammox and methanotrophic abundances. In general, both functional groups increased downstream towards the Estuary mouth (**Figure IV-12A, B and C**), where greater $\delta^{15}\text{N}$ values and nutrients were found during both winter and summer (**Figure IV-4A**). Moreover, methanotrophic abundance in the estuary was significantly correlated with methanogens and with nitrifiers (**Figure IV-13**), indicating the potential metabolic interaction between these three functional groups. This is the case of nitrite-dependent anaerobic methane oxidation by bacteria, including Methylomirabilaceae (**Figure IV-11**) that could benefit from the nitrite produced by ammonia-oxidation linking carbon and nitrogen cycling in wetlands as previously reported [83,84].

Benthic OM considering nitrogen availability could be relevant for the resistance or tolerance of the functional microbial groups to the WWTP discharge

in the estuary. Winter conditions were unfavorable for methanotrophs and nitrifying assemblages, potentially reducing their role in nutrient and GHG recycling. Nutrients accumulation towards the mouth was evidenced during both seasons, confirming the pervasive eutrophic conditions of El Sauce Estuary [31].

These results together with previous studies on the same ecosystem encourage further research on the current effects of WWTP discharges, an issue that has aroused wide public concern, especially in rural areas, since more infrastructure, resource and higher regulatory standards are often oriented towards increasing wastewater treatment capacity of cities [28, 85]. This situation is even worse in countries like Chile, where a relevant fraction of rural residents has lower incomes, less education, and more importantly, less access to public utilities, compared to their urban counterparts [86]. The effects on the water availability and quality caused by these discharges and other sources of contamination are often suffered by more vulnerable rural communities that lose fisheries resources, agricultural production, and health [87].

This is the case of Laguna Verde and the community residing near the El Sauce Estuary, which is an area of rapid population growth in the Valparaíso area, and precarious urban development and highly dependent on agriculture [88]. Thereby, gaining insights into the interaction of the negative impacts that these discharges have on water quality is necessary to optimally address not only their consequences on the environment at the local level, but also their effects on greenhouse gas dynamics.

5. Conclusions

In summary, our results confirm that the El Sauce Estuary is an anthropogenic impacted ecosystem characterized between 40 – 60 % of the variables with poor-quality conditions. Among other anthropogenic pressures, including leachate from a municipal landfill and domestic water inputs that have permanently contributed to this eutrophic system, our study concluded that WWTP effluents play a major role in shaping microbial and biogeochemical processes

ending in GHG accumulation. For instance, WWTP effluent was found to have a great impact at the upstream area compared to the pre- and post-discharge sites in the physicochemical conditions and the dissolved GHG concentration in the water especially for CH₄ and N₂O by one order of magnitude. The organic matter quality varied spatially along the Estuary shifting from upstream towards the mouth associated with terrestrial ($\delta^{13}\text{C} = -28$ to -29.5 and $\delta^{15}\text{N} = 4$ to 8.7) vs estuarine ($\delta^{13}\text{C} = -25.3$ to -26.5 and $\delta^{15}\text{N} = 8.4$ to 14.2) origins. Benthic microbial community composition in the estuary followed the same trend as OM, characterized by the enrichment and enhancement of functional groups of methanotrophs and methanogens reaching up to 6.5×10^5 and 2.1×10^5 abundance towards the mouth. However, the anthropogenic impact of the WWTP-discharge effluent upstream contributed to reshaping the benthic microbial community composition even at phylum level by introducing allochthonous phyla, and changing the relative contribution of core wetland taxa potentially limiting the growth of nitrifying and methanotrophic bacteria during winter. Therefore, the spatio-temporal (winter vs summer) natural conditions and anthropogenic sources of perturbations strongly drive the dynamics of the benthic microbial community composition and potentially the way nutrients and GHG get recycled in the El Sauce Estuary.

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8. Supplementary Material

8.1 Supplementary Figures

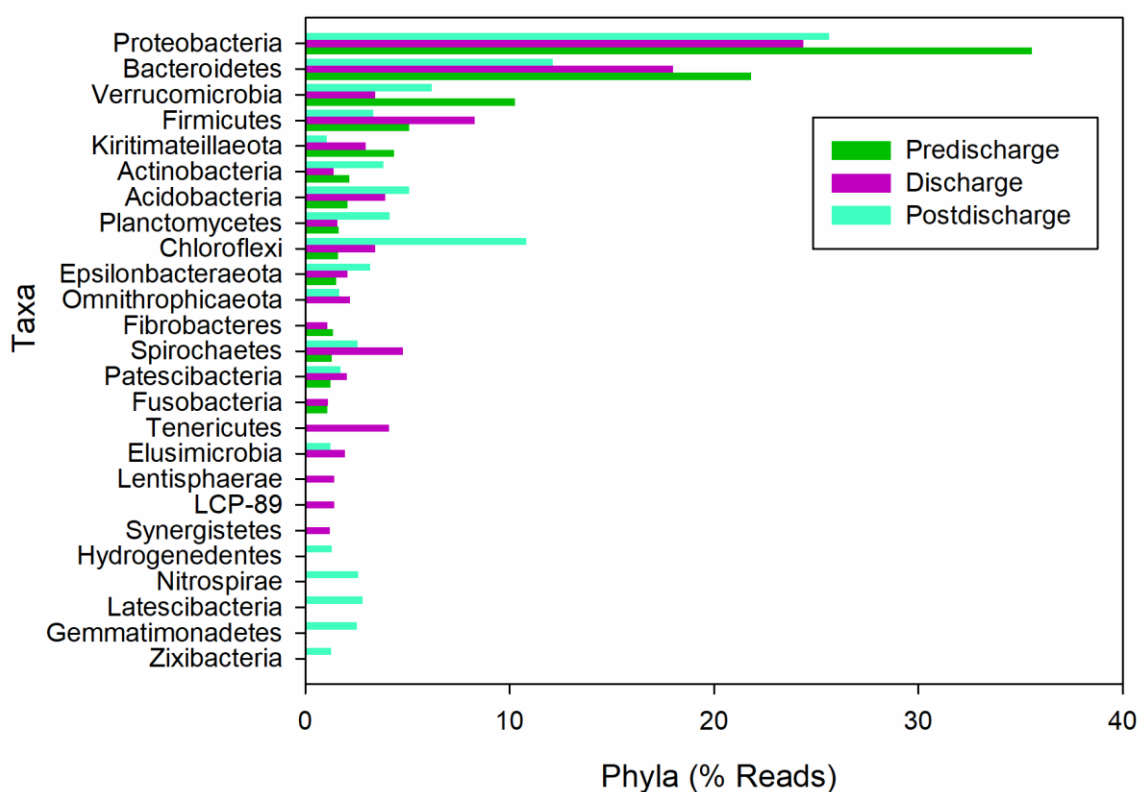


Figure IV-S1. Graph representation of SIMPER analysis comparing the most dominant phyla between pre discharge, discharge and post discharge stations.

8.2 Supplementary Tables

Table IV-S1. Taxonomic classification at the phylum level.

Phylum	F2W	F2S	F3W	F3S	E7W	E10W	E10S	E11W	E11S
Altiarchaeota	0	0	0	0	0	2	0	7	2
Asgardaeota	0	0	24	13	0	47	559	59	180
Crenarchaeota	0	0	25	18	5	18	313	169	122
Diapherotrites	0	0	145	2	3	0	75	112	46
Euryarchaeota	3	46	2217	296	109	11	884	621	1076
Hadesarchaeaeota	0	0	0	2	0	0	0	0	2
Nanoarchaeaeota	31	120	774	972	149	601	1783	2109	2586
Thaumarchaeota	0	0	11	0	43	0	749	27	67
Acetothermia	0	0	10	0	0	0	0	3	2142
Acidobacteria	388	545	5006	2833	4322	6928	11231	5844	4732
Actinobacteria	288	132	6496	541	3519	5308	2642	2685	3085
Aegiribacteria	0	0	288	0	17	0	11	8	22
Armatimonadetes	6	16	471	117	65	174	510	127	253
Atribacteria	0	0	7	0	0	0	0	0	44
BRC1	4	7	65	57	36	78	200	121	243
Bacteroidetes	44002	8323	55008	54948	58332	30231	30453	53409	25385
CK-2C2-2	0	0	0	5	0	0	21	0	14
Caldiserica	0	4	1022	22	60	0	21	0	67
Calditrichaeota	0	0	162	4	3	0	50	11	308
Chlamydiae	43	15	392	158	277	97	258	64	93
Chloroflexi	177	547	8725	1862	1971	38728	18664	25509	20005
Cloacimonetes	2	78	307	785	7	6	33	72	76
Cyanobacteria	82981	3137	1945	21366	10416	23644	13876	20707	16261
Dadabacteria	0	0	0	0	2	0	0	4	0
Deinococcus-Thermus	0	0	0	0	0	0	0	0	47
Dependentiae	6	0	1092	41	217	188	256	56	196
Elusimicrobia	61	92	2277	747	244	153	487	595	752
Epsilonbacteraeota	154	49	9541	739	3112	4602	638	2976	2952
FBP	0	0	4	6	14	2	16	0	5
FCPU426	0	12	162	103	8	8	42	79	61
Fibrobacteres	88	47	214	519	27	155	410	331	420
Firmicutes	1539	1615	14477	10825	1825	1405	2696	2652	3406
Fusobacteria	106	30	172	284	161	29	45	71	71
GN01	0	0	17	0	0	0	6	0	0
Gemmatimonadetes	44	10	133	134	1738	1478	2165	2031	747

Continuation of Table IV-S1

Phylum	F2W	F2S	F3W	F3S	E7W	E10W	E10S	E11W	E11S
Halanaerobiaeota	0	0	0	0	0	8	10	0	11
Hydrogenedentes	63	47	56	448	378	194	461	190	458
Kiritimatiellaeota	1204	437	1502	3056	195	256	278	356	831
LCP-89	10	138	332	1012	13	0	33	68	132
Latescibacteria	19	47	270	172	145	755	4869	2185	1945
Lentisphaerae	47	47	428	496	118	91	242	487	138
Margulisbacteria	0	7	194	48	5	0	42	9	41
SAR406 clade	0	0	0	0	0	0	0	0	2
Modulibacteria	2	0	20	0	0	0	0	0	18
Nitrospinae	9	0	0	15	74	0	45	0	183
Nitrospirae	28	173	27	192	197	862	5187	2312	911
Omnitrophicaeota	14	100	3464	817	311	359	1122	606	1419
PAUC34f	0	0	0	0	0	0	15	26	22
Patescibacteria	659	131	4272	1287	1199	967	1831	2739	2418
Planctomycetes	182	145	383	1145	816	4440	5959	3014	4070
	7999	2395	10016	11960	15152	14498	9689	14076	10087
Proteobacteria	3	1	4	1	3	3	0	4	6
Rokubacteria	2	9	50	44	13	55	249	16	63
Spirochaetes	112	552	7861	4014	368	488	4350	1876	2719
Synergistetes	2	34	519	194	29	17	5	53	93
TA06	0	0	0	0	0	0	0	0	2
Tenericutes	54	320	10145	2525	1103	13	40	166	399
Thermotogae	0	0	86	0	0	0	5	0	10
	1401								
Verrucomicrobia	7	1859	1804	12730	6731	7565	5362	7356	6256
WOR-1	0	3	340	55	53	2	48	29	233
WPS-2	0	20	0	107	57	5	0	13	0
WS1	0	0	9	7	0	6	96	25	41
WS2	0	0	4	0	0	0	29	0	12
WS4	0	0	85	0	2	5	17	0	46
Zixibacteria	0	20	730	45	42	103	1170	649	2247

Table IV-S2. Spearman correlation analyses between functional group and physico-chemical variables studied. The different color represent significant level (p < 0,05 = violet, p < 0,01 = lilac, p < 0,001 = purple).

Variable	Ammonia Oxidizers	Comammox	Methanotrophs	Methanogens
Water Temperature (°C)	0,385	0,711	0,661	0,559
pH (pH unit)	0,250	0,200	0,300	0,236
Conductivity (µS/cm)	0,033	0,683	0,650	0,552
Dissolved Oxygen (mg/L)	0,183	0,283	0,317	0,406
DTS (mg/L)	0,033	0,683	0,650	0,552
Transparency (cm)	-0,050	-0,201	-0,251	0,215
Turbidity (NTU)	-0,400	0,067	0,050	0,103
Color (Pt/C)	-0,233	-0,100	-0,117	-0,018
Sulfate (mg/L)	-0,433	0,283	0,233	0,600
Chlorides (mg/L)	-0,075	-0,142	-0,142	0,280
Total Coliforms (NMP)	-0,025	-0,050	-0,067	-0,134
Fecal Coliforms (NMP)	0,101	0,050	0,067	0,061
BOD ₅ (mg/L)	0,367	0,833	0,850	0,103
Chlorophyll a (mg/L)	0,500	0,550	0,517	0,091
CO ₂ (µM)	-0,133	-0,367	-0,383	-0,224
CH ₄ (nM)	-0,083	0,167	0,033	0,455
N ₂ O (nM)	-0,200	-0,033	-0,083	0,139
Nitrate (mg/L)	0,483	0,217	0,233	-0,418
Nitrite (mg/L)	0,350	0,417	0,450	-0,079
Phosphate (mg/L)	-0,050	-0,600	-0,533	-0,927
Silicate (mg/L)	-0,250	-0,433	-0,450	0,018
Nitrate/Phosphate	0,483	0,517	0,533	0,370
Total Organic Matter (g)	0,333	0,700	0,667	0,576
C/N	-0,083	-0,317	-0,267	0,018
δ ¹⁵ N	0,833	0,717	0,700	0,346
Gravel (g)	0,100	0,033	0,117	-0,261
Very Coarse Sand (g)	0,267	0,300	0,333	0,236
Coarse Sand (g)	-0,500	-0,033	-0,150	0,358
Medium Sand (g)	-0,583	-0,367	-0,417	0,127
Fine Sand (g)	-0,433	-0,333	-0,317	-0,164
Very Fine Sand (g)	0,433	0,533	0,567	0,139
Silt (g)	0,450	-0,083	-0,050	-0,079

CAPÍTULO V. DISCUSIÓN GENERAL

Discusión General

Esta tesis doctoral se enfocó en determinar el impacto de la salinidad, entre otros factores físico - químicos, naturales y antrópicos sobre la dinámica espacio-temporal de la estructura y composición del microbioma bentónico y su relación con los GEI en humedales costeros de la ZSACHC. Conocer estas relaciones son cruciales para generar instancias de predicción, conservación y/o manejo sustentable de humedales costeros, considerando su rol ecosistémico en el secuestro de carbono, su vulnerabilidad y sobre todo considerando escenarios de cambio climático que predicen un aumento en el nivel del mar, que lleva a un incremento en la intrusión de aguas salinas en los humedales costeros. Además es importante mencionar que la disminución del aporte de agua dulce desde las cuencas por su uso en actividades antrópicas y sequía también ha generado un impacto sobre los humedales costeros. La salinidad es uno de los factores reconocidos por alterar a la comunidad microbiana y los procesos biogeoquímicos que realizan [17], como es la disminución de las tasas de descomposición de la materia orgánica en los sedimentos de humedales costeros [27, 105] entre otros procesos relacionados con el reciclaje de nutrientes y GEI [59], sin embargo su efecto puede ser variable de acuerdo a las condiciones presentes en cada ecosistema.

El primer resultado de esta tesis doctoral fue informado en Pozo-Solar et al. (2022), un estudio que abordó una escala latitudinal $N \rightarrow S$ ($29^{\circ}82' - 33^{\circ}77'$), considerando un grupo de 24 humedales costeros en invierno y verano. Este enfoque de estudio no tiene precedente en Chile, siendo además una de las pocas investigaciones que combina dos áreas multidisciplinarias del conocimiento, i.e., la ecología microbiana y la biogeoquímica. En general, este tipo de investigaciones se han realizado en estuarios del hemisferio norte considerando análisis a lo largo de un mismo estuario o la dinámica temporal tomando en cuenta los ciclos mareales. La importancia de este tipo de estudio se sustenta en que los humedales costeros que además de disminuir hacia el norte del país [106], son ecosistemas reguladores que proveen diversos servicios ecosistémicos a la

población, como por ejemplo, hábitat para la pesca o avistamiento de aves, el secuestro de carbono, funcionar como barreras antes tsunamis, fuentes de agua, sumideros de sedimento, entre otros [107]. Por lo tanto es esencial aumentar la comprensión sobre cómo funcionan los humedales costeros y qué factores y procesos reguladores claves operan en estos ecosistemas. En el caso de la zona de estudio, el área donde se encuentran situados los humedales presenta una mega sequía acentuada hacia el norte [108], como consecuencia de un efecto combinado del cambio climático [98] y al impacto antrópico por uso de agua para diversas actividades [90].

Los resultados de esta investigación dan cuenta de dos grandes grupos de humedales; 1) humedales ubicados en cuencas áridas “high aridity basins, HAB” asociadas a un menor grado de impacto antrópico ($n = 12$) y 2) humedales ubicados en cuencas con mayor disponibilidad hídrica, pero con un mayor impacto antrópico “low aridity basins, LAB” ($n = 12$) [109]. Los resultados a modo general de las características de las cuencas y sus humedales se esquematizan en la **Figura V-1**. Los humedales emplazados en HAB se caracterizaron por una mayor presencia de humedales mesosalinos con altas temperaturas y bajas concentraciones de nitrito y nitrato. Al contrario con esto, los humedales emplazados en LAB fueron mayormente hiposalinos y caracterizados por temperaturas más bajas y altas concentraciones de nitrito y nitrato. Este contraste fisicoquímico entre los humedales HAB y LAB se debe principalmente a las diferencias geográficas y climáticas que ocurren en cada región de cada grupo de humedales. La región árida de Coquimbo, lugar donde se distribuyen los humedales HAB, es una zona de transición entre la porción sur de la zona mediterránea de Chile central y el desierto hiperárido de Atacama. Su clima va a estar determinado por una combinación de factores atmosféricos, oceánicos y orográficos, que dan origen a la formación de neblinas [110, 111] que son una de las principales fuentes de agua para el paisaje y la flora de la región. A diferencia de esto la región de Valparaíso es principalmente influenciada por un clima mediterráneo que se caracteriza por un régimen estacional de precipitaciones y temperaturas, con inviernos fríos y húmedos y veranos cálidos y secos [112]. Por

lo tanto las contrastantes condiciones climáticas observadas en los humedales HAB y LAB nos dan cuenta del por qué presentan diferentes condiciones en su disponibilidad de agua, salinidad y temperatura. Por otro lado, las mayores concentraciones de nitrato y nitrito observadas en los humedales del grupo LAB, nos dan cuenta de un potencial mayor impacto antrópico hacia el sur de la ZSACHC, por diversos usos industriales, domésticos, aguas residuales y un potencial mayor desarrollo de actividades agrícolas. Estudios previos refrendan el fuerte impacto agrícola por altas concentraciones de nitrato encontradas en los ríos Aconcagua [113] y Maipo [114], lo cual, potencialmente estimularía una mayor acumulación de N_2O en los humedales LAB, en comparación con los humedales HAB donde potencialmente podrían estar actuando como sumidero de N_2O [109].

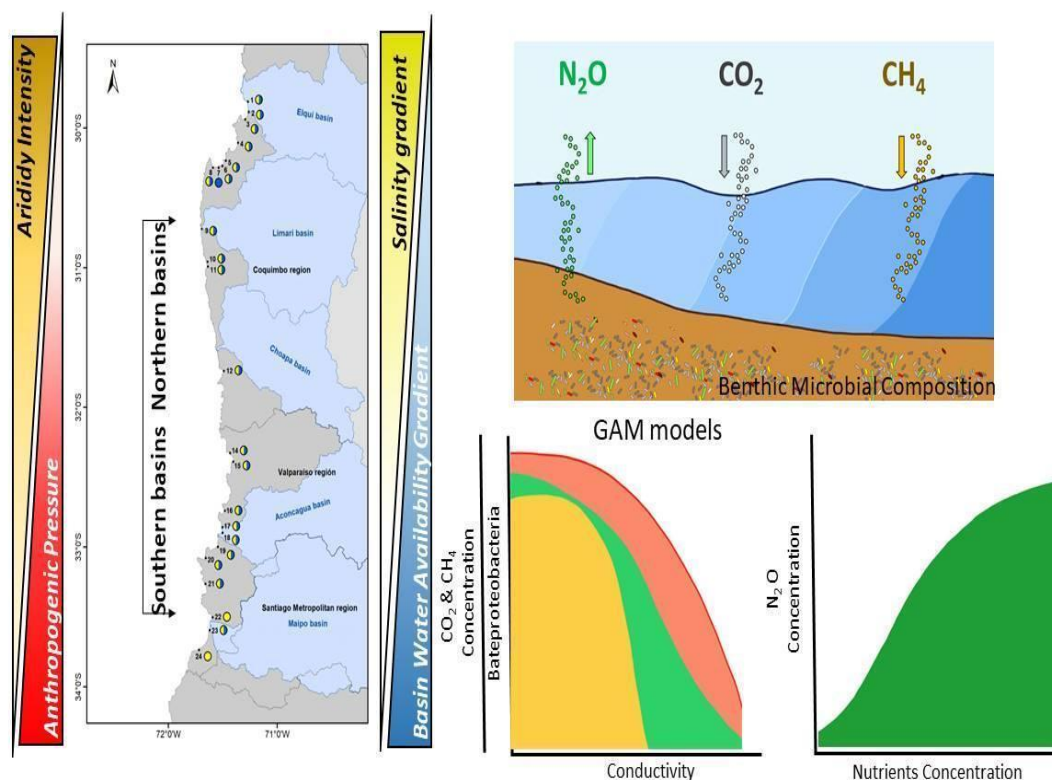


Figura V-1: La salinidad es un factor clave sobre la distribución de GEI y su microbioma bentónico en humedales costeros de la ZSACHC, que cuenta con gradientes de salinidad, aridez, disponibilidad de agua y el nivel de impacto antrópico.

La identificación de la importancia de los factores físico-químicos, como la salinidad además de los cambios en la composición microbiana con énfasis en grupos funcionales asociados a la distribución de los GEI se exploró mediante modelos GAM y SEM. Ambos modelos multivariados son utilizados para entender el efecto o causalidad de un conjunto de variables por sobre variables de respuesta, mediante relaciones lineales significativas con sus respectivos ajustes. En este campo de estudio, estos modelos se utilizan con un enfoque multidisciplinario, en donde variables ambientales, incluyendo diversos factores físicos, químicos y biológicos, son contrastados con estudios ecológicos, o viceversa, buscando dilucidar la causalidad y sus efectos en los distintos procesos biogeoquímicos que toman lugar en el ecosistema estudiado. Para la aplicación de estos modelos en el presente estudio, se realizó una secuencia progresiva, en donde se (i) evaluó correlaciones entre factores físico-químicos, GEI y los grupos funcionales, (ii) evaluó la significancia espacial y temporal de los factores ambientales (EF), los nutrientes (Ntr) y la materia orgánica MO, (iii) chequeó mediante un NMDS que los datos de invierno y verano de los grupos funcionales no se agruparan, (iv) evaluó a través de modelos GAM el efecto de la conductividad y otros factores ambientales sobre los GEI y los grupos funcionales y (v) evaluó mediante un modelo SEM el efecto causal de los factores ambientales por sobre los grupos funcionales y los grupos funcionales por sobre los GEI (**Figura V-2**). A partir de los resultados obtenidos con los modelos GAM, se pudo observar que con el aumento de la salinidad disminuyen las concentraciones de CH₄ y CO₂. Nuestros resultados son consistentes con estudios previos donde una disminución en los flujos de CH₄ y CO₂ se observan con un aumento en la salinidad en el estuario del río Yellow River Delta [115] y el estuario Yangtze [116]. Además, estos cambios en las concentraciones de CH₄ y CO₂ son mejor explicados cuando se suman variables predictoras como la latitudinal y el pH, respectivamente. Esta relación entre el pH y el CO₂ tiene sentido ya que las variaciones en el pH (H⁺/HO⁻) van a estar directamente relacionadas con cambios en la conversión neta de moléculas disueltas asociadas al metabolismo del carbono como por ejemplo el CO₂, el H₂CO₃ (ácido carbónico) el HCO₃⁻

(bicarbonato) el CO_3^{2-} (carbonato) y el CaCO_3 (carbonato de calcio) en los ecosistemas acuáticos [117]. Por otro lado, desde los modelos GAM también se desprende que la salinidad no fue el mejor predictor para explicar los cambios en las concentraciones de N_2O , siendo mejores variables predictoras las concentraciones de nitrato y la latitud [109]. Por lo tanto, el cambio latitudinal de la zona semiárida de Chile central destaca como una variable que resume la condición de las cuencas que involucra no sólo un mayor aporte de agua o disponibilidad hacia el sur, sino que también un incremento en actividades antrópicas que se traducen en una mayor producción de GEI. Estos resultados se corresponden con los resultados obtenidos para el CH_4 y el CO_2 en estuarios [42].

Por otro lado, la estructura del microbioma bentónico que alberga el sedimento de estos humedales es altamente diversa (3.7 - 5.6 Índice de Shannon) y dominada por Phylum, incluyendo Proteobacteria, Cianobacterias, Bacteroidetes, Actinobacteria y Acidobacteria. Desde el modelo GAM se pudo observar que el orden Betaproteobacteria (incluido dentro de la clase Gammaproteobacteria y que contiene a comunidades metanotróficas y nitrificantes), disminuye con el aumento de la salinidad y la materia orgánica [109]. Se ha observado mediante microcosmos que el aumento de la salinidad puede tener efectos inhibidores sobre el crecimiento microbiano, la respiración del suelo y la remineralización del nitrógeno [118]. Además, se han observado altos flujos de CO_2 en hábitat de agua dulce en comparación con humedales afectados por la salinidad [119]. Podría ser que las mayores concentraciones de MO observadas en los humedales mesosalinos HAB [109] se debería a menores tasa de remineralización inducidas por el estrés salino, sin embargo, nosotros no medimos tasas de remineralización y por lo tanto quedaría como una pregunta abierta para próximas investigaciones. El modelo SEM arrojó que variables fisicoquímicas como la conductividad, las concentraciones de nitrato, los contenidos de MO y la latitud, explican los cambios de grupos funcionales claves como aquellos pertenecientes a las comunidades metanotróficas/metilotróficas (*Methylothermobacter*) y nitrificantes (*Notrosomonas*, *Nitrospira*) y que a su vez, influyen sobre el reciclaje de GHG [109]. Por lo tanto, la

variación latitudinal observada en la salinidad, la disponibilidad de agua y la presión antrópica, son factores claves sobre las comunidades microbianas involucradas en el reciclaje de GEI en humedales costeros de la ZSACHC [109]. De esta forma, los resultados obtenidos por este estudio concuerdan con la hipótesis testeada, indicando que el aumento de la salinidad a lo largo del transecto que incluyó 24 humedales costeros de las ZSACHC disminuye la abundancia de comunidades asociadas al reciclaje de GEI y las concentraciones de GEI.

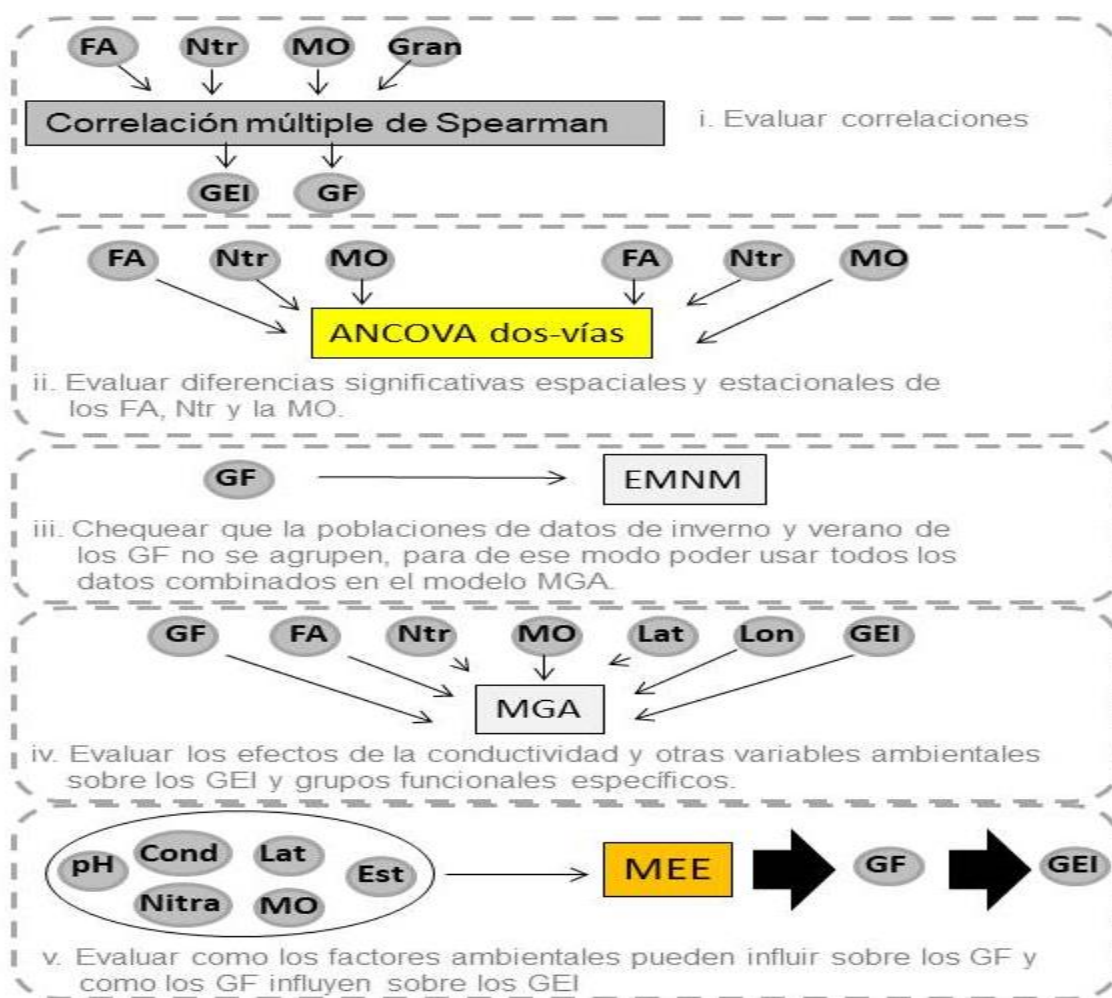


Figura V-2: Resumen general de la estadística usada en el capítulo III. Las abreviaciones usadas son las siguientes: FA = Factores Ambientales, Ntr = concentración de nutrientes, GEI = concentración de GEI, GF = grupos funcionales tales como, nitrificantes metanogénicos y metanotróficos, MO = materia orgánica, Gran = granulometría, Lat = latitud, Lon = longitud; Est = Estación, Nitra = nitrato, Cond = conductividad, MGA = modelo generalizado aditivo, EMNM = modelo de escala multidimensional no métrica, MEE = modelo de ecuaciones estructurales.

El segundo resultado de la investigación de tesis doctoral se focalizó en la variabilidad espacio-temporalidad de las comunidades microbianas bentónicas, incluyendo la cuantificación específica de grupos funcionales mediante genes marcadores relacionados a microorganismos amonio oxidantes, comammox, metanogénicos y metanotróficos a lo largo del estero y humedal El Sauce. Este humedal es parte de los humedales correspondientes al grupo LAB caracterizado por presentar las mayores concentraciones de nitrito además de elevadas concentraciones de GEI [109]. En este estuario la salinidad no fue un factor determinante, como sí lo fue la calidad del agua y cambios en el origen de la materia orgánica (MO). El estero “El Sauce” recibe descargas desde la planta de tratamiento de aguas servidas ESVAL (PTAS), que además de cambiar las condiciones fisicoquímicas del agua (pH, temperatura y la concentración de nutrientes), contribuyen con una acumulación de GEI y MO con señal isotópica diferenciada potencialmente de origen terrestre y antrópica (Pozo-Solar et al. Capítulo IV). Evidencias previas ya han dado cuenta de su condición de humedal eutrofizado y su baja calidad del agua [104] y en efecto, la eutrofización promueve mayores emisiones de GEI en ecosistemas de agua dulce [120]. Adicional a esto, los resultados de esta investigación doctoral indicaron que la descarga desde la PTAS altera la estructura y composición del microbioma bentónico a nivel de phylum en el estero “El Sauce”, contribuyendo con phylum foráneos como Tenericutes, Lentisphaerae, LCP-89 y Synergistetes y disminuyendo phylum vinculados a procesos claves como Cianobacterias, Nitrospirae y Chloroflexi (Pozo-Solar et al. Capítulo IV). La menor abundancia de Cianobacterias se debe a la alta turbidez del agua que al no dejar pasar la radiación solar se inhibe el proceso de fotosíntesis [121]. Además, la baja presencia de Chloroflexi se debe al aporte de otros potenciales compuestos tóxicos liberados desde la descarga y a la baja carga nutritiva que induce la descarga de la PTAS, afectando sobre su crecimiento, como ha sido previamente sugerido por Drury & Kelly (2013) [122]. De este modo, los cambios fisicoquímicos producidos por las descargas de aguas residuales en esteros y/o humedales costeros, pueden alterar la estructura del microbioma bentónico nativo mediante la incorporación de nuevos taxa o bien la

inhibición de otros. Por otro lado, la descarga de aguas residuales de la PTAS, promueven un aumento de la riqueza de comunidades metanogénica, al igual que su abundancia basada en la cuantificación del gen funcional marcador (mcr) mediante qPCR (Pozo-Solar et al., Capítulo IV). De acuerdo con esto Guo et al., 2019, reportaron un aumento de comunidades metanogénicas principalmente asociadas al phylum Euryarchaeota en zonas impactadas por PTAS en el estuario Yangtze (China) [123]. El aumento de comunidades metanogénicas en la zona impactada por la PTAS se debe a la incorporación de taxa que vienen en la misma descarga, contribuyendo a su diversidad y modificando su estructura comunitaria. Por otra parte, otros grupos funcionales como nitrificantes y metanotrofos se desfavorecen en el punto de descarga principalmente en invierno cuando existe una menor disponibilidad de nutrientes, en comparación a otros sitios especialmente en la desembocadura y durante el período de verano (Pozo-Solar et al., Capítulo IV). De acuerdo con esto resultados, una disminución de comunidades metanotróficas fue observada en una zona impactada por una PTAS en el estuario Yangtze (China) [123]. La menor abundancia observada de comunidades metanotrofas y nitrificantes en el punto de descarga durante invierno se explica por la menor disponibilidad de nutrientes disponible para el crecimiento y mantenimiento de sus poblaciones. Además los pH observados en el punto de descarga en invierno y verano (6.8-6.9), no coinciden con los óptimos (8.2) reportados para la nitrificación en sedimentos humdales [124]. Por lo tanto el pH también puede funcionar como una variable clave sobre el proceso de nitrificación, además del oxígeno y la disponibilidad de nutrientes. Sería interesante en investigaciones futuras mediante experimentos de microcosmos y trasplantes recíprocos poder entender mejor como responden las comunidades microbianas a diversos factores que regulan procesos claves asociados al reciclaje de GEI en humedales costeros.

Análisis comparativo entre el capítulo III y IV

Con el propósito de comprender el grado de perturbación del estero El Sauce y su implicancia sobre los GEI y las comunidades microbianas, se generó

una comparación detallada de los resultados del estero El Sauce con respecto a un grupo de humedales pertenecientes a LAB. Desde un análisis de Mann-Whitney comparando las concentraciones de GEI entre los humedales más impactados de la ZSACHC (LAB) y el conjunto de datos obtenidos desde el estero “El Sauce”, se pudo corroborar que las aguas del estero presentaron concentraciones de GEI significativamente altas ($p < 0.001$) en comparación con el resto de humedales impactados en LAB (**Figura V-3**). Las concentraciones significativamente mayores observadas en “El Sauce”, dan cuenta del gran de impacto y de la pobre calidad de agua que presenta todo el estero y estuario “El Sauce”. El efluente de la PTAS se considera la principal fuente de agua que recibe el estero “El Sauce” (~ 70 %), por lo tanto, es posible que los tratamientos secundarios realizados previo a la descarga, no eliminan los GEI, aportando con grandes concentraciones de estos gases y materia orgánica diluida hacia la cuenca. Complementario a esto, el análisis de componentes principales (PCA) de los factores de calidad de agua, incluyendo los GEI, indicó que la estación F3 del estero “El Sauce” (punto de descarga de la PTAS) presenta una marcada separación con los humedales LAB y el resto de las estaciones del estero “El Sauce” (**Figura V-4**). Este modelo nos explica dos cosas, primero que la estación impactada por la PTAS presenta condiciones fisicoquímicas totalmente diferentes a sus otras estaciones y a los humedales LAB, lo cual es resultado del crónico impacto que recibe la cuenca producto de la constante descarga. En segundo lugar la similitud fisicoquímica entre el Maipo en invierno y verano y el Aconcagua en invierno con las otras estaciones del estero “El Sauce” (F2 – E11), reflejan el alto grado de impacto que presentan el Maipo y el Aconcagua en la región de Valparaíso. Además el Maipo y el Aconcagua junto con las otras estaciones del estero “El Sauce”, muestran fuertes correlaciones con los nutrientes y los GEI (**Figura V-4**). Estudios previos ya ha reportado una alta carga orgánica y de nutrientes en el Maipo debido al alto porcentaje de actividad industrial humana [114]. En el Aconcagua ya se ha observado que concentra altos niveles de nitratos [113] y que producto de la intensa actividad agrícola su calidad de agua también se ha visto afectada [125]. Por lo tanto además del estero “El Sauce” las cuencas

del Maipo y el Aconcagua también se han visto fuertemente impactadas por la presión antrópica que presenta la región. De este modo se considera esencial tomar medidas de conservación en estos ecosistemas con el fin de protegerlos, cuidarlos y preservarlos.

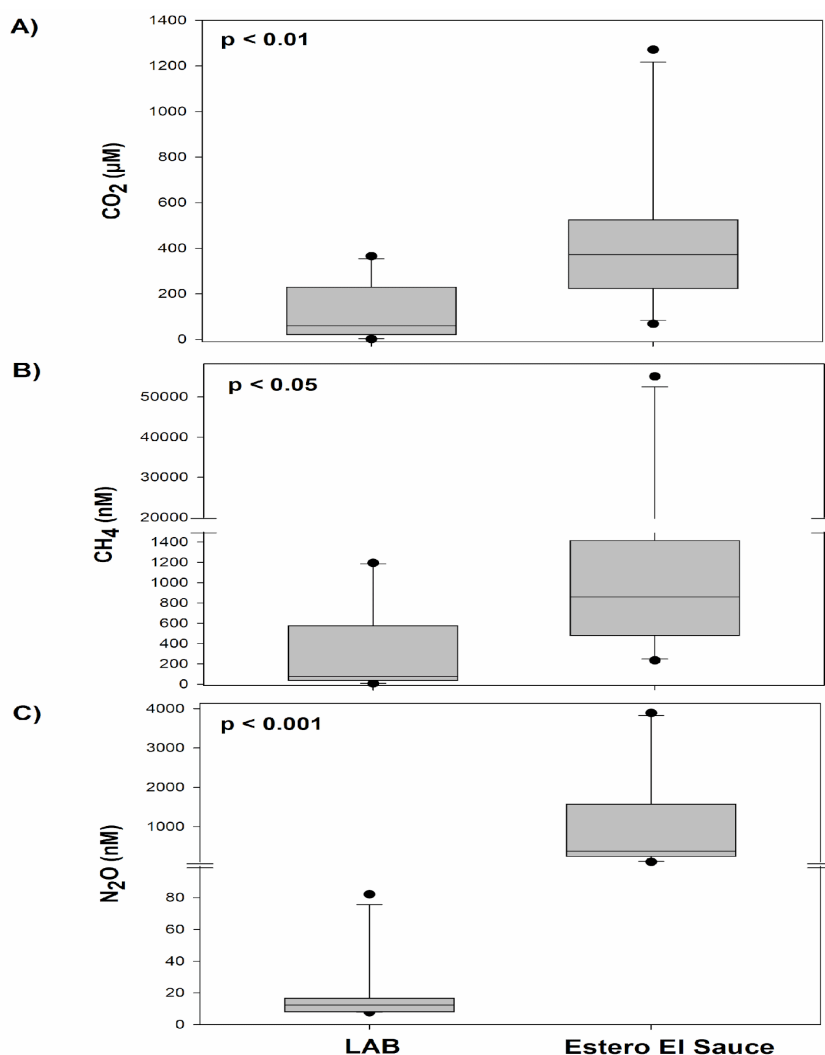


Figura V-3: Comparación de las concentraciones de CO₂ (A), CH₄ (B) y N₂O (C) entre los humedales LAB de la ZSACHC y las estaciones del Estero El Sauce que ha sido impactado por una planta de tratamiento de aguas residuales.

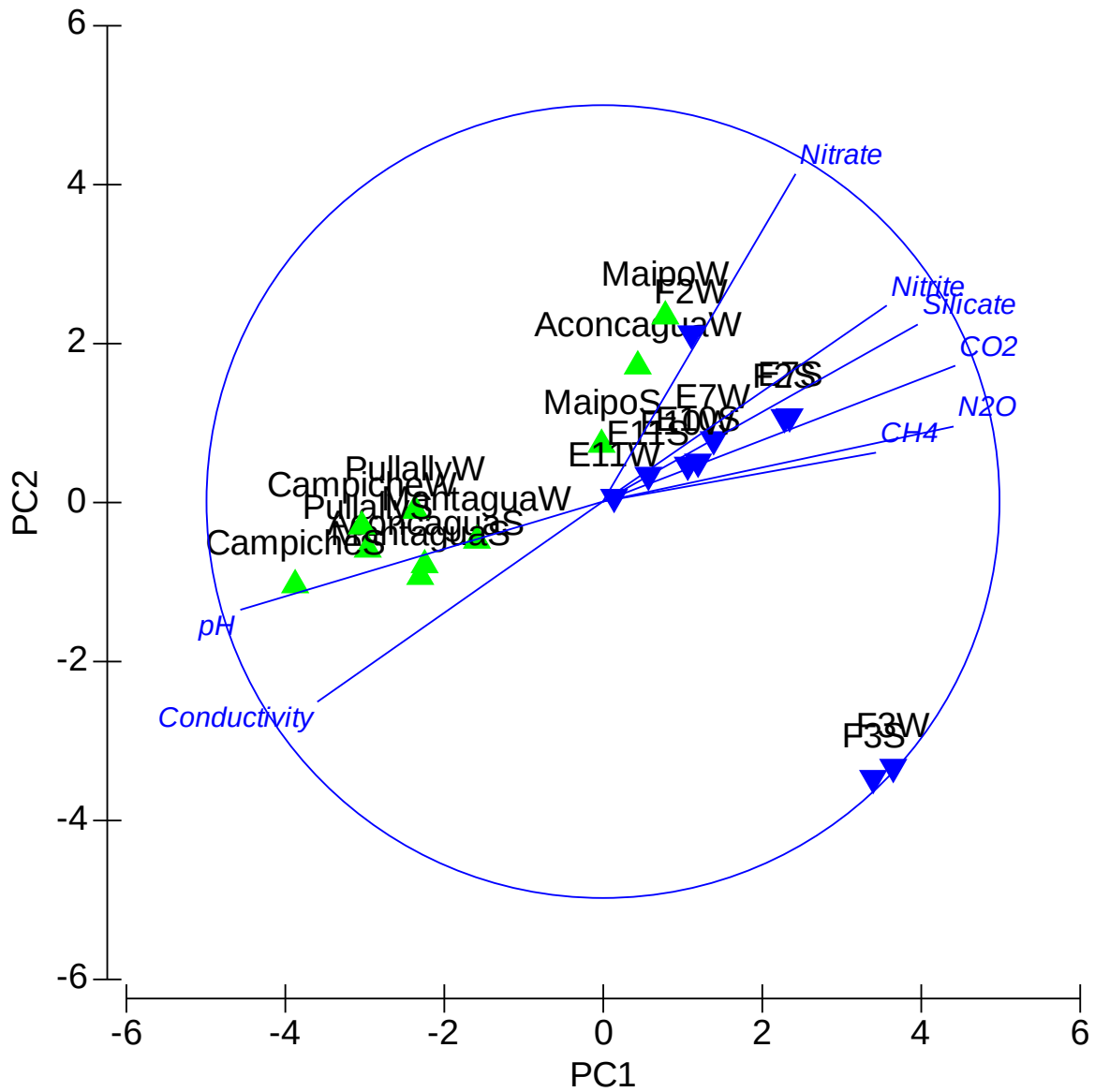


Figura V-4: Análisis de componentes principales de las variables de la calidad de agua y GHG en los humedales LAB de la ZSACHC (triángulos verdes) y las estaciones del Estero El Sauce (triángulos azules).

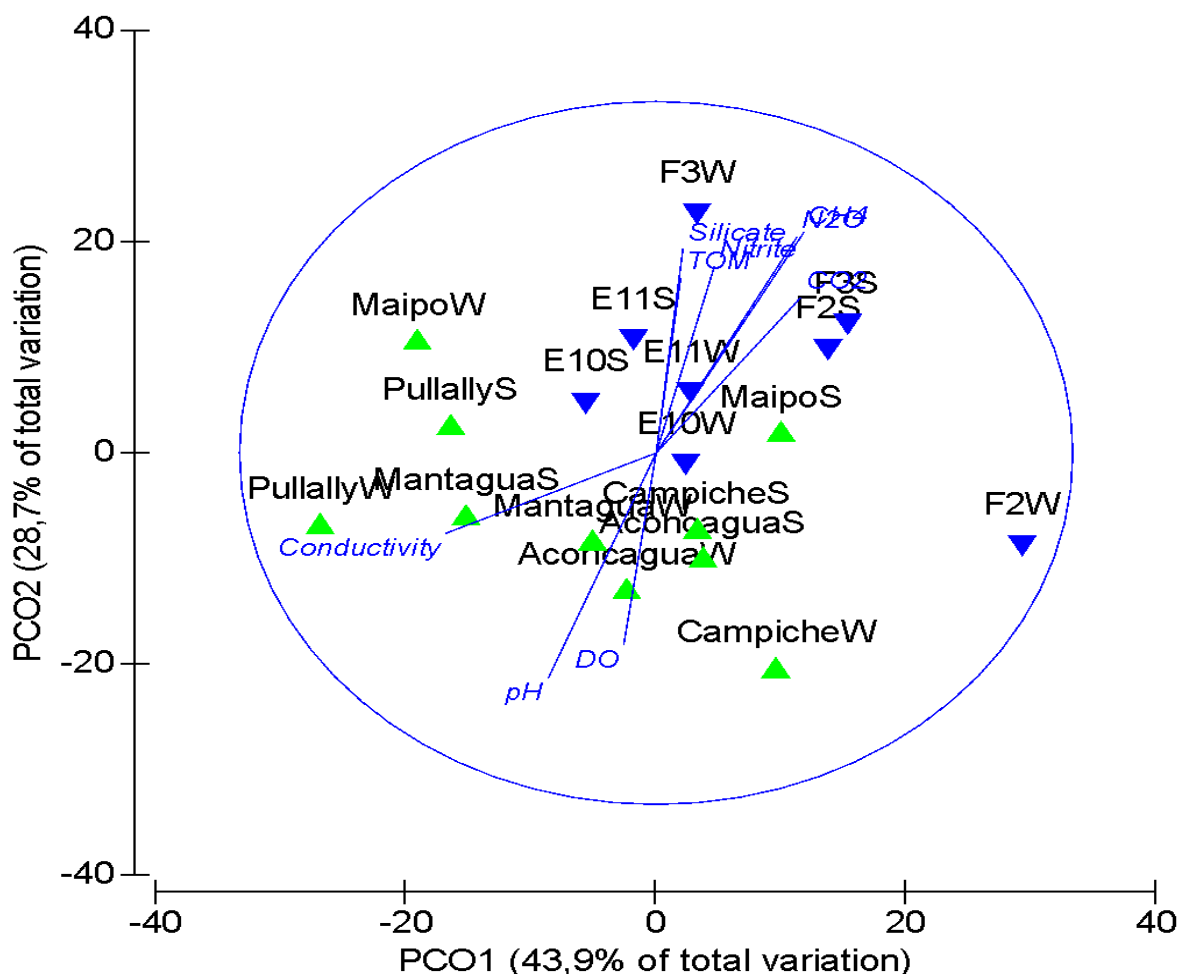


Figura V-5: Análisis de coordenadas principales (PCO) que muestra la similitud entre los microbiomas bentónicos obtenidos desde los humedales LAB (triángulos verdes) y las estaciones del estero El Sauce (triángulos azules) y su relación con factores del sedimento y del agua.

Con respecto al microbioma bentónico, mediante un análisis de coordenadas principales (PCoA), las comunidades microbianas dan cuenta de una marcada separación entre la estructura comunitaria microbiana bentónica de los humedales LAB y las estaciones del estero “El Sauce” (PCoA, **Figura V-5**). Además y se observa una correlación positiva y significativa ($r > 0.45$) entre la MO, el silicato, el nitrito, y los GEI con el eje 2, dando cuenta sobre cómo influyen estos factores sobre el microbioma de las estaciones del estero El Sauce (**Figure V-5**). De manera interesante, el Maipo en verano se agrupa con las estaciones de “El Sauce” en el análisis PCA y PCoA, lo cual nos da a entender que el Maipo en verano debido a su alto grado de impacto, presentaría condiciones similares con

las estaciones del estero “El Sauce”, ya que tanto su estructura microbiana (**Figure V-5**) y condiciones fisicoquímicas (**Figura V-4**) lo muestran. A nivel de Phylum, se observó una menor representatividad de Planctomycetes y Gemmatimonadetes en El Sauce en comparación a los humedales LAB, mientras que en el primero dominaron Firmicutes y Kiritimatiellaeota (**Figure V-6**). Además desde un análisis DNA-seq que sirve para determinar diferencias significativas entre microbiomas bentónicos de distintos lugares, a nivel de clase (**Figure V-7**) y orden (**Figure V-8**), mostro un aumento y una disminución de diversos órdenes y clases de microbios que habitan en el estero “El Sauce” e comparación con los humedales LAB. Por ejemplo a nivel de clase Omnitrophia, Holophagae, Elusimicrobia, Clostridia, Cloacimonadia, y a nivel de orden Betaproteobacteriales, Frankiales, Bacteroidales, Clostridiales, Beggiaotoales, fueron significativamente más abundantes en las estaciones del estero “El Sauce” en comparación con los humedales LAB (**Figure V-7** y **Figure V-8**). Los órdenes Bacteroidales y Clostridiales han servido como buenos bioindicadores del impacto de PTAS [126], así como también de la calidad del agua [127] en ambientes acuáticos, por lo tanto, la mayor abundancia de Bacteroidales y Clostridiales observada en las estaciones del estero El “Sauce” se debe a un aporte directo de la PTAS por sobre el estero. Desde estas observaciones se hace evidente que además de inducir un claro impacto fisicoquímico sobre la calidad del agua, las PTAS también impactan notablemente la estructura del microbioma bentónico en ambientes acuáticos.

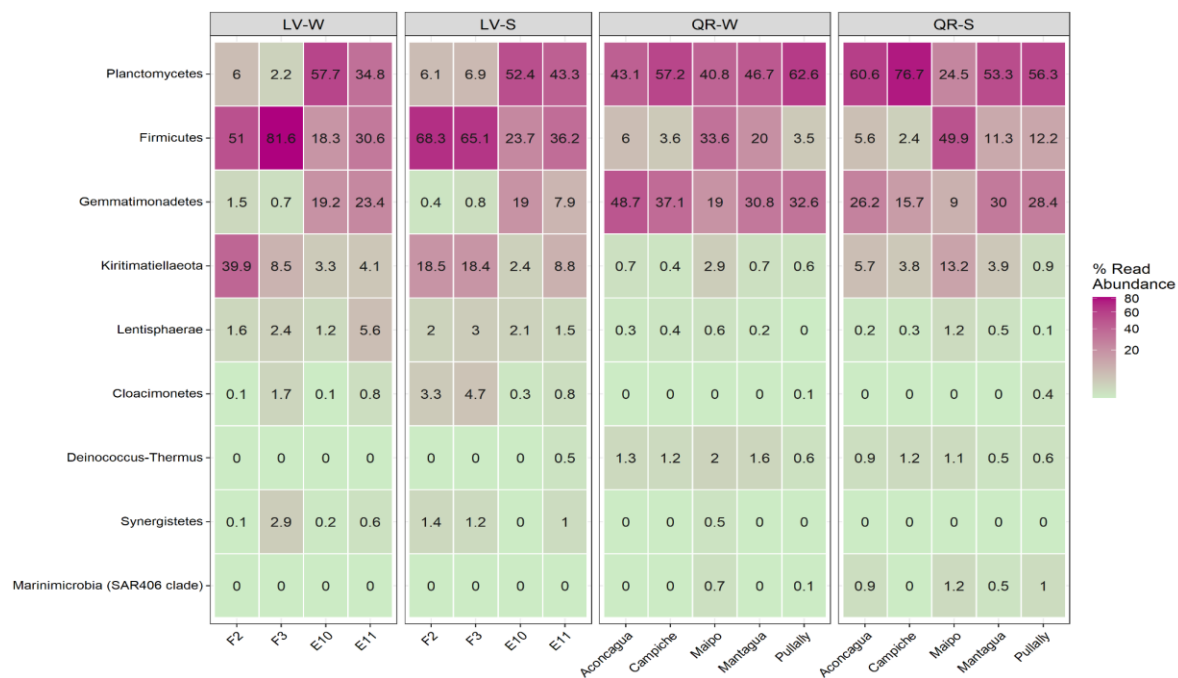


Figure V-6: Mapa de calor a nivel de phylum que compara el % de la abundancia de secuencias entre las estaciones del estero el Sauce y los humedales LAB durante invierno y verano.

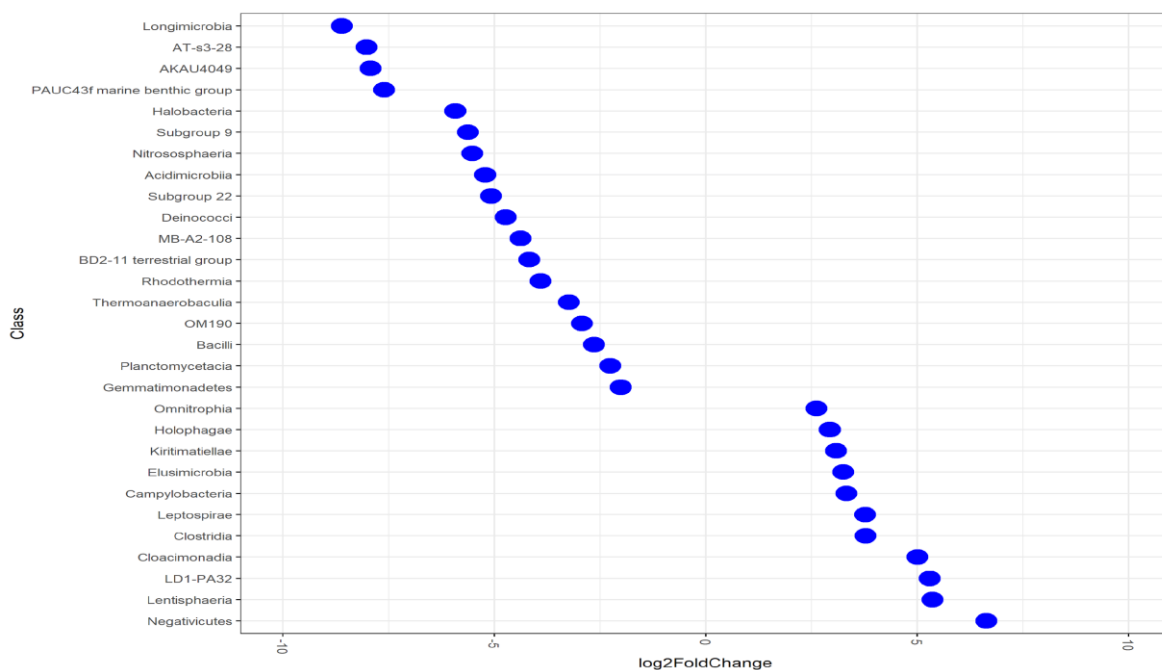


Figura V-7: Análisis DNA-seq que muestra a nivel de clase las diferencias significativas entre los microbiomas de los humedales LAB y las estaciones del estero El Sauce.

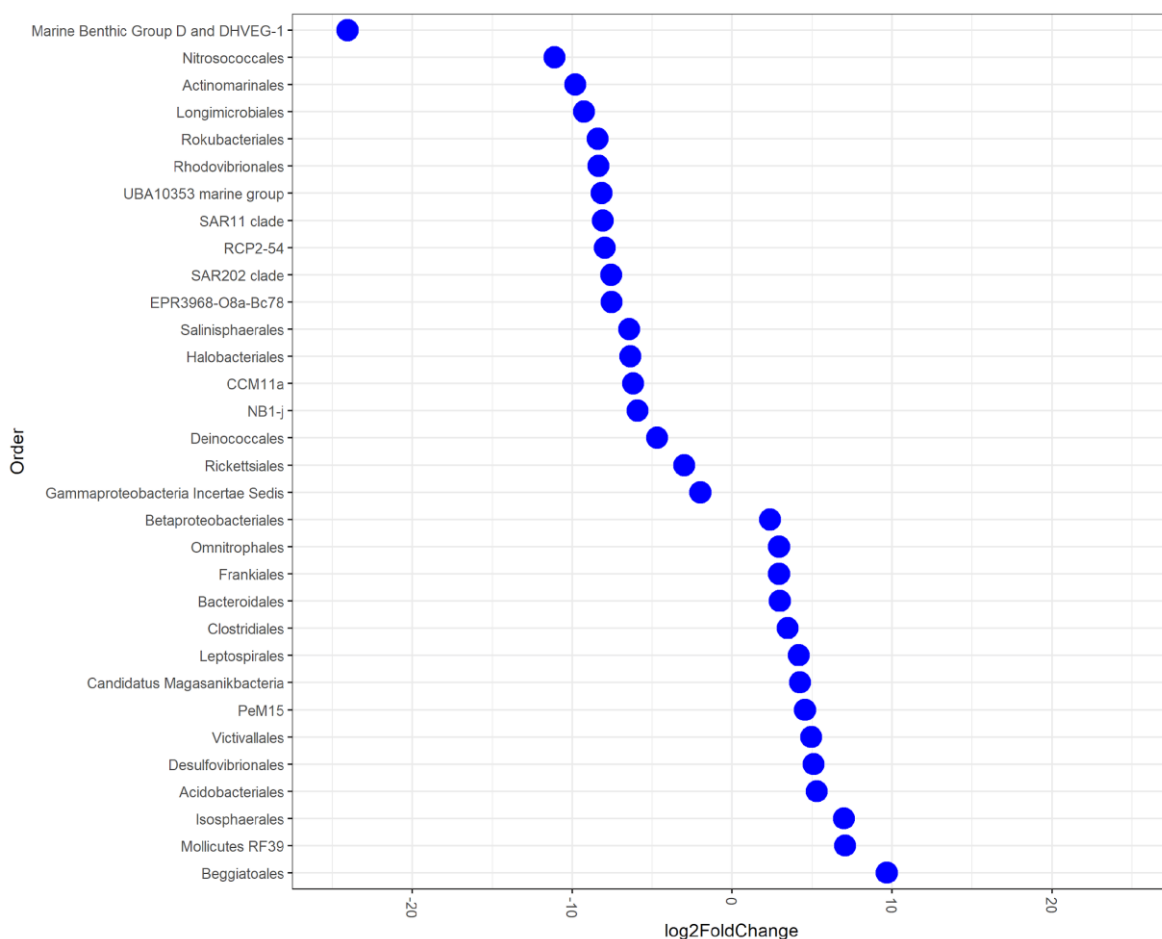


Figura V-8: Análisis DNA-seq que muestra a nivel de orden las diferencias significativas entre los microbiomas de los humedales LAB y las estaciones del estero El Sauce.

Este es el primer estudio en Chile que caracteriza como cambia la distribución de los GEI y el microbioma bentónico de humedales costeros en lo largo de una costa de 400 km. Como se pudo explicar en el capítulo 3, la zona sur de la ZSACHC que es representada por los humedales LAB es la más afectada por la presión antrópica y por lo tanto es necesario ampliar medidas de conservación y manejo que nos permita poder proteger y cuidar estos ecosistemas ante diversas actividades humanas, como por ejemplo lo es el caso del estero “El Sauce” en laguna verde como también el Maipo y el Aconcagua. Por otro lado y a una escala climática, la sequía presente en toda la zona y que se acentúa hacia el norte, es otro problema importante a considerar cuando se tomen medidas de conservación y manejo, ya que el agua es cada vez más escasa y sin ella tanto la población

humana así como también los humedales y su biodiversidad se varían seriamente impactados.

CAPÍTULO VI. CONCLUSIONES GENERALES

Conclusiones Generales

Los humedales costeros de la ZSACHC se caracterizan por presentar una heterogeneidad de condiciones físico - químicas, gases de efecto invernadero y microbioma bentónico relacionadas a una variabilidad espacio-temporal (invierno-verano) en una escala sinóptica latitudinal (Zona Centro-Norte) y en el curso de agua de un mismo humedal como en el caso del Estero “El Sauce” ratificado como uno de los humedales entrópicamente más impactados de la Región particularmente por el vertido de aguas servidas desde una planta de tratamiento.

En cuanto a la escala sinóptica latitudinal que se abordó en el Capítulo 1 de la tesis se concluye que la distribución de las características físico-químicas de los humedales costeros de la ZSACHC presentan gradientes de aridez de las cuencas observándose una prevalencia de mayor temperatura y salinidad hacia el norte (High Aridity Basin, HAB), a diferencia de los humedales del sur (Low Aridity Basin, LAB). Todos los humedales presentan una acumulación de CH_4 y CO_2 , sin embargo el grupo HAB se comportó como sumidero potencial de N_2O en comparación al grupo LAB, concentraciones que cambiaron entre invierno y verano, caracterizado por una menor acumulación de CO_2 en los LAB. Mediante un modelo GAM (generalized additive model) se identificó que la salinidad junto con otros factores ambientales explican los cambios en las concentraciones de GEI. El aumento de la salinidad explica la distribución del CO_2 y CH_4 , lo cual se acentúa al sumar el pH y la latitud, respectivamente, como variables predictoras. Por otro lado, el aumento en las concentraciones de nitrato y la materia orgánica explican mejor la distribución de las concentraciones de N_2O .

La comunidad microbiana bentónica de los humedales presentó una dominancia de Betaproteobacterias, las cuales reúnen a grupos funcionales como amonio y metano oxidantes cuya variabilidad se explica por factores ambientales como la conductividad, concentración de nitrato y materia orgánica y la latitud, a su vez, los grupos microbianos influyen sobre la concentración de GEI

lo cual fue ratificado mediante un modelo estructural SEM (Factor Ambiental → Grupos Microbianos Funcionales → GEI).

En cuanto a la escala espacial a lo largo de un mismo humedal (estero El Sauce) impactado por el vertido desde una planta de tratamiento de aguas residuales abordado en el Capítulo 2 de la tesis se concluye que El estero “El Sauce” representa un modelo de estudio de un humedal impactado eutrófico con una mala calidad de sus aguas, donde se evidencia el impacto de una planta de tratamiento de aguas residuales cuyos efluentes modifican las condiciones físicas, químicas y biológicas del Estero, alterando la estructura y composición del microbioma bentónico incluyendo grupos funcionales que participan en el reciclaje de GEI el cual se acumula en sus aguas.

CAPÍTULO VII. REFERENCIAS

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