UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

CENTRO DE INVESTIGACIONES EN ECOSISTEMAS

Ecología

OLIGOTROFÍA AL EXTREMO

Implicaciones de las estrategias bacterianas para promover el reciclaje de nutrientes en el suelo

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

YUNUEN TAPIA TORRES

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MÉXICO, D.F. **Marzo, 2015.**

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Dr. Isidro Ávila Martínez
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Por medio de la presente, me permito informar a usted, que en reunión ordinaria del Subcomité por Campo de Conocimiento (Ecología y Manejo Integral de Ecosistemas) del Posgrado en Ciencias Biológicas, se aprobó el siguiente jurado para el examen de grado de **Doctora en Ciencias Biológicas** de la alumna **Yunuén Tapia Torres** con número de cuenta **508010851** con la tesis titulada: **"OLIGOTROFÍA AL EXTREMO: Implicaciones de las estrategias bacterianas para promover el reciclaje de nutrientes en el suelo"** bajo la dirección del **Dr. Felipe F. García Oliva,** Tutor Principal.-

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Sin otro particular, quedo de usted.

A t e n t a m e n t e
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, D.F., a 11 de febrero de 2015

Dra. María del Coro Arizmendi Arriaga
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AGRADECIMIENTOS

Al Posgrado en Ciencias Biológicas de la Universidad Nacional Autónoma de México (UNAM), al Consejo Nacional de Ciencia y Tecnología (CONACyT)-México (No. de beca 210603) y a la Dirección General de Estudios de Posgrado (DGEP) de la UNAM, por el apoyo económico otorgado para la realización de los estudios de doctorado de los cuales forma parte esta tésis.

Este proyecto fue financiado por PAPIIT DGAPA-UNAM (Análisis de la vulnerabilidad de la dinámica de nutrientes en un ecosistema árido de México, IN204013)

A la Fundación Carlos Slim: Alianza WWF-FCS, por el apoyo otorgado.

A los miembros de mi comité tutoral: Dr. Felipe García Oliva, Dr. Carlos Montaña Carubelli y Dra Valeria Souza. Gracias por las invaluables enseñanzas a lo largo de estos años.

"Cuando decidí estudiar un doctorado estaba llena de ilusiones, expectativas y motivaciones. Pronto todos esos sentimientos se volvieron un reto, el reto que felizmente termina cuatro años y medio después con la escritura de esta tesis. ¿Qué es lo que realmente permitió terminar este reto? ¿Qué hizo esta tésis posible? Fue el apoyo de los múltiples tutores que creyeron en mi, que me apoyaron, me orientaron, me alegraron los días, me levantaron en los momentos de debilidad, que me obsequiaron lo más valioso que te pueden dar; su tiempo y sus enseñanzas. Por ahí leí, que los tutores pueden venir en múltiples formas y tamaños y ahora lo creo. Desde académicos muy prestigiados hasta compañeros de cubículo, amigos, hermanos, administrativos, extraños que te hacen reflexionar con preguntas escépticas sobre la importancia de tu trabajo para el mundo"

Primero quiero agradecer enormemente a todos los tutores gracias a los cuales se realizó esta tesis. Investigadores de diferentes instituciones, los cuales me abrieron las puertas de sus laboratorios, sin saber qué esperar.

Al **Dr. Felipe García Oliva.** Laboratorio de Biogeoquímica de Suelos, Centro de Investigaciones en Ecosistemas, UNAM. Gracias Felipe gracias por aceptar ser tutor de esta tesis, por darme esa libertad para plantear mis inquietudes en ideas y por caminar conmigo para realizarlas. Por darme esa confianza que me ayudo a terminar esta etapa. Gracias por las largas horas de discusiones, por los viajes realizados, por la amistad brindada. Gracias por ser mi maestro!! Porque una gran "relación de largo tiempo" de estudiante-tutor es realmente importante.

A la **Dra. Valeria Souza Saldivar**. Laboratorio de evolución molecular y experimental, Instituto de Ecología, UNAM. Por que siempre haz confiado en mi. Por abrirme tu corazón y hacerme sentir en todo momento que el camino elegido era el correcto. Por llevar el estandarte de "jefa de jefas" con eso que te caracteriza. Por haberme permitido trabajar y conocer ese maravilloso sitio terrenal "Cuatro Ciénegas" y por saborear conmigo de una manera especial los resultados de esta tesis. Gracias por los años de enseñanzas y por los abrazos y mimos dados.

Al **Dr. Carlos Montaña Carubelli**. Instituto de Ecología A.C. Por formar parte de los tutores que me guiaron y por todos los valiosos comentarios durante estos cuatro años. Por ser el investigador ejemplar que eres, por facilitarme las cosas cuando estaba en tus manos y por ser un modelo de persona e investigador a seguir.

A la **Dra. Gabriela Olmedo Álvarez**. Laboratorio de Ecología Microbiana, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV) unidad Irapuato. Por ser la científica entusiasta e incansable que te caracteriza. Por que tu capacidad de asombro y de maravillarte de las pequeñas cosas de la vida (los microorganismos creciendo), me hicieron maravillarme a mi también. Gracias por abrirme las puertas de tu laboratorio y por compartir tus

conocimientos; por que lo poco que ahora se de microorganismos lo aprendí de ti. Por tu apoyo y confianza incondicional durante estos años.

Al **Dr. Antonio González Rodríguez**. Laboratorio de Genética de la conservación, Centro de Investigaciones en Ecosistemas, UNAM. Por recordarme en todo momento que "la vida es dura" y que si no trabajas tú, nadie lo va a hacer por ti. Por que día y noche, cansado o no, discutiste conmigo cada una de mis inquietudes. Por que en cada idea planteada siempre me diste el beneficio de la duda, y al final, con palabras mudas poco a poco las dudas te abandonaron. Porque esta tesis, tiene el sello de tus enseñanzas.

Al **Dr. Gerardo Vázquez Marrufo**. Centro Multidisciplinario de Estudios en Biotecnología, Universidad Michoacana de San Nicolás de Hidalgo. Por ser el primero en haberme recibido en su laboratorio en esta etapa. Por proporcionarme las herramientas para iniciar mi carrera en una de las áreas más fascinantes (la enzimología). Porque cuando dudé, amablemente discutiste todo conmigo. Por ser el investigador jovial, amigable y optimista que eres. Por mantener esa chispa curiosa que muchos pierden en el camino.

Al **Dr. James Elser**. Ecology, Evolution, and Environmental Science, School of Life Sciences, Arizona State University. Por aceptar mi estancia en tu laboratorio a pesar de las múltiples firmas y papeles escaneados demandados por el CONACyT. Por haberme enseñado el camino de la estequiometria ecológica y por ayudarme a cerrar con broche de oro. Gracias a ti Jim, me llevo una gran enseñanza de vida. "Todos podemos contar una historia, pero no todos sabemos contarla bien; implica tiempo, esfuerzo, paciencia y colaboración"

Gracias por el tiempo obsequiado, por las enseñanzas regaladas, por las horas de discusiones disfrutadas y por la confianza brindada.

A los técnicos de laboratorio por su enorme ayuda. Rodrigo Velázquez Duran, por la ayuda con los análisis de laboratorio de biogeoquímica de suelos. Africa Islas, por su apoyo y ayuda con las extracciones de ADN, por el apoyo para aislar las bacterias del suelo, por facilitarme la vida en el laboratorio de microbiología. Víctor Rocha, por siempre tener lo que me faltaba para realizar mis experimentos, por su ayuda técnica en el laboratorio de genética.

A Dolores Rodríguez!!! Lola qué habría sido de mi, sin ti? Gracias por sacarme siempre de problemas con la parte administrativa del posgrado.

A Janik por la ayuda en toda la cuestión administrativa y logística para poder graduarme.

A Atzimba López, Heberto Ferreira y Alberto Valencia por el apoyo en el área de computo. Atzimba, gracias por todo tu apoyo y por prestarme libros a pesar de que siempre los regresé con retrasos.

Al Dr. Ken Oyama, por permitirme la entrada y el uso de su laboratorio.

Y además…

A todos mis compañeros del laboratorio de "Biogeoquímica de suelos", UNAM: Alberto, Bruno, Cristina, Dainiz, Natali, Pamela. Por la retroalimentación en todo momento, por compartir sus conocimientos conmigo.

A los compañeros del laboratorio de "Ecología Microbiana", CINVESTAV: Africa, Ismael, Lolis, Paul, Toño, Zule. Por que amablemente me ayudaron durante mi estancia con ellos. Lolis gracias por apoyarme tanto!!!!!

A los compañeros de "Arizona State University": Amisha, thanks for your help with the computers programs. Jess, thanks for invited me to know Arizona. Marcela, por las buenas pláticas durante tu estancia. Neg Ion, thanks for your company. Zarraz, thanks amiga for all your lab help, for being my teacher!!!!

A mi familia de Arizona: Elizabeth, thanks for your originality. For the nice picture that convinced me to be part of your family, for all your help! Christopher, for the success moments and for sharing your coffee with me, every morning. Thanks for the nice trip to the Grand Canyon. Mitza ¡principessa! Thanks for being my sister, for share your car, your house, your family, your friends, your clothes, with me!!! Cynthia, to begin this journey with me. Love you prima.

Agradezco a los que confiaron en mi y me dieron la oportunidad de aprender juntos. Natali, gracias por haber sido la primera en confiar en mi, por todos los buenos y malos momentos y por las enseñanzas de vida! Pam y Beto, por ser tan entusiastas, trabajadores y honestos. Por esa confianza que me dan y por el grupo que hemos formado. Gracias por compartir conmigo esos momentos de discusión y ojalá que podamos materializar poco a poco todas las locuras que se nos ocurren. Por las enseñanzas que cada alumno nos deja!

A mis migas del alma cosechadas en el camino. Nuria, Dainiz, Fany, Monica, Maribel, Laura, Liz, Anel, Claudia, Xochiquetzal, Yuris, Oli. Por las largas horas de pláticas, locuras y buenos momentos. Por estar siempre, siempre, siempre.

A mis hermanos, por el amor que siempre me demuestran. Jaime, por ser tan tu y por siempre pensar en que debes de cuidarnos. Por la aventura en la que nos metiste y que casi termina con mi hígado. Caro por ser mi hermana mayor que siempre me apoya y cuida. Alis, por tus palabras certeras en mis peores momentos, por haber estado ahí y ayudarme a salir adelante. Los amo!!!

A Itza, Casael y Jhony, porque sólo ustedes logran completar el gran grupo de hermanos. Por las buenas reuniones, aventuras y compañía.

A los que iluminan mi corazón de una manera especial, a mis niños: Iktan, Jaimito y Emi.

A mi familia González Rodríguez por aceptarme como un miembro mas de la familia, por los viajes y buenos momentos cuando estamos juntos.

A mis papás por su cariño incondicional.

A Toño, por los años juntos. Por todo lo compartido, por todo lo que hemos aprendido juntos, por saber sacar lo mejor de mi. Por la gran enseñanza de vida!!! Gracias por ayudarme a ser una mejor persona y caminar a mi lado en esta aventura que es la vida.

Dedicada a

J.J. Tapia y A.H. Torres

Por acompañar mi camino y guiar mis pasos

A. González-Rodríguez

Por compartirme tu gran corazón

A los que inspiraron mi camino

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RESUMEN

Las zonas áridas cubren más de un tercio de la superficie terrestre continental y por lo tanto constituyen el bioma terrestre más extenso del planeta. Las estimaciones sobre el almacenamiento de carbono en las regiones áridas, indican que estos ecosistemas contribuyen con el 36% del total de almacenamiento de carbono (C) a nivel mundial. Una alta proporción de estas zonas áridas está cubierto por pastizales, los cuales representan un importante almacén (8%) de los almacenes de C a nivel global. Estas zonas desérticas, generalmente se caracterizan por condiciones de estrés debido a la baja disponibilidad de energía, nutrientes (Nitrógeno-N y fósforo-P) y agua para los microorganismos. Por lo tanto, la baja disponibilidad de N y P generalmente limita la productividad primaria, así como la actividad de los microorganismos. Sin embargo, se ha observado que en ecosistemas desérticos Mexicanos, el P es el nutriente que generalmente limita la productividad debido a su baja disponibilidad y alta reactividad en el suelo.

El P es un elemento esencial para muchas biomoléculas y juega un papel fundamental en muchos procesos biológicos. Es un elemento fundamental para la producción de alimentos, actualmente no existe un sustituto de este elemento para el crecimiento de las plantas y microorganismos, además de que no puede manufacturarse; condición que ha generado una preocupación a nivel mundial. Sólo pocos países tienen el control de las reservas globales del fosfato, los cuales incluyen a: Marruecos con un 70%, Iraq, China, Argelia y Siria (Jasinski, 2012). De esta manera, asegurar la disponibilidad y accesibilidad del P a largo plazo, principalmente para la seguridad alimentaria, es un reto global. Sin embargo, la adquisición biológica del P no está acoplada con la adquisición de C y N; por lo que el balance elemental C:N:P puede variar entre organismos.

La pregunta principal de esta tesis es ¿Cuáles son las estrategias de las comunidades bacterianas edáficas para promover la disponibilidad y adquirir el nitrógeno y fósforo en un ecosistema halófilo ultra-oligotrófico? Entre los resultados mas relevantes obtenidos en esta tesis, encontramos que el sistema formado por la vegetación-suelo controla los mecanismos de transformación de nutrientes en el sistema desértico estudiado. Sin embargo, las distintas especies vegetales promueven de manera diferencial esta transformación. Se observó una relación entre la biomasa radical, los nutrientes del suelo y la composición de la comunidad

de bacterias, donde uno de los sistemas estudiados (pastizal-suelo) promueve un ciclo cerrado del N, favoreciendo así su conservación, mientras que el sistema de matorral-suelo presentó un ciclo abierto, siendo el N vulnerable a la pérdida de este nutriente. En estos mismos sistemas de pastizal y matorral–suelo; se cuantificó la distribución del P en las diferentes fracciones del suelo, y se evaluó la capacidad del suelo para retener el P. Los resultados obtenidos demuestran, que la microbiota es el factor principal en la regulación de la disponibilidad de este nutriente, principalmente en el pastizal, ya que a pesar de que la proporción de P disponible es muy baja en ambos sistemas, el suelo del pastizal tiene una mayor capacidad de ocluir el P, principalmente en sus formas orgánicas. La importancia relativa de la microbiota se reduce en el suelo del matorral.

Debido a que la obtención microbiana de C, N y P requiere de la síntesis de exoenzimas; otro de nuestros objetivos fue evaluar la estequiometria de las exoenzimas (a la cual nos referimos como estequiometría ecoenzimática a lo largo de este trabajo) y su relación con la disponibilidad de energía y nutrientes para los microorganismos del suelo del sistema oligotrófico estudiado. Obteniendo como resultados los valores mas bajos de actividad ecoenzimática reportados para diferentes ecosistemas a nivel mundial. Sin embargo, la estequiometria ecoenzimática coincide con la reportada para diferentes ecosistemas. Así mismo, encontramos que las comunidades microbianas están co-limitadas por C, N y/o P, la cual fue diferente en los dos sitios estudiados dentro del valle de Cuatro Ciénegas. Además, los patrones ecoenzimáticos sugieren que la comunidad microbiana del suelo dedica más energía para adquirir el nutriente limitante, más que para el incremento de la biomasa microbiana. Por otro lado, ésta habilidad de sintetizar las ecoenzimas necesarias para obtener el nutriente limitante no está distribuida en todos las especies de los diferentes taxa microbianos. Por el contrario, la síntesis de ecoenzimas es una estrategia que puede ser considerada como un “bien común” a nivel de la comunidad; así si algún individuo de la comunidad posee este bien (estrategia), los demás individuos se pueden beneficiar de él.

ABSTRACT

Drylands cover more than one third of the continental land surface and therefore constitute the largest terrestrial biome on the planet. Estimates of carbon storage in arid regions indicate that these ecosystems contribute to 36% of total carbon storage (C) worldwide. A high proportion of these arid areas is covered by grasslands, which represent an important reservoir (8%) of C globally stores. These desert areas are generally characterized by stressful conditions due to the low availability of energy, nutrients (nitrogen-N and phosphorus-P) and water for microorganisms. Therefore, the low N and P availability generally limits primary productivity and microorganism activity. However, it has been observed that in Mexican desert ecosystems, P is the nutrient that limits productivity, generally due to its low availability and high reactivity in the soil.

Phosphorus is an essential element for many compounds and plays a key role in many biological processes. It is a key element in food production, there is currently no substitute for this element for the growth of plants and microorganisms, plus it cannot be manufactured; condition that has generated worldwide concern. Only few countries have control of global reserves of phosphate, which include: Morocco with 70%, Iraq, China, Algeria and Syria (Jasinski, 2012). Thus, ensuring the availability and accessibility of P for long term, mainly for food security is a global challenge. However, acquisition of the biological P is not coupled with the acquisition of C and N; so the elemental balance of C:N:P may vary between organisms.

The main question of this thesis is: What are the strategies of soil bacterial communities to promote the availability and acquire nitrogen and phosphorus in an ultra-oligotrophic ecosystem? Among the most relevant results obtained in this thesis, we found that the system formed by vegetation-soil controls the mechanisms of nutrients transformation in the desert system studied. However, different plant species differentially promote this transformation. A relationship between root biomass, soil nutrients and the composition of the bacterial community, was observed. Wherein, one of the studied systems (grassland-soil) promotes a closed N cycle, thus promoting their conservation; while the system scrubland-soil presented an open cycle, being vulnerable to the loss of this nutrient. In these same systems grassland and scrubland; P distribution in the different soil

fractions was quantified, and the soil's ability to retain P was evaluated. The results showed that the microbiota is a major factor in regulating the availability of this nutrient, mainly in the grassland. The relative importance of the microbiota is reduced on the scrubland soil.

Because the microbial C, N and P acquisition requires the exoenzymes production; another objective was to evaluate the stoichiometry of exoenzymes (which we refer to as ecoenzyme stoichiometry) and its relation to the energy and nutrients availability for soil microorganisms in the oligotrophic system studied. In this study we report the lowest ecoenzymatic activities yet quantified in soil. Nevertheless, activities for both organic N and organic P acquisition enzymes scale with C acquisition with a slope of ~1.0, indicating that the soil microbial communities of this ultra-oligotrophic desert ecosystem follow the global ecoenzymatic stoichiometry patterns. Soil microbial communities were co-limited by C and either by N or P but this co-limitation played out differently in different parts of the valley studied as indicated by microbial ecoenzymatic shift to allocate more resources to acquire and immobilize the scarcer nutrient. By extending ecoenzymatic analyses to these ultra-oligotrophic soils, our findings support the broad utility of the approach in illuminating how microbes acquire limiting resources in arid ecosystems.

Capítulo I

Introducción general

1.1. INTRODUCCIÓN

Ecosistemas desérticos

La UNEP en el Atlas Mundial para la Desertificación (UNEP, 1992) define a las regiones áridas como aquellas áreas donde la relación entre la precipitación media anual y la evapotranspiración potencial media anual varía entre 0.05 y 0.20, y las regiones semi-áridas son aquellas donde la relación varía entre 0.2 y 0.5. Estas zonas representan aproximadamente el 30% del área total mundial y ahí habitan aproximadamente el 20% de la población total mundial (Sivakumar y Brunini, 2005).

México cuenta con una gran proporción de zonas áridas y semiáridas, representando aproximadamente el 40% de su territorio (Challenger, 1998). La principal vegetación que domina en estas zonas son matorrales y pastizales desérticos. Debido a las características principalmente ambientales que poseen, estas zonas son reservorios de una gran cantidad de especies vegetales y animales endémicos (Rzedowski,1983).

Los pastos en climas desérticos pertenecen a la familia de los Poaceae, los cuales representan probablemente la familia de plantas más importante en el mundo, tanto económica, como ecológicamente. Existen alrededor de 650 géneros y casi 10,000 especies de pastos y estos tienen una distribución ecológica prácticamente ubicua, únicamente están ausentes en montañas de gran altitud y en las regiones polares (Provan et al. 2004). Además los pastos forrajeros también son de gran importancia económica, después de los cereales como el maíz, arroz y trigo, que representan la principal fuente de alimento para prácticamente la mayoría de la población mundial.

Un Poaceae desértico de gran importancia ecológica es *Sporobolus airoides Torr* (zacate alcalino), el cual es pasto perene ampliamente distribuido en Norte América

occidental (Mealor y Hild, 2006). Este pasto es usado en prácticas de reforestación debido a su habilidad de tolerar suelos salinos (Aldon, 1981) y por su capacidad de acumular compuestos potencialmente tóxicos del suelo (Retana et al. 1993). Después del establecimiento, *S. airoides* es tolerante tanto a la sequía, como a la inundación por agua (Johnson 2000), además, se ha observado que en ambientes productivos *S. airoides* es más competitivo que otros pastos de zonas áridas (Novplansky y Goldberg 2001).

En México, *S. airoides* presenta una amplia distribución geográfica. Actualmente se tienen registros de que este pasto se encuentra en sitios con altitudes que van desde 1 – 2,560 msnm (REMIB-CONABIO, 2012), distribuidos entre los estados de Sonora, Chihuahua, Coahuila, Nuevo León, Durango, Zacatecas, San Luis Potosí, Jalisco, Hidalgo y Veracruz (Figura 1).

Figura 1. Área de distribución geográfica para *Sporobolus airoides* (Poaceae) en México. Coordenadas geográficas obtenidas de REMIB-CONABIO, 2012. Elaborado por H. Rodríguez-Correa.

La demanda actual de alimentos ha generado que un porcentaje de los pastizales desérticos se destinen a zonas agrícolas (FAO, 2013). Sin embargo, para que la producción agrícola en los sistemas desérticos sea rentable, se requiere de mayores entradas de materia (agua, fertilizantes, pesticidas, combustibles) y energía en comparación con los sistemas templados, debido a la baja disponibilidad de nutrientes y agua que caracteriza a los sistemas desérticos.

La intensificación agrícola genera a largo plazo una disminución de la calidad del suelo (van Bruggen y Semenov, 2000), lo cual afecta procesos que alteran la dinámica natural de los ciclos biogeoquímicos, tales como la disminución de la materia orgánica y de la diversidad biológica, además de un aumento en la degradación del suelo como la salinización, la compactación y la erosión (van Bruggen y Semenov, 2000).

Aunado a lo anterior, el desconocimiento de las tecnologías apropiadas para llevar a cabo la agricultura en zonas áridas, ha llevado a un deterioro creciente de los recursos naturales de estas zonas (Díaz, 2007). Además, la rápida degradación del suelo en zonas áridas provoca la disminución de la productividad de los cultivos, condición que lleva al abandono de las tierras y al continuo incremento de las zonas desertificadas a nivel mundial.

Dinámica de nutrientes y la estequiometría ecológica

El carbono (C), nitrógeno (N) y fósforo (P) son los principales elementos que forman parte de todos los organismos vivos y su adquisición a partir del ambiente en el que se desarrollan, es fundamental para todos los seres vivos. En el suelo, la principal entrada de C se da mediante la fotosíntesis de las plantas, las cuales suministran el C orgánico que

utilizan los organismos descomponedores (hongos y bacterias), quienes a su vez regulan la disponibilidad de nutrientes para las plantas y otros microorganismos (Díaz, 2007). Por otro lado, la principal entrada de N al suelo se da mediante la fijación biológica del N atmosférico (N_2), por acción de la enzima nitrogenasa (Zehr et al. 2003). Se ha reportado la presencia de la nitrogenasa en Cianobacterias, Proteobacterias de vida libre y Firmicutes, bacterias simbióticas como *Rhizobium* y en Archaeas, las cuales fijan el N en forma de amonio (NH_4^+). Posteriormente, organismos como *Nitrosomonas* y *Nitrobacter* pueden oxidar el NH_4^+ a nitrato (NO_3^-); proceso que requiere la participación de las enzimas (amonio monoxigenasa, hidroxilaminaoxidoreductasa y nitrito oxidasa (López-Lozano et al., 2012). Finalmente, representantes de los generos como *Pseudomonas* o *Alcalígenes* reducen el NO_3^- a una de las formas gaseosas (N_2O o N_2) con ayuda de las enzimas (nitrato reductasa, nitrito reductasa, oxido nítrico reductasa y una oxido nitroso reductasa (Ferguson, 1998), completando de esta manera el ciclo del N.

A diferencia del C y del N, el P es un nutriente que carece de una fase gaseosa estable, por lo que debe ser suministrado casi en su totalidad por el intemperismo del material parental, debido a que presenta un bajo retorno atmosférico (Walker y Syers, 1976). A consecuencia de esto, la fuente inicial del P son los minerales primarios, principalmente la apatita. El intemperismo de estos minerales suministra iones fosfato (HPO^- y HPO^{2-}) a la solución del suelo (Cross y Schlesinger, 1995; Whalem

(Cross y Schlesinger, 1995). Se ha observado que microorganismos de los géneros *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium*

y *Erwiniaé* juegan un papel muy importante en la disponibilidad del P mediante la solubilización de los fosfatos (Rodríguez y Fraga, 1999). Además, se ha reportado que la mineralización de ésteres de fosfato mediante fosfomonoesterasas o fosfodiesterasas, así como la mineralización de fosfonatos por acción de fosfonatasas y C-P liasas, es llevada a cabo por microorganismos de los géneros *Bacillus*, *Flavobacterium, Agrobacterium y Achromobacter,* principalmente (Quinn et al. 1989; Liu et al. 1991; Kertesz et al. 1994).

El suelo de los ecosistemas desérticos se caracteriza por las bajas concentraciones de C, N y P. Se ha estimado que el N almacenado en el suelo de estos ecosistemas es muy bajo (0.2 Kg m^{-3}) comparado con otros ecosistemas como tundra (2 Kg m^{-3}) o bosques subtropicales lluviosos (1.6 Kg m^{-3}), además en ecosistemas áridos se ha observado que el C almacenado en el suelo sigue el mismo patrón que el observado para N (Post, et al. 1985). A pesar de que e

(PT) (Cross y Schlesinger, 2001; Buckingham et al., 2010), en estos ecosistemas se ha observado un amplio rango de concentraciones, las cuales oscilan entre 70 μ g^{-1} y 1000 μ g^{-1} (Tapia-Torres y García-Oliva, 2013). Sin embargo, la concentración de PT no es el mejor indicativo de disponibilidad del nutriente a consecuencia de la alta reactividad de este elemento.

Debido a que los mecanismos que los organismos utilizan para obtener tanto el C como el N y P son distintos, la adquisición de estos elementos generalmente no está acoplada (van de Wall, 2010), por lo que el balance elemental C:N:P puede variar entre organismos. El campo de la estequiometría ecológica (Sterner y Elser, 2002), se centra en entender este balance elemental de C y nutrientes para describir la compleja relación que existe entre los organismos y su ambiente.

De esta manera, la relación C:nutrientes generalmente refleja la disponibilidad de los recursos en el ambiente y la demanda energética y nutricional de los sistemas vivos. Por ejemplo, cuando la disponibilidad del P es muy baja, la relación C:P en la biomasa microbiana del suelo tiende a ser relativamente alta. Sin embargo, se ha observado que existen microorganismos que tienen gran capacidad de inmovilizar el P cuando es el nutriente limitante, con lo que la relación C:P en la biomasa microbiana disminuye debido a que se favorece la inmovilización del P y por lo tanto se incrementa la protección del recurso (como se observó en el capítulo V de esta tesis). Debido a la baja disponibilidad y alta reactividad del P, este nutriente es el que generalmente limita la productividad de los ecosistemas.

El problema del nutriente limitante: el fósforo y la seguridad alimentaria

El P es un elemento esencial para la síntesis de muchas biomoléculas (e.g. ADN, fosfolípidos, ATP)y juega un papel fundamental en muchos procesos biológicos (Huang et al. 2005). Debido al incremento de la disponibilidad del P en el suelo producto de la sobreexplotación de las minas de P, se ha aumentado nuestra habilidad para producir alimento, sin embargo, no existe sustituto de este elemento para el crecimiento de las plantas y los microorganismos, además de que no puede manufacturarse (Johnston, 2000). Desafortunadamente, en la mayoría de los suelos del mundo existe poca disponibilidad de P, ya que éste es rápidamente ocluido con otras moléculas del suelo y, por tanto, existe poca disponibilidad de este elemento (como es el caso en el suelo del sitio de estudio donde se desarrolló esta tesis que corresponde a *Gipsisoles* y *Calcisoles*). Esta situación es común en suelos donde se desarrolla la agricultura marginal, principalmente en países en desarrollo, donde los agricultores poseen pocos recursos económicos para acceder al

mercado de los fertilizantes (Cordell et al. 2009; Cordell, 2010). Además, solo pocos países tienen el control de las reservas globales de las minas de fosfato, los cuales incluyen: Marruecos, con un 70%, Iraq, China, Argelia y Siria (Jasinski, 2012). De esta manera, asegurar la disponibilidad y accesibilidad del P a largo plazo, principalmente para la seguridad alimentaria, es un gran reto global.

La presente tesis está centrada en contribuir a responder la pregunta ¿cómo es que los microorganismos adquieren y reciclan los nutrientes C, N y P? Enfocándonos en la dinámica de comunidades microbianas de suelos oligotróficos desérticos de México, donde hay baja disponibilidad de C, N y P. En este trabajo logramos conocer los factores que determinan las relaciones C:N:P entre el suelo y las comunidades microbianas.

Los sistemas ideales para entender las relaciones energéticas y nutricionales entre el suelo y las comunidades microbianas, son los desiertos. Debido a que las presiones evolutivas generadas por la baja disponibilidad de nutrientes en estos sistemas han generado una diversidad de estrategias para adquirir los nutrientes limitantes, por lo cual se pueden presentar una mayor diversidad de estrategias en comparación con los sistemas con mayor disponibilidad de nutrientes.

1.2. ÁREA DE ESTUDIO

El estudio se llevó a cabo en el valle de Cuatro Cienégas, Coahuila (VCC), dentro del C (6°45’ ’ ’ 7° ’ ’ ’ N ; ° 7’53’ ’ O . É v z 74 m.s.n.m, entre los dos macizos montañosos más grandes de México; al este la Sierra Madre Oriental y al oeste la Sierra Madre Occidental (Figura 2). Estos dos macizos montañosos

generan un cerco que evita que la humedad que proviene del Golfo de México y del océano Pacífico entre al valle, fenómeno que en gran medida explica la formación de este desierto. Además, el VCC se encuentra rodeado por diferentes Sierras: al norte por La Madera y La Menchaca, al sur por San Marcos y Pinos, al sureste La Fragua y al oeste La Purísima y San Vicente.

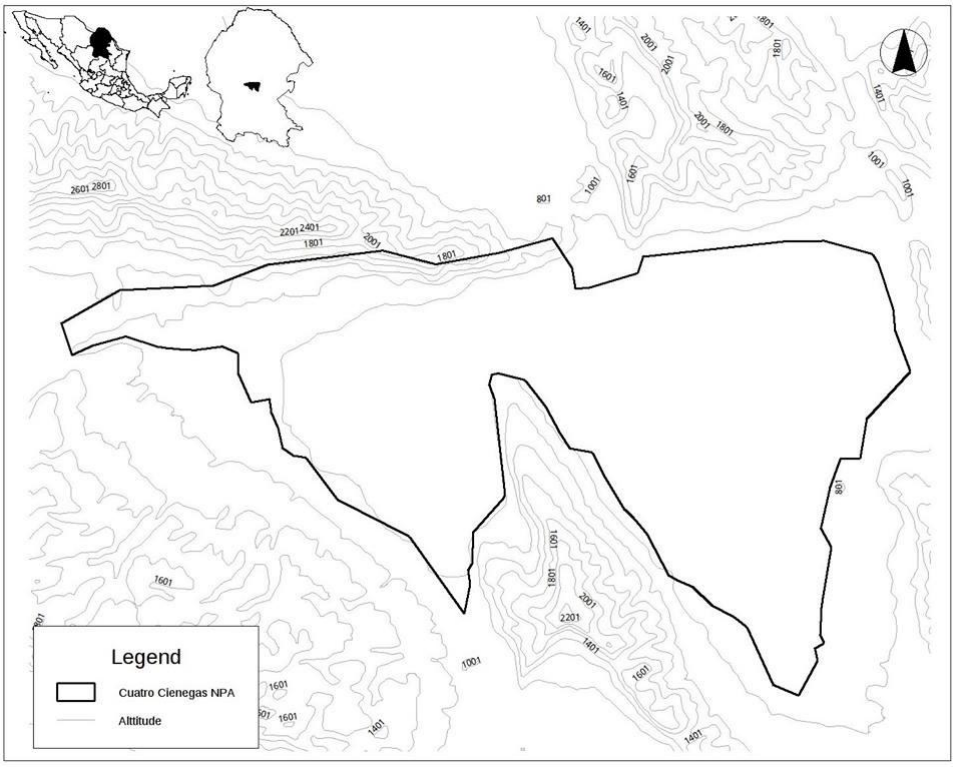

Figura 2. Mapa del valle de Cuatro Ciénegas de Carranza, Coahuila. Elaborado por J.L. Peña-Mondragón.

El clima del valle es árido, y a pesar de que la temperatura media anual es de 21ºC, se han registrado temperaturas que exceden los 45ºC, principalmente en julio, así como temperaturas por debajo de 0ºC en enero (SMN, CONAGUA 2013). La precipitación media anual es de 253 mm pero es altamente variable entre años (Figura 3; SMN, CONAGUA 2013). En la parte Occidental del valle, el material parental que domina es yeso derivado

del Jurásico, mientras que en la parte Este, domina la caliza (McKee*et al.* 1990). De acuerdo a la clasificación de la World Reference Base for Soil Resource (WRB, 2007), los tipos de suelos dominantes del valle son los *Gipsisoles* y C*alcisoles* para la parte Oeste y Este, respectivamente. En ambas partes del valle, se pueden encontrar matorrales xerófilos, sin embargo, los pastizales halófilos son la vegetación nativa dominante, con una gran dominancia de la especie *Sporobolus airoides* (Torr.) (Perroni *et al.* 2014b).

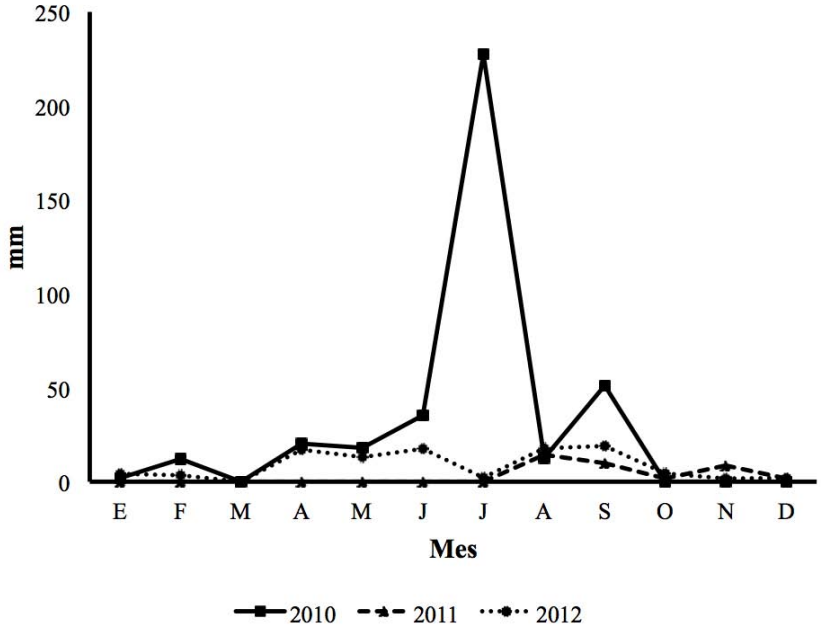

Figura 3. Precipitación mensual reportada para tres años (2010, 2011 y 2012) en el valle de Cuatro Ciénegas, Coahuila. Estos datos corresponden a los años de muestreo de esta tesis. Datos obtenidos del SMN, CONAGUA (2013).

Los hábitats tanto acuáticos (pozas, ríos y manantiales), como terrestres del VCC, se caracterizan por ser ultra-oligotróficos (con muy bajas concentraciones tanto de N, como de P) reportándose cocientes C:N:P muy bajos 15820:157:1 (Peimbert et al. 2012), condición que ha generado un alto estrés nutricional y energético, para las comunidades microbianas

(Souza et al. 2012). Debido a esto, se ha reportado una composición elemental principalmente en N:P, distinta a la reportada en otros ambientes. En el VCC, estos cocientes van en un rango que oscila entre 157:1 (cuando hay muy poca disponibilidad de P) y 1.8:1 (cuando hay poca disponibilidad de N (Souza et al. 2012). Mientras que para ecosistemas oceánicos, Redfield (1934), propuso la relación N:P de 16:1 como una relación que aseguraba el balance estequiométrico para la vida. Los efectos de esta desviación del balance en la estequiometria en los procesos que son llevados a cabo en el suelo, serán abordados en los capítulos II al VI de esta tesis.

1.3. PREGUNTA GENERAL

¿Cuales son las estrategias de las comunidades bacterianas edáficas para promover la disponibilidad y adquirir el nitrógeno y fósforo en un ecosistema halófilo ultra-oligotrófico?

1.4. OBJETIVO GENERAL

Describir los procesos involucrados en la disponibilidad de nutrientes del suelo en un ecosistema oligotrófico en el norte de México

1.5. OBJETIVOS PARTICULARES

- Hacer una revisión de la literatura actual sobre los procesos que determinan la dinámica del P en los ecosistemas terrestres, con énfasis en ambiente oligotróficos.
- Examinar el efecto de dos sistemas de vegetación-suelo (pastizal y matorral) en la transformación potencial del N y su disponibilidad en el suelo dentro del valle de Cuatro Ciénegas.

- Cuantificar la distribución del P en las diferentes fracciones del suelo, así como evaluar la capacidad de la retención de P en el suelo mineral por medio de la estimación de la capacidad de oclusión del P en los dos principales tipos de vegetación (pastizal y matorral) dentro del valle de Cuatro Ciénegas.

- Evaluar la estequiometria eco-enzimática y su relación con la disponibilidad de energía y nutrientes para los microorganismos del suelo

- Bajo un enfoque experimental, determinar las estrategias bacterianas para la adquisición y uso eficiente de P comparando las comunidades microbianas de sedimentos y suelos.

1.6. ESTRUCTURA DE LA TESIS

Para cumplir con estos objetivos, la tesis se estructuró en siete capítulos que se resumen en la siguiente sección.

CAPITULO II. ***La disponibilidad del fósforo es producto de la actividad bacteriana en el suelo en ecosistemas oligotróficos: una revisión crítica (TERRA Latinoamericana, 31: 231-242, 2013).***

Este capítulo es una revisión de la literatura actual sobre los procesos que determinan la dinámica del P en los ecosistemas terrestres, con principal énfasis en los ecosistemas oligotróficos y el papel de las bacterias en el control de la disponibilidad de este nutriente. Los temas abordados son la evolución del P en estos ecosistemas, los principales almacenes del P y las formas químicas en que se puede encontrar este nutriente. La última parte de esta revisión está dedicada a analizar la importancia de las bacterias en la mineralización de los ésteres de fosfato y de los fosfonatos, ya que pueden representar la principal fuente de P disponible en los ecosistemas oligotróficos.

CAPITULO III. ***Vegetation-soil system controls soil mechanisms for nitrogen transformations in an oligotrophic Mexican desert (Journal of Arid Environment, 114: 62-69, 2015).***

El objetivo de este capitulo fue examinar el efecto de dos sistemas de vegetación-suelo (pastizal y matorral) en la transformación potencial del N y su disponibilidad en el suelo dentro del valle de Cuatro Ciénegas Coahuila. La hipótesis de este trabajo es que la mineralización y la inmovilización en la biomasa microbiana del N dominan en el pastizal y por tanto, el N está protegido en un ciclo cerrado, mientras que la nitrificación es el proceso dominante de la transformación de N en el matorral, favoreciendo la pérdida de este nutriente. Para probar esta hipótesis se cuantificó la concentración de C, N y P en la biomasa radical de ambos sistemas vegetación-suelo. Así mismo, se midió la disponibilidad y la transformación potencial del C, N y P del suelo por medio de la comunidad microbiana, la cual fue caracterizada utilizando librerías de clonas del gen que codifica para la subunidad 16S del rRNA. Los resultados de este trabajo sugieren una relación entre la biomasa radical, los nutrientes del suelo y la composición de la comunidad de bacterias, donde el sistema de pastizal promueve un ciclo cerrado del N favoreciendo su conservación, mientras que el sistema de matorral presentó un ciclo abierto, siendo más vulnerable a la pérdida de este nutriente.

CAPITULO IV. ***Relationship between soil P fraction and microbial biomass in an oligotrophic grassland-desert scrub system (Ecological Research, 29: 463-472, 2014).***

El objetivo de este capitulo fue cuantificar la distribución del P en las diferentes fracciones del suelo, así como evaluar la capacidad de retención de P en el suelo mineral por medio de la estimación de la capacidad de oclusión del P en dos tipos de vegetación (pastizal y

matorral) dentro del valle de Cuatro Ciénegas. La hipótesis fue que la concentración de N y P en la biomasa microbiana puede estar positivamente correlacionada con la fracción dominante de P, la cual puede ser un indicador de los mecanismos que tiene la microbiota para acceder al P ocluído en los minerales del suelo. Para ello se realizó el fraccionamiento de P del suelo en ambos sistemas estudiados, así como la concentración de C y P en la biomasa microbiana y la capacidad de oclusión de P por los minerales del suelo. Los resultados obtenidos demuestran que la proporción de P disponible es muy baja en ambos sistemas y el suelo del pastizal tiene una mayor capacidad de ocluir el P, principalmente en sus formas orgánicas. Estos resultados sugieren que la microbiota es el factor principal en la regulación de la disponibilidad de este nutriente en el pastizal, mientras que su importancia relativa se reduce en el suelo del matorral.

CAPITULO V. ***Ecoenzymatic stoichiometry at the extremes: How microbes cope in an ultra-oligotrophic desert soil (Enviado a Soil Biology and Biochemistry).***

Este trabajo tiene como objetivo evaluar la estequiometria ecoenzimática y su relación con la disponibilidad de energía y nutrientes para los microorganismos del suelo en un ecosistema desértico oligotrófico. Para ello se estimaron los cocientes C:N:P del suelo y biomasa microbiana, así como la actividad ecoenzimática de β-glucosidasa, N-acetil-glucosaminidasa y fosfatasa alcalina, para determinar si el metabolismo de las comunidades microbianas está limitado por energía o por nutrientes. Nuestros resultados presentan los valores mas bajos de actividad ecoenzimática reportados para suelo. Sin embargo, la estequiometría ecoenzimática coincide con una relación cercana a 1, la cual ha sido reportada para diferentes ecosistemas. Así mismo, encontramos que las comunidades microbianas pueden estar co-limitadas por C, N y/o P, la cual fue diferente en los dos sitios

estudiados dentro del valle de Cuatro Ciénegas. Por último, los patrones ecoenzimáticos sugieren que la comunidad microbiana del suelo dedica más energía para adquirir el nutriente limitante, mas que para el incremento de la biomasa microbiana.

CAPITULO VI. ***Diverse scavenging strategies for phosphorus: Lessons from phosphorus limited bacteria (para ser enviado a Environmental Microbiology)***

El principal objetivo de este capítulo fue determinar bajo un enfoque experimental, si los aislados bacterianos obtenidos de un ecosistema ultra-oligotrófico, comparten las estrategias para usar y reciclar el P; o si estas estrategias pueden considerarse como bienes públicos para la comunidad de microorganismos del suelo. Para esto se obtuvieron 1160 aislados provenientes de suelo y de sedimento del valle de Cuatro Ciénegas y se sometieron a un experimento de crecimiento utilizando seis diferentes fuentes de P. Entre las fuentes probadas, se encuentran: fosfato de potasio, fosfato de calcio, fosfito, 2-fosfonoacetaldehído, 2-amino-etil-fosfonato y ADN. Cada una de estas fuentes fue probada como única fuente de P con cada uno de los 1160 aislados obtenidos. Los resultados muestran que las estrategias presentes en las bacterias aisladas de los sedimentos difieren de las estrategias aisladas del suelo. Observando un mayor porcentaje de crecimiento en todas las fuentes de P en las bacterias de los sedimentos. Sin embargo, se pudo observar que todas las estrategias para obtener tanto ésteres de fosfatos, como fosfonatos, están presentes en las comunidades microbianas de ambos hábitats. Podemos concluir que las enzimas necesarias para obtener el P de cualquiera de las fuentes probadas, puede considerarse como un bien público y si algún individuo de la comunidad posee esta estrategia, los demás individuos pueden beneficiarse de ella.

CAPITULO VII. *Conclusiones*

En este capítulo se resaltan las conclusiones generales de la tesis, en las que se hace referencia en los aportes más sobresalientes en el campo de la ecología de suelos.

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Capítulo II

La disponibilidad del fósforo es producto de la actividad bacteriana en el suelo en ecosistemas oligotróficos: una revisión crítica

Yunuen Tapia-Torres y Felipe García-Oliva

**** Publicado en: TERRA Latinoamericana, 31: 231-242, 2013***

LA DISPONIBILIDAD DEL FÓSFORO ES PRODUCTO DE LA ACTIVIDAD BACTERIANA EN EL SUELO EN ECOSISTEMAS OLIGOTRÓFICOS: UNA REVISIÓN CRÍTICA

Phosphorus Availability is a Product of Soil Bacterial Activity in Oligotrophic Ecosystems: a Critical Review

Yunuen Tapia-Torres[1] y Felipe García-Oliva[2‡]

RESUMEN

El fósforo (P) es un elemento esencial para la vida, por lo cual entender los mecanismos que permiten su disponibilidad en el suelo es prioritario. Debido a la complejidad de la dinámica de este nutriente, aún existen varios procesos que no están claramente entendidos, principalmente en los ecosistemas oligotróficos. En la presente revisión se analiza literatura relacionada con procesos involucrados en la disponibilidad del P, dándole énfasis al papel de las bacterias. La forma química disponible del P es el ortofosfato, pero por su alta reactividad y demanda de la biota, esta forma es rápidamente disminuida de la solución del suelo. Por lo que es necesario que la biota adquiera este elemento de otras formas químicas. Entre ellas, las formas orgánicas representan la principal fuente de este nutriente mediante la mineralización bioquímica producto principalmente de la comunidad bacteriana del suelo. Entre los compuestos organofosforados, los ésteres de fosfatos son los más fáciles de mineralizar, por la poca demanda energética en la producción de las enzimas involucradas en dicho proceso. Por otro lado, la mineralización de los fosfonatos puede representar una fuente alternativa de P disponible, a pesar de que se habíaconsiderado que esta forma química no era accesible por la complejidad de sus moléculas. En general, una estrategia exitosa para la adquisición de P en ecosistemas donde dicho elemento es limitado, depende de la presencia de una maquinaria genética capaz de sintetizar las diferentes enzimas que mineralizan compuestos orgánicos con demandas energéticas diferentes (fosfohidrolasas, fosfonatasas y C-P liasas), además de la presencia de transportadores específicos de membrana y la disponibilidad de C como fuente de energía. Aún faltan estudios integrados que permitan elucidar el movimiento del P en los ecosistemas y cómo esto puede ser controlado y llevado a cabo por las bacterias que habitan en el suelo.

Palabras clave: *fosfatasa, fosfonatasa, ésteres, fertilidad, fosfonatos.*

SUMMARY

Phosphorus (P) is an essential element for life; thus, understanding the mechanisms associated with soil P availability is primordial. There are several processes of P dynamics that are not clearly understood, mainly in oligotrophic ecosystems. In the present review, current literature on the P availability process is analyzed, emphasizing the role of bacteria in the soil. Orthophosphate is the form in which available P is found, but due to its high reactivity and demand by the biota it rapidly diminishes in the soil solution. For this reason, it is necessary that the biota acquire P from other chemical forms. Among these, organic forms represent the main source for P through biochemical mineralization, which is mainly accomplished by the soil bacterial community. Among the organophosphorus compounds, phosphate esters are easier to mineralize due to the low energy demand for production of enzymes involved in this process. However, mineralization of organic phosphonate represents an alternative source of available P, although it has been considered that this chemical form was not available due to its molecular complexity. In general, a successful strategy for P acquisition in limited ecosystems depends on the presence of the genetic machinery capable of synthesizing different enzymes which mineralize organic compounds with different energy demands (phosphohydrolases, phosphonatases and C-P

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Recibido: marzo de 2013. Aceptado: julio de 2013.
Publicado como revisión en
Terra Latinoamericana 31: 231-242.

lyases), as the presence of specific membrane transporters and the availability of C as an energy source. More integrated studies are needed to elucidate the movement of P in ecosystems and how it can be controlled and carried out by soil bacteria.

Index words: *phosphatase, phosphonatase, ester, fertility, phosphonates.*

INTRODUCCIÓN

El fósforo (P) es un elemento esencial para la vida, siendo fundamental en el metabolismo de los organismos. Además de participar en innumerables rutas metabólicas, el P es un componente de las moléculas esenciales de la célula, tales como los fosfolípidos, ARN, ADN y del principal cofactor nucleotídico (ATP), requerido para la transferencia de energía y catálisis celular (White y Metcalf, 2007). En particular, las plantas y los microorganismos edáficos obtienen este elemento de la solución del suelo, principalmente de las formas inorgánicas más disponibles (ortofosfosfato PO_4^{3-}), pero debido a su alta reactividad química y su demanda, su disponibilidad se reduce rápidamente. Cuando el ortofosfato no está disponible, es necesario adquirir el P de otras formas químicas, entre las cuáles, las formas orgánicas son la principal fuente alternativa. Los microorganismos pueden adquirirlo de formas orgánicas lábiles, como los ésteres de fosfato que son fácilmente hidrolizables, los cuales contienen P en su mayor estado de oxidación, +5 (Kononova y Nesmeyanova, 2002). Estos ésteres se caracterizan por tener uniones entre carbono-oxígeno-fósforo (C-O-P), los cuales son fáciles de romper. Así mismo, se sabe que algunas bacterias y hongos tienen la capacidad de utilizar como fuente de P algunos compuestos organofosforados con menor estado de valencia, +3 (White y Metcalf, 2007), principalmente los fosfonatos, los cuales se caracterizan por tener un enlace directo carbono-fósforo (C-P), pero se requiere de mayor energía para romper este enlace y por lo tanto, dejar disponible al P.

El presente trabajo es una revisión de la literatura sobre los procesos que determinan la dinámica del P en los ecosistemas terrestres, con principal énfasis en los sistemas oligotróficos y el papel de las bacterias en el control de la disponibilidad de este nutriente. Los temas abordados son la evolución del P en estos ecosistemas, los principales almacenes del P y las formas químicas en que se puede encontrar este nutriente. La última parte de esta revisión está dedicada a analizar la importancia de las bacterias en la mineralización de los ésteres de fosfato y de los fosfonatos, ya que puede representar la principal fuente de P disponible en los ecosistemas oligotróficos.

Evolución del Fósforo en los Ecosistemas Terrestres

En el suelo, los principales componentes de la materia orgánica son el Carbono (C), Nitrógeno (N), P y Azufre (S). El P es el nutriente que debe ser suministrado casi en su totalidad por el intemperismo del material parental, debido a que presenta un bajo retorno atmosférico (Walker y Syers, 1976). A consecuencia de esto, la principal fuente inicial del P son los minerales primarios, principalmente la apatita. El intemperismo de estos minerales suministra iones fosfato ($H_2PO_4^-$ y HPO_4^{2-}) a la solución del suelo (Cross y Schlesinger, 1995; Whalen y Sampedro, 2010) y posteriormente, las bacterias, hongos y plantas incorporan estos iones fosfato en su biomasa, iniciando con esto la ruta biológica del P (Cross y Schlesinger, 1995).

Walker y Syers (1976) propusieron un modelo que aún en la actualidad es ampliamente aceptado sobre la evolución del P en el suelo durante la pedogénesis. Este modelo sugiere que la disponibilidad y la oclusión de este nutriente depende de la edad del suelo. Por lo tanto, propone que en los diferentes estadios del suelo cambiará la proporción de las diferentes formas químicas del P. Por ejemplo, este modelo sugiere que para suelos jóvenes poco intemperizados, la proporción de P en minerales primarios (apatita) será alta en comparación con suelos viejos muy intemperizados, donde dominarán las fracciones de P orgánico (Po) y P inorgánico ocluido (Poc), reduciendo así su disponibilidad.

Generalmente, la concentración de iones fosfato en la solución del suelo es muy baja debido a su alta reactividad química. Estos iones pueden tener diferentes destinos y por lo tanto, formar parte de diferentes fracciones en el suelo: a) ser rápidamente asimilados por la biota, formando parte de la fracción de Po, una vez que retorna la materia orgánica al suelo, b) pueden reaccionar rápidamente quedando adsorbidos en la superficie de partículas órgano-minerales a través de fuerzas electrostáticas, la cual representa la fracción de P adsorbido (Pad) o c) precipitarse en minerales secundarios, con aluminio, hierro y calcio (Al, Fe, y Ca), lo que representaría al Poc.

En los ecosistemas donde el suministro de iones fosfato a partir de la fracción geoquímica no es suficiente para satisfacer los requerimientos de las plantas y microorganismos, el almacén orgánico puede representar la principal fuente de P disponible. Los residuos orgánicos de las plantas, animales y biomasa microbiana muerta contienen compuestos de fósforo orgánico que pueden ser hidrolizados y mineralizados por acción de enzimas (fosfomono-, fosfodi- y fosfotri-esterasas) de origen tanto microbiano, como vegetal. A este proceso se le conoce como mineralización bioquímica, debido a que se realiza extracelularmente. El producto de la mineralización bioquímica es la liberación de iones fosfato a la solución del suelo. Por lo que en los suelos intemperizados, la disponibilidad de P depende principalmente de la mineralización bioquímica (Walker y Syers, 1976; McGill y Cole, 1981; Cross y Schlesinger, 2001) y por lo tanto, está regulada por la actividad de los microorganismos que sintetizan las enzimas necesarias para llevar a cabo este proceso.

Formas Químicas del Fósforo

El P en el suelo puede encontrarse en diferentes formas químicas, cada una de las cuales juega un papel diferente y fundamental en el reciclado del P, influyendo además en la dinámica de otros nutrientes, tales como el C y el N (McGill y Cole, 1981; Lathja y Schlesinger, 1988; Cross y Schlesinger, 2001; Buckingham *et al.*, 2010; Selmants y Hart, 2010). Se puede encontrar al P en compuestos inorgánicos y orgánicos que pueden ser desde iones en la solución del suelo hasta compuestos altamente estables (Negassa y Leinweber, 2009). El grado de estabilización depende de la complejidad de la molécula o el elemento de unión. De esta manera, los compuestos de Pi casi siempre se encuentran unidos a diferentes formas de Al, Fe y Ca, dependiendo del pH del suelo (Buckingham *et al.*, 2010). Por su parte, los compuestos de Po están asociados a moléculas orgánicas que pueden variar en la complejidad del compuesto (Negassa y Leinweber, 2009; Buckingham *et al.*, 2010).

En la dinámica del P en el suelo, se pueden identificar dos almacenes principales en los cuales se agrupan los compuestos orgánicos y los inorgánicos: a) el almacén biológico, representado por plantas, microorganismos y Po edáfico y b) el almacén geoquímico, representado por minerales primarios, secundarios, Pad y Poc (Walker y Syers, 1976; Johnson *et al.*, 2003; Whalen y Sampedro, 2010). Sin embargo, ambos almacenes están fuertemente relacionados debido a que la acción de los mecanismos biológicos puede modificar el balance químico del suelo y éste a su vez, puede afectar los procesos biológicos. Por ejemplo, algunos microorganismos pueden sintetizar ácidos orgánicos con lo que se acidifica el suelo (en su proximidad) y se pueden liberar iones fosfato de las superficies de intercambio, aumentando la concentración de estos iones en la solución del suelo y por lo tanto, su disponibilidad (Coyne, 1999).

Una alta concentración de iones fosfato en la solución del suelo, está relacionada con una alta disponibilidad de P para la biota. Sin embargo, esta forma química del P es rápidamente ocluida por las partículas del suelo, lo cual limita su disponibilidad. El grado de estabilización funcional entre el P y las partículas del suelo ha sido ampliamente estudiado en diferentes ecosistemas que van desde desérticos (Cross y Schlesinger, 2001; Buckingham *et al.*, 2010) hasta húmedos (Giardina *et al.*, 2000; Tiessen *et al.*, 1983).

En la mayoría de los estudios se pueden identificar tres niveles de estabilización del P, que va desde el P unido débilmente a las partículas del suelo (el cual es disponible), hasta el P que es prácticamente inaccesible para la biota debido a la complejidad de la unión. A estos grados, por simplicidad se les conoce como: a) lábil, b) moderadamente lábil y c) ocluido (Selmants y Hart, 2010). Para conocer cuáles son los procesos involucrados en la disponibilidad del P es necesario determinar en qué grado (lábil u ocluido) y en qué fracción (orgánica o inorgánica) se encuentra en el suelo.

El P en Ecosistemas Oligotróficos

Los ecosistemas oligotróficos son aquellos que presentan bajas concentraciones de algún nutriente y que a su vez no está disponible (Elser *et al.*, 2005). Las relaciones estequiométricas de C:N:P se han utilizado como un indicador para saber si alguno de estos elementos es limitante. Por ejemplo, los valores promedio reportados de estos cocientes para suelo en sistemas pastoriles y forestales son 166:12:1 y 212:15:1, respectivamente (Cleveland y Liptzin, 2007).

En los suelos de los ecosistemas áridos se han reportado las concentraciones más bajas de P total (PT) (Cross y Schlesinger, 2001; Buckingham *et al.*, 2010). Sin embargo, se ha observado un amplio rango de concentraciones en el mismo tipo de ecosistema (Figura 1). Por ejemplo, en una zona árida de Utah con vegetación de pastizales o matorrales y una precipitación

media anual de 215 mm, la concentración de PT en el suelo oscila entre 500-1000 µg g^{-1}; mientras que para una zona desértica de México con aproximadamente la misma precipitación (el valle de Cuatro Ciénegas), los pastizales presentan concentraciones más bajas que oscilan entre 70-90 µg g^{-1} y los suelos con matorrales presentan concentraciones de alrededor de 200 µg g^{-1}.

A pesar de los estudios llevados a cabo en diferentes sistemas oligotróficos, ha sido difícil asignar un valor de concentración del PT a partir del cual considerar si un sistema es oligotrófico o no, debido a la complejidad de la dinámica de este nutriente. Por ejemplo, a pesar de las diferencias de PT entre algunos ecosistemas áridos, la mayoría de ellos se caracterizan por presentar una baja proporción de Po y una mayor dominancia de Pi (Figura 2). Los ecosistemas desérticos donde la fracción de Po es mayor o igual a la de Pi son raros, como es el caso de los pastizales de Churince en Cuatro Ciénegas, México (Pastizal CH, Figura 2), donde a pesar de su baja concentración de PT, presentan una alta proporción de Po (Figura 2).

Los patrones de fraccionamiento secuencial de P muestran una marcada diferencia entre sitios. Por ejemplo, en los suelos que presentan una mayor concentración de PT (Buckingham *et al.*, 2010; Figura 3 f-i) dominan las fracciones de Pi ocluido (extraídas con HCl y H_2SO_4), las cuales son prácticamente inaccesibles para la biota. Sin embargo, en los pastizales y matorrales de Cuatro Ciénegas, donde la concentración de PT es baja, las fracciones de Pi y Po lábil y moderadamente lábil (extraídas con $NaHCO_3$ y NaOH) pueden llegar a representar en algunos casos hasta el 15 % del valor total (Figura 3 a, b, d). Además, en este mismo sitio, la fracción de Po extraída con HCl que es una de las más recalcitrantes, también puede representar una fuente de P para la biota. A pesar de que este P no está en la fracción más disponible, puede ser accesible para la biota mediante la mineralización bioquímica. Lo cual no sucede con el Poc (extraído con HCl), el cual está formado por compuestos muy estables.

En ecosistemas con características similares a las del valle de Cuatro Ciénegas (Po ≥ Pi), la mineralización del P es fundamental por la disponibilidad para las plantas y los microorganismos. En estos casos, la disponibilidad de P depende casi exclusivamente de la mineralización de las formas orgánicas y la rápida adquisición de

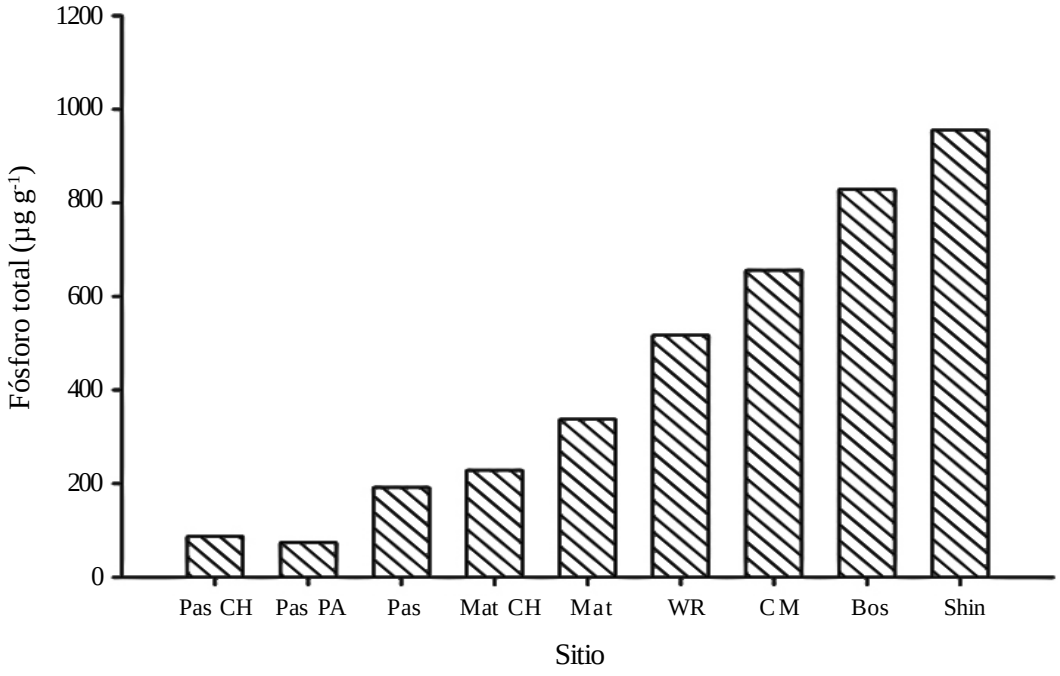

Figura 1. Concentración de fósforo total (PT, µg g^{-1}) para 9 ecosistemas desérticos con diferentes coberturas vegetales. Los cuales corresponden a Pastizal y Matorral en Churince, Coahuila (Pas CH y Mat CH, respectivamente), Pastizal en Pozas Azules, Coahuila (Pas PA), Pastizal y Matorral (Pas y Mat, respectivamente, Cross y Schlesinger, 2001) y 4 sitios con cobertura vegetal que puede ser pastizal, matorral o juníperos, White Rim, Cedar Mesa, Bosque y Shinarump (WR, CM, Bos, Shin, respectivamente, Buckingham *et al.*, 2010).

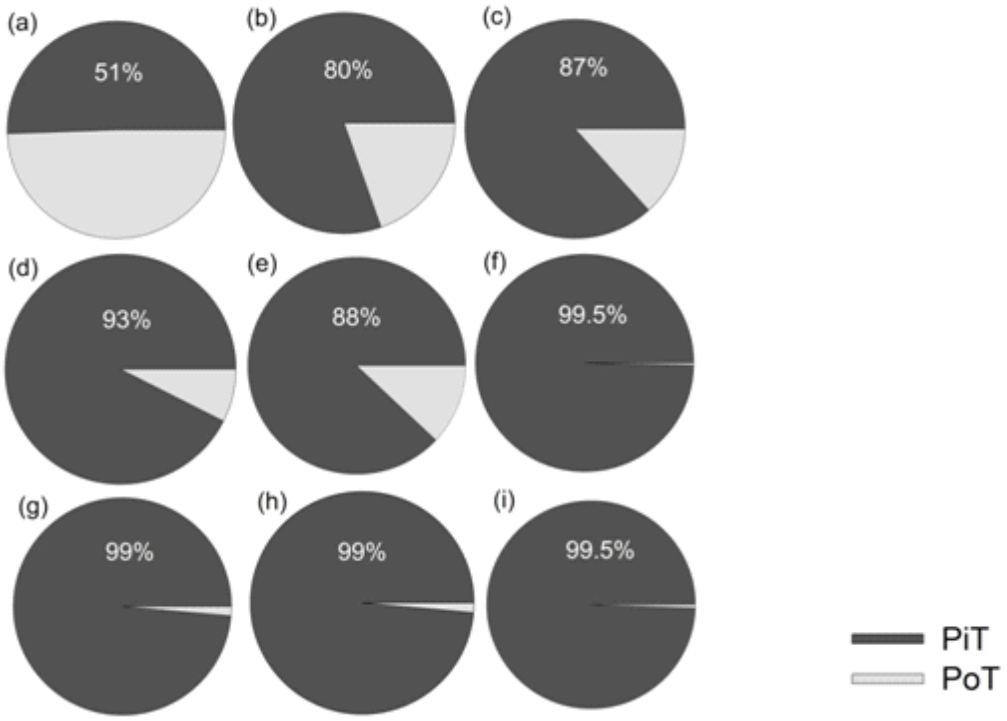

Figura 2. Proporción de fósforo inorgánico total (PiT) y fósforo orgánico total (PoT) en el suelo de 9 ecosistemas desérticos con diferente cobertura vegetal. (a) Pastizal en Churince, Coahuila, (b) Pastizal en Pozas Azules, Coahuila (Montiel-González, (c) Pastizal (Cross y Schlesinger, 2001), (d) Matorral en Churince, Coahuila, (e) Matorral (Cross y Schlesinger, 2001) y 4 sitios con cobertura vegetal que puede ser pastizal, matorral y/o juníperos, (f) Shinarump, (g) White Rim, (h) Cedar Mesa y (i) Bosque (Buckingham *et al.*, 2010).

las formas inorgánicas por la biota, como lo sugiere el modelo de Walker y Syers (1976).

Compuestos Organofosforados y su Mineralización

Entre los compuestos organofosforados que se conocen, los ésteres de fosfato son los más abundantes en la biósfera, por lo que han sido los más ampliamente estudiados. Sin embargo, la deficiencia de P en sistemas oligotróficos ha permitido estudiar otros compuestos organofosforados, tales como los fosfonatos que pueden representar una fuente alternativa de P (Clark *et al.*, 1998; Kolowith, 2001).

La liberación del P de estos compuestos orgánicos debe ser mediante exoenzimas (fosfatasas, fosfonatasas y C-P liasas). Los genes codificantes de estas enzimas se cree que están mediados casi exclusivamente por el regulón *pho* (Quinn *et al.*, 2007). Dentro de este regulón, se encuentran los genes específicos de lasfosfatasas mediados por el operón *pho* y los genes para las otras enzimas están mediados por el operón *phn* (Wackett *et al.*, 1987; Metcalf y Wanner, 1991).

Ésteres de fosfato. En la biósfera, el P se encuentra principalmente en su mayor estado de oxidación (+5), como el ortofosfato inorgánico (PO_4^-), y los ésteres de ácido fosfórico (Quinn *et al.*, 2007). Estas formas químicas de Po generalmente consisten en fosfomono ésteres de inositol, fosfolípidos y ácidos nucleicos que derivan de plantas y microorganismos (Figura 4) (Dao, 2011).

Debido a que las plantas y microorganismos adquieren el P como Pi y no como Po, la disponibilidad del P depende de la mineralización de los ésteres de fosfato. Estos compuestos son mineralizados por la acción de enzimas hidrolíticas extracelulares, principalmente fosfatasas (Dinkelaker y Marschner, 1992). En el suelo, la mayor actividad de mineralización de Po ocurre en la rizósfera, ya que tanto las raíces de las plantas (López-Gutíerrez *et al.*, 2004), como los microorganismos (Quan

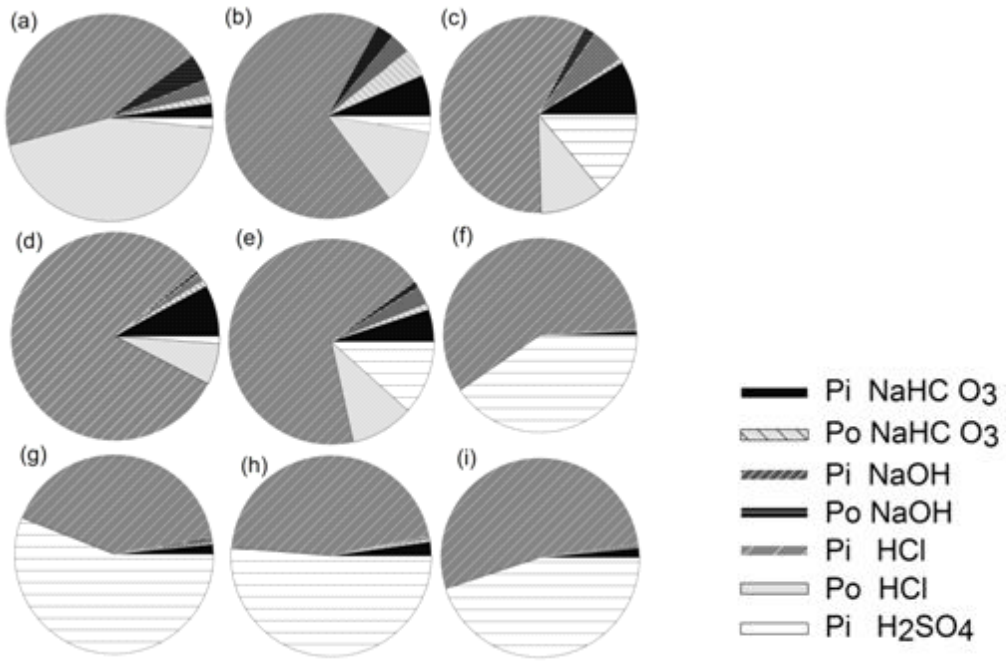

Figura 3. Fraccionamiento secuencial de P en el suelo realizado en 9 ecosistemas desérticos con cobertura vegetal distinta. (a) Pastizal en Churince, Coahuila, (b) Pastizal en Pozas Azules, Coahuila, (c) Pastizal (Cross y Schlesinger, 2001), (d) Matorral en Churince, Coahuila, (e) Matorral (Cross y Schlesinger, 2001) y 4 sitios con cobertura vegetal que puede ser pastizal, matorral o juníperos, (f) Shinarump, (g) White Rim, (h) Cedar Mesa y (i) Bosque (Buckingham *et al.*, 2010).

et al., 2003; Dao y Hoang, 2008) liberan fosfohidrolasas extracelulares (Ezawa *et al.*, 2005).

Las raíces de las plantas y los hongos producen fosfatasas ácidas, mientras que las bacterias pueden producir fosfatasas alcalinas (Tarafdar y Marschner, 1994). La alta diversidad de enzimas extracelulares, garantiza el éxito en la adquisición del recurso (Pi) en los ecosistemas. Una vez que se libera el Pi en la solución del suelo por la acción de fosfohidrolasas extracelulares, es necesario introducirlo a la célula por mecanismos pasivos o activos a través de la membrana (Dao, 2011). Existen muchos transportadores de fosfato ampliamente estudiados presentes en células de raíces de plantas y en microorganismos (Kulaev y Kulakovskaya, 2000). Los transportadores son proteínas componentes de las membranas celulares, con la capacidad de absorber

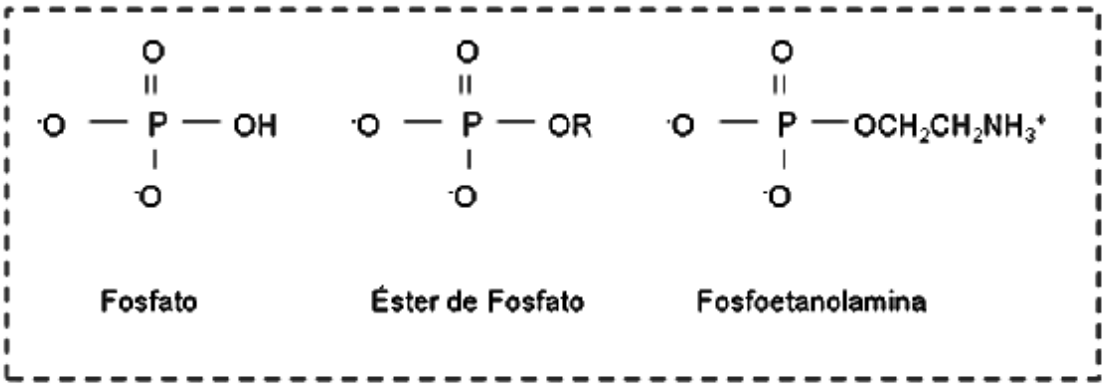

Figura 4. Compuestos orgánicos en donde el P (fósforo) presenta su mayor estado de valencia, +5 (Modificado de White y Metcalf, 2007).

al fosfato inorgánico y a moléculas estructuralmente simples (Dao, 2011).

Fosfonatos. En las últimas décadas ha crecido el interés por el estudio y entendimiento de la bioquímica del P cuando se encuentra en un menor estado de valencia, +3 (ej. Fosfonato, Aminoetilfosfonato, Fosfito; Figura 5), principalmente a partir del trabajo de Horiguchi y Kandatsu (1959), el cuál describió la presencia de fosfonatos en organismos vivos.

Los fosfonatos son una clase de compuestos organofosforados que contienen un enlace directo carbono-fósforo (C-P), en lugar del enlace éster más común C-O-P (Martínez *et al.,* 2010; Sviridov *et al.,* 2012). Debido a la estabilidad del enlace, estos compuestos son altamente resistentes a la biodegradación por factores químicos, térmicos, fotolíticos y enzimáticos (Hayes *et al.,* 2000; Sviridov *et al.,* 2012). Entre los fosfonatos conocidos se incluyen compuestos biogénicos y xenobióticos (Ternan *et al.*, 2000), ambos de gran importancia en los ecosistemas. Los organofosfonatos sintéticos son ampliamente utilizados en la industria química como detergentes, anticongelantes y pesticidas, entre otros (Hayes *et al.,* 2000). Por otro lado, dentro de los organofosfonatos naturales se incluyen una gran variedad de antibióticos de origen microbiano (Hayes *et al.,* 2000; White y Metcalf, 2007).

Algunos ejemplos de bacterias con la habilidad de biosintetizar fosfonatos son *Actinobacteria* (Quinn *et al.,* 2007), *Pseudomonas* y *Bacillus*, por lo que se ha sugerido que muchos microorganismos hayan adquirido esta habilidad durante su evolución (Kugler *et al.*, 1990). Entre los fosfonatos de origen natural, el más común es el aminoetilfosfonato (AEPn), el cual se ha encontrado formando parte de fosfonolípidos, polisacáridos y glicoproteínas en numerosos procariontes y eucariontes (Horiguchi y Kandatsu, 1959; White y Metcalf, 2007). Una vez que los organofosfonatos entran al suelo, independientemente de la vía, se ha encontrado que la actividad microbiana es casi exclusivamente la única responsable de su degradación.

La habilidad que tienen algunas bacterias para utilizar a los fosfonatos como fuente de P presume la presencia de enzimas necesarias para romper el enlace C-P. Actualmente, se conocen dos estrategias para este fin: a) una que involucra hidrolasas con alta afinidad por el sustrato y b) otra basada en la acción menos específica de C-P liasa (Kononova y Nesmeyanova, 2002). Hasta hoy se conocen 3 diferentes hidrolasas que pueden ser las encargadas de romper el enlace C-P: fosfonoacetaldehído hidrolasa conocida como fosfonatasa (Morais *et al.*, 2004), fosfonoacetato hidrolasa y fosfonopiruvato hidrolasa (Ternan *et al.*, 2000).

El 2-aminoetilfosfonato (2AEP) es el organofosfonato biogénico más abundante en la naturaleza y se ha especulado que las bacterias pueden tomar y degradar esta molécula. Esta hipótesis surge de los trabajos realizados por Rosenberg y La Nauze (1967), quienes describieron el transporte de 2AEP en *Bacillus cereus*. Posteriormente, Lacoste *et al*. (1976) describieron el transporte de 2AEP en *Pseudomona aeruginosa* A237. Estos trabajos fueron los primeros en arrojar evidencia de que la degradación de 2AEP se lleva a cabo en una ruta de dos pasos (Figura 6).

La primera reacción en la ruta involucra la transaminación del 2AEP a 2-fosfonoacetaldehído (PAA) (OHC-CH2-PO3H2). La enzima responsable de catalizar esta reacción es conocida como la 2AEP-

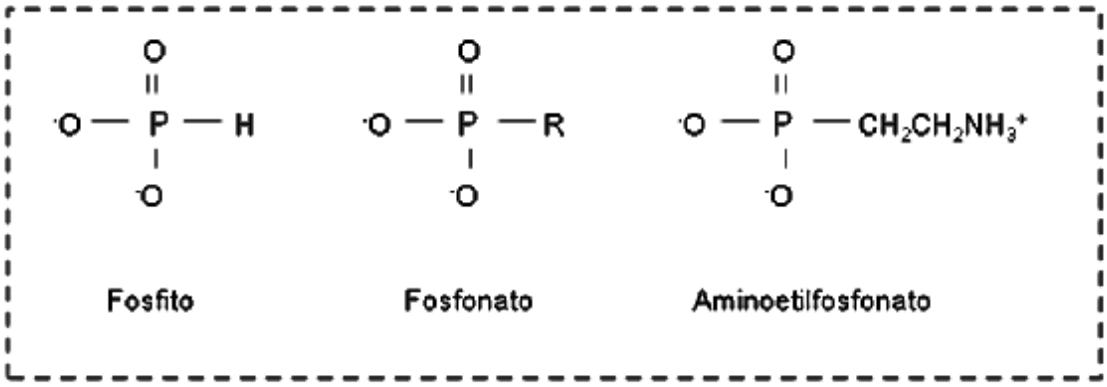

Figura 5. Compuestos orgánicos en donde el P presenta un estado de valencia de +3 (Modificado de White y Metcalf, 2007)

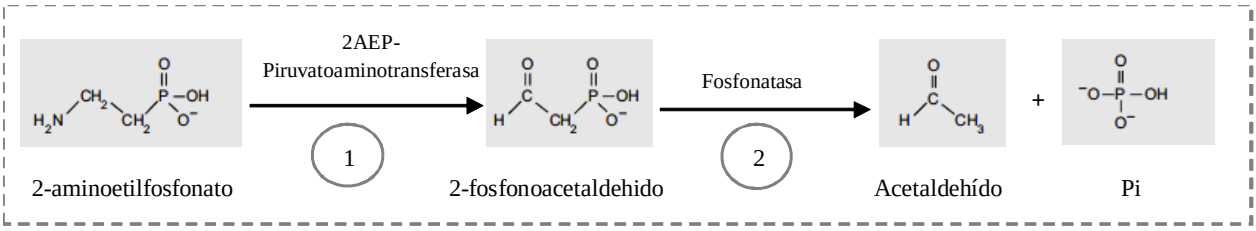

Figura 6. Ruta degradativa del 2-Aminoetilfosfonato (2AEP). Modificado de White y Metcalf (2007).

piruvato aminotransferasa, la cual tiene alta afinidad por los sustratos 2AEP y ácido pirúvico. En el segundo paso de la ruta degradativa, el PAA es dividido hidrolíticamente a fosfato inorgánico y acetaldehído. La enzima que cataliza esta reacción es la fosfonoacetaldehído hidrolasa comúnmente conocida como fosfonatasa, la cual fue aislada de *B. cereus* (La Nauze *et al.*, 1970) y *P. aeruginosa* (Dumora *et al.*, 1989). Fue gracias al entendimiento de esta ruta que se pudo aclarar el mecanismo por el cual se puede dividir o romper el enlace C-P (La Nauze *et al.*, 1977; Olsen *et al.*, 1988; Olsen *et al.*, 1992).

Hasta hoy, existen pocos trabajos que evalúen la importancia cuantitativa de los fosfonatos naturales como fuente de P en la biósfera terrestre, por lo que aún no está bien comprendida. Algunos de estos trabajos se han hecho en ecosistemas acuáticos y se sabe que el P contenido en los fosfonatos representa más del 25% del P orgánico disuelto de alto peso molecular en las columnas de agua del pacífico (Clark *et al.*, 1998) y otros océanos (Kolowith *et al.*, 2001). Además, en los organismos en donde se han encontrado es claro que los fosfonatos desempeñan un papel fundamental debido a su abundancia. Por ejemplo, algunas especies de anémonas marinas tienen más del 50% de su P total en forma de fosfonatos (Quin, 1965), en *Tetrahymena* el 30% de sus membranas lipídicas están en forma de fosfonolípidos (Hilderbrand, 1983). Aunque los fosfonolípidos también se han encontrado en vertebrados (Hilderbrand, 1983), son más abundantes en las formas de vida más sencillas.

De esta manera, los compuestos C-P pueden representar un recurso clave, tanto en los ecosistemas marinos, como en los terrestres, en donde la productividad puede estar limitada por la disponibilidad de P y de esta manera, podrían jugar un papel importante en el ciclo global del P (Benítez-Nelson y O'Neill, 2004; Dyhrman *et al.*, 2006). Es evidente que los organofosfonatos pueden representar una fuente alternativa de P, sobre todo en ecosistemas terrestres deficientes de este nutriente. Sin embargo, para conocer el papel real que pueden desempeñar los fosfonatos en el suelo, es necesario realizar estudios enfocados a identificar y cuantificar la actividad de las enzimas (fosfonatasas), así como los mecanismos que pudieran limitar o favorecer esta actividad. Así mismo, es importante conocer a los grupos bacterianos en los que está presente el regulón *pho*, los cuales pueden proveer claves sobre los factores que han permitido su evolución y su expresión.

Estrategias Bacterianas para la Adquisición y el Uso de P en Ambientes Oligotróficos

Para poder vivir en ambientes oligotróficos, las bacterias han desarrollado diferentes estrategias para la adquisición, inmovilización, reemplazo y uso eficiente del P (Adams y Wall, 2000; Tetu *et al.*, 2009). El Pi es transportado al interior de la célula bacteriana mediante proteínas de membrana, conocidas como transportadores específicos de fosfatos, las cuales están codificadas en el operón *pst* (Figura 7). Este operón comprende diferentes subunidades codificadas por distintos genes (Hirota, *et al.* 2010).

Debido a que en la membrana bacteriana únicamente existen transportadores de Pi y de fosfonatos de bajo peso molecular (Pn BPM) codificados por los genes *phn CDE* (Figura 7), los organofosfatos y los fosfonatos de alto peso molecular deben ser mineralizados por reacciones catalizadas por enzimas de origen bacteriano (Wackett *et al.*, 1987). Las enzimas para degradar a los ésteres de fosfato, están codificadas en el conjunto de genes conocido como *pho*, mientras que las enzimas para degradar fosfonatos están codificadas en el conjunto genético conocido como *phn*, actualmente formado por 17 genes (Figura 7; Wackett *et al.*, 1987; Metcalf y Wanner, 1991).

No todos los grupos bacterianos de ecosistemas oligotróficos presentan la habilidad para mineralizar

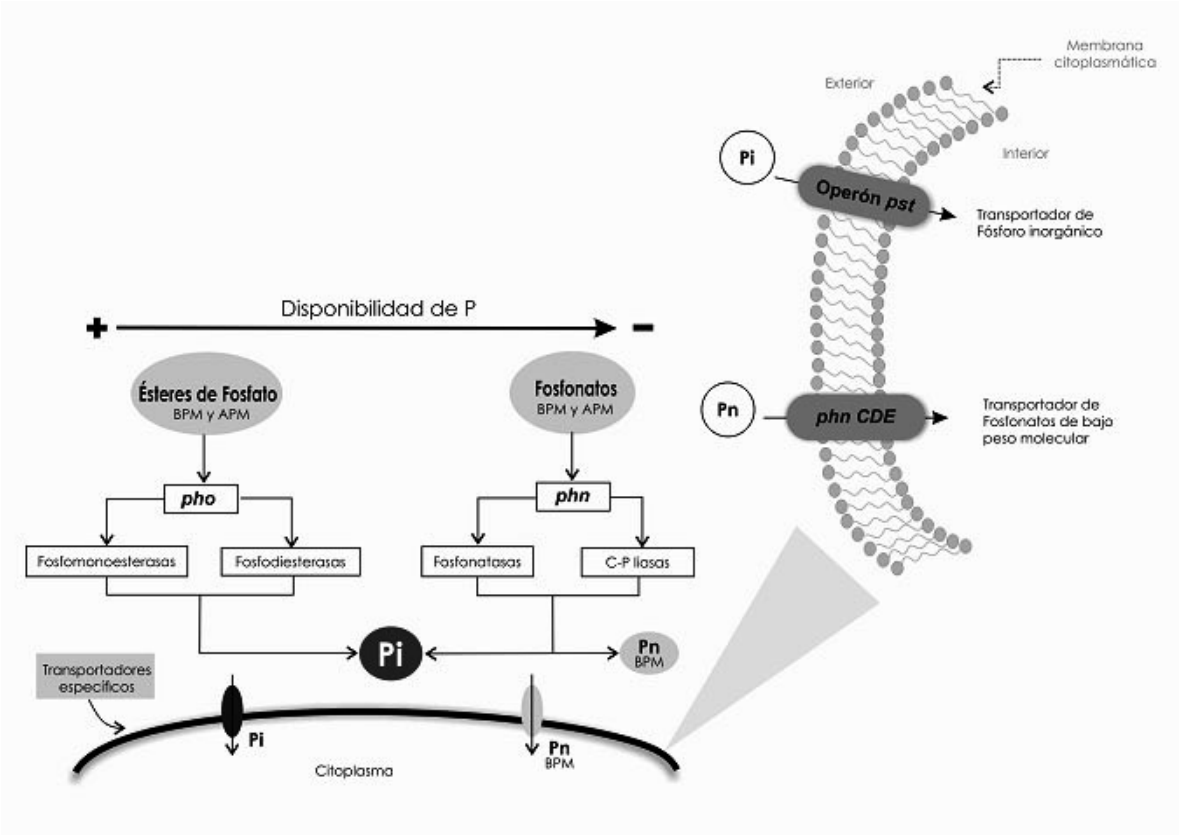

Figura 7. Estrategias bacterianas para la utilización de P (Adaptado de Hirota *et al.* 2010). *pho*: operón que codifica a los genes de fosfatasas. *phn*: operón que codifica a los genes de fosfonatasas y C-P liasas. Pi: fósforo inorgánico. Pn: fosfonato. APM: Alto peso molecular. *phn CDE*: genes codificantes para transportadores de membrana de fosfonatos de bajo peso molecular (BPM). Operón *pst*: conjunto de genes que codifican a los transportadores de membrana de Pi.

diferentes formas orgánicas de P. Por lo que a continuación se describen las estrategias que utilizan las bacterias tanto edáficas como acuáticas para satisfacer las demandas biológicas del P.

Inmovilización. La inmovilización es la adquisición de nutrientes por la comunidad de microorganismos. Existen bacterias como los Gemmatimonadetes con la capacidad metabólica para remover el Pi de la solución del suelo y acumularlo dentro de la célula en forma de polifosfatos (García-Martín *et al.*, 2006; Fukushima *et al.*, 2010). El polifosfato es un polímero lineal que puede contener hasta cientos de fragmentos de fosfatos unidos por enlaces de alta energía conocidos como fosfoanhidridos (Kulaev y Kulakovskaya, 2000). Los polifosfatos le confieren al organismo un aumento en la resistencia a las fluctuaciones ambientales, ayudan en la regulación de la actividad enzimática y son fuente de fosfato, con lo cual se satisfacen las demandas energéticas internas (Dao, 2011). Si el P disponible en el suelo es muy limitado, los microorganismos pueden inmovilizar en su biomasa entre el 20-50% del Po contenido en las superficie del suelo (Walbridge, 1991).

Adquisición. Algunas bacterias representantes de grupos como Firmicutes y Gamma proteobacterias tienen la capacidad de sintetizar ácidos orgánicos que se utilizan para desplazar a los iones fosfato de los sitios de intercambio y ponerlos disponibles (Mehment *et al.*, 2010).

Reemplazo. Tanto en bacterias terrestres (Dörmann y Benning, 2002; Alcaraz *et al.*, 2008) como en acuáticas (Van Mooy *et al.*, 2006, 2009) se ha observado que reemplazan al P por otros nutrientes en moléculas orgánicas esenciales, tales como fosfolípidos por sulfo-, galacto-, o fosfonolípidos con el objetivo de mantener la funcionalidad de la membrana.

Uso eficiente. Una de las estrategias bacterianas más sorprendentes es la capacidad de la reducción del genoma. Por ejemplo, la síntesis de ADN genómico

puede representar más de la mitad de la demanda de P en picocianobacterias (Bertilsson *et al.*, 2003). *Prochlorococcus*, una picocianobacteria que domina en ambientes oligotróficos en P, posee el genoma más pequeño de todos los organismos fotosintéticos reportados (Bertilsson *et al.*, 2003). Otro ejemplo es el género *Bacillus*, donde *Bacillus coahuilensis* (aislado de Cuatro Ciénegas) tiene el genoma más pequeño de todas las especies de *Bacillus* hasta ahora secuenciados (3640 Mpb) (Alcaraz *et al.*, 2008; Cerritos *et al.*, 2008).

CONCLUSIONES

- En la mayoría de los ecosistemas, se había considerado que el fósforo (P) contenido en moléculas orgánicas complejas (fosfonatos) era inaccesible para la biota, con lo cual se limitaba la productividad en los ecosistemas con poca disponibilidad de P (oligotróficos). Sin embargo, actualmente se ha reconocido la importancia de las bacterias en liberar el P contenido en estas moléculas.
- Para que la comunidad bacteriana sea capaz de sintetizar las diferentes enzimas que mineralizan estos compuestos orgánicos, es necesaria la presencia de una maquinaria genética capaz de sintetizar dichas enzimas. En ecosistemas oligotróficos, las comunidades bacterianas pueden estar formadas por linajes filogenéticamente distantes, con una alta diversidad de metabolismos para utilizar diferentes sustratos de P, asociados a genomas funcional y estructuralmente distintos. Sin embargo, no todos los grupos bacterianos tienen esta maquinaria genética, por lo cual han desarrollado una diversidad de estrategias para optimizar la adquisición y el uso del P. Este conjunto de estrategias ha permitido la permanencia y evolución de las bacterias, a pesar de la poca disponibilidad del P.
- Para poder entender los procesos que explican la disponibilidad del P en el suelo en estos ecosistemas es necesario realizar estudios integrales que consideren herramientas bioquímicas, biogeoquímicas y moleculares. Aún faltan estudios con análisis integrados que permitan elucidar el movimiento del P en los ecosistemas y como éste puede ser controlado por los microorganismos del suelo. Con estos estudios integrados será posible entender como se mantiene la vida en sistemas fuertemente limitados por el P, como es el caso de varios desiertos en el planeta.

AGRADECIMIENTOS

Al Posgrado en Ciencias Biológicas, UNAM. Al financiamiento otorgado por el Papitt-DGAPA, UNAM. Al CONACYT por la beca otorgada (210603) para realizar estudios de doctorado de los cuales forma parte este trabajo. Al Dr. Antonio González-Rodríguez y a dos revisores anónimos por los comentarios al presente trabajo. A Y. Perroni y C. Montiel por permitirnos presentar sus datos de fraccionamiento de fósforo(P).

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Capítulo III

Vegetation-soil system controls soil mechanisms for nitrogen transformations in an oligotrophic Mexican desert

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**** Publicado en: Journal of Arid Environment, 114: 62-69, 2015***

Journal of Arid Environments 114 (2015) 62–69

Contents lists available at ScienceDirect

Journal of Arid Environments

journal homepage: www.elsevier.com/locate/jaridenv

Vegetation-soil system controls soil mechanisms for nitrogen transformations in an oligotrophic Mexican desert

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ARTICLE INFO

Article history:
Received 17 February 2014
Received in revised form
15 September 2014
Accepted 19 November 2014
Available online

Keywords:
Vegetation
Dissolved organic carbon
Soil bacterial community
16S rRNA

ABSTRACT

Vegetation communities with high soil carbon (C) inputs, e.g. grassland ecosystems, promote N protection via microbial communities in the soil whereas communities with low soil C inputs, e.g. desert scrub ecosystems, promote nitrification and are therefore susceptible to N loss. This study examines this relationship more closely by assessing the effects of two vegetation-soil systems on soil N transformation, in a grassland-desert scrub in Cuatro Ciénegas Basin, Mexico. Metrics used in our study include: the belowground biomass of C, N and phosphorus (P) in both vegetation types; the availability of C, N and P in the soil; and the potential transformation of these nutrients by the microbial community which was characterized by 16S rRNA clone libraries. We found: (1) a higher NH_4^+ and microbial N concentration in the grassland soil than in the desert scrub soil, and (2) a different bacterial soil communities between both vegetation-soil systems. These findings suggest an interrelationship between nutrients in the belowground biomass, soil nutrient dynamics, and the soil bacterial community whereby grasslands promote a closed system that conserves N, whereas desert scrub vegetation exhibits an open system that sheds N.

1. Introduction

Drylands constitute the most extensive terrestrial biome on the planet, covering more than one-third of Earth's continental surface (Pointing and Belnap, 2012). Grasslands and desert scrub are the predominant vegetation types in drylands (Epstein et al., 2002), and are known to influence patterns of soil carbon (C) and nitrogen (N) distribution within the soil (West and Klemmedson, 1978). Grasses allocate more of their biomass to their roots and the majority of this root biomass is concentrated in the top 0.5 m of the soil. By contrast, desert scrub plants allocate less of their total biomass below ground (Goodale and Davidson, 2002). The two vegetation types found in these ecosystems correspond with varying quantities of organic C (OC) and N inputs to the soil.

Nutrient dynamics in desert ecosystems is also closely linked to seasonal variation in air temperature and moisture (Burke, 1989). As a result, organic matter (OM) decomposition, microbial immobilization and N mineralization are largely confined to the growing season, when the moisture and temperature of the desert are amenable to microbial activity (O'Brien, 1978). After this initial summer boom, however, microbial activity typically decreases as the rate of soil-nutrient transformations slows down and sources of C in the soil diminish (Montaño et al., 2007; Schimel and Weintraub, 2003).

Although the soil is an important reservoir of OC, only a low concentration is readily available to microorganisms (Daniel, 2005). Indeed, the available form of C, termed dissolved organic carbon (DOC), typically accounts for only 0.05–4% of soil organic carbon (SOC) and varies in concentration according to soil and vegetation types (Haynes, 2005). Once a microbe has taken up the organic molecules, the availability of inorganic molecules within the ecosystem is entirely determined by the metabolism of the microbiota (Schimel and Schaeffer, 2012). For example, C availability promotes N immobilization by heterotrophic microorganisms, decreasing the N budget in the soil and thereby reducing N availability for plants and microbes (Chapin et al., 2002; Montaño et al., 2007). The enzymes involved in N transformation include hydrolases, oxidases, deaminases and lyases, all of which are

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http://dx.doi.org/10.1016/j.jaridenv.2014.11.007
0140-1963/

produced by plants, animals and microorganisms (Jones et al., 2004). These enzymes act intra-cellularly, decomposing the organic nitrogen (ON) until only the N in excess of microbial demand is released to the soil as ammonium (NH_4^+). This is understood to be a closed system because all N is entirely conserved through the activities of biota in the ecosystem. Similar observations have been made in studies of desert soils (López-Lozano et al., 2012), as well as in other ecosystems (tropical deciduous forest; Montaño et al., 2007).

By contrast, when the availability of DOC is low, autotrophic pathways, such as nitrification, are favored, and nitrate (NO_3^-) accumulates (Vitousek, 2002). Finally, NO_3^- is released to the atmosphere by denitrification or leached from the soil. These autotrophic strategies are associated with an open system, where N can be lost from the ecosystem. In this way, C availability affects both soil N transformation and soil bacteria composition by favoring the activities of taxa adapted to grow on resources supplied by soil organic matter (SOM) inputs (Thomson et al., 2010).

López-Lozano et al. (2012) conducted a study on two sites with different humidity in the gypsum rich Cuatro Ciénegas basin (CCB), a Mexican desert oasis ecosystem. They found that the site with higher DOC concentration also exhibited higher quantities of NH_4^+, microbial C (Cmic) and N concentration. As expected, a higher bacterial taxonomic diversity at this site was associated with denser vegetation and more water availability. This humid and richer site had a closed N cycle with greater N immobilization. However, in the dry site with poor plant coverage and lower DOC, nitrification dominated over N microbial immobilization which resulted in N losses. This last study was the first to characterize the soil microbial community and its relationship to nutrient dynamics in CCB.

Nutrient dynamics is direct function of microbial diversity in the soil. For example, in a wide sampling of soils in North America, Fierer et al. (2007) found a negative correlation between Acidobacteria abundance and C mineralization rates, whereas the abundances of β-Proteobacteria and Bacteroidetes positively correlated with C mineralization rates. No such correlation was observed, however, with other groups like α-Proteobacteria, Firmicutes, and Actinobacteria. However, in the desert soil of CCB, Lopez-Lozano et al. (2013) found a positive correlation between the anaerobic photosynthetic Cloroflexi and TM6 with total organic C (TOC) availability. This suggests that in this site soil microbes also fix C, having a key role in soil productivity.

The main objective of this study was to examine the effects of a vegetation-soil system on potential soil N transformation and availability in a grassland-desert scrub in the Cuatro Ciénegas basin during two contrasting seasons. Our hypothesis was that mineralization and immobilization processes are more important in the grassland ecosystem and thus that N is more protected in a closed cycle. This contrasts with the desert scrub ecosystem where nitrification could be the most important process in N transformation, given the scarcity of OM inputs to the soil. Additionally, we expected a higher microbial immobilization and mineralization in the warmer and moist season (summer) than in the cold and dry season (winter).

To test the hypothesis, we measured the C, N and P concentration in the root biomass of the two vegetation-soil systems. We also quantified soil C, N and P availability and determined the potential transformation of these nutrients by the microbial community, which was characterized by 16S rRNA clone libraries. We observed different potential N transformation in each site along with contrasting microbiota. As expected, we found these microbial transformations were higher during the summer season than in the winter season.

2. Materials and methods

2.1. Site description and soil-vegetation sampling

This study was carried out in the Cuatro Ciénegas basin (26°50′N and 102°8′W) located at 740 m above sea level, in the Chihuahuan desert, Mexico. The climate is hot and arid, with an annual precipitation of 252 mm, which occurs mainly in summer and occasionally in winter. The average annual temperature is 21 °C, with July as the hottest (28 °C) and January as the coldest month (15 °C; Cuatro Ciénegas weather station). According to the world reference base for soil resources (WRB, 2007), the dominant soils are gypsisols and leptosols, as opposed to Jurassic gypsum and limestone (McKee et al., 1990). The main vegetation types are grassland dominated by *Sporobolus airoides* (Poaceae) and *Allenrolfea occidentalis* (Amaranthaceae); and desert scrub dominated by *Jatropha dioica* (Euphorbiaceae) and *Larrea tridentata* (Zygophyllaceae) (Perroni et al., 2014). Each vegetation type is growing over different bedrock and consequently defines the two vegetation-soil systems studied: grassland over gypsisol and desert scrub over leptosol. The grassland site was located in the valley bottom on gypsum without the influence of lime-stone from Sierra de San Marcos. In contrast, this lime-stone influence the desert scrub soils, which was located on the foot slope of this ridge. There are five plant species in the grassland and 21 species in the desert scrub, with only one species present in both habitats (*Prosopis laevigata*; Perroni et al., 2014).

A sampling site was selected for each vegetation-soil system. These two sites are separated by 3.5 km. In August 2007 (summer sampling) and February 2008 (winter sampling), a 100 by 50 m plot was demarcated at each sampling site. The plots were divided into 10 transects, separated from each other by a distance of 10 m. A sampling subplot of 2 × 2 m (4 m^2) was selected randomly on each transect; 15 cm deep soil samples were taken from five locations (one central point and four corners) within each sampling subplot, and then were mixed to form one composite sample. In total, 10 composite samples were taken at each site during each sampling date. Afterwards, each sampling subplot was divided into four parts for sampling aboveground vegetation. In two of these four parts, all aboveground biomass was collected and stored in black plastic bags before laboratory analyses of total carbon (TC) and total nitrogen (TN). Five root samples were collected in each of the 10 transects per plot and stored in the same way, and the same analyses were performed. The vegetation sampling was conducted only in one season (August 2007). Soil for biogeochemical analysis was stored in black plastic bags and refrigerated at 4 °C for laboratory analyses. As a way of characterizing the bacterial community at each site, 100 g samples were taken from each subplot and used to prepare one composite sample from each site and each sampling season. The resulting four samples were immediately stored in liquid nitrogen until DNA extraction.

2.2. Biogeochemical analyses

Soil pH was measured in deionized water (1:2 w: v) with a digital pH meter (Corning). A subsample (100 g) was oven-dried at 75 °C to constant weight for soil moisture determination using the gravimetric method. All C forms analyzed were determined with a Total Carbon Analyzer (UIC Mod. CM5012; Chicago, E.U.A), while N and P forms determined by colorimetrical analyses using a Bran-Luebbe Auto Analyzer III (Norderstedt, Germany).

Before the analyses of total nutrient forms, soil samples were dried and then ground with a pestle and mortar. Total carbon and inorganic carbon were determined by combustion and coulometric detection (Huffman, 1977). TOC was calculated as the difference between total C (TC) and inorganic C (IC). For total N (TN) and TP

determination, the samples were acid digested with H_2SO_4, H_2O_2, K_2SO_4 and $CuSO_4$ at 360 °C. N was determined by the macro-Kjeldahl method (Bremmer, 1996), while P was determined by the molybdate colorimetric method after ascorbic acid reduction (Murphy and Riley, 1962).

The available, dissolved and microbial nutrient forms were extracted from field moist soil samples. Inorganic nitrogen forms (NH_4^+ and NO_3^-) were extracted with 2 M KCl, followed by filtration through a Whatman No. 1 paper filter, and determined colorimetrically by the phenol-hypochlorite method. Inorganic phosphorous (IP) was extracted with sodium bicarbonate (pH 8.5) and was determined colorimetrically by the molybdate-ascorbic acid method (Murphy and Riley, 1962).

Dissolved nutrients were extracted with deionized water after shaking for 45 min and then filtering through a Millipore 0.42 μm filter (Jones and Willet, 2006). Before acid digestion, one aliquot of the filtrate was used to determine dissolved ammonium (DNH_4^+) and IP in deionized water extract. Total dissolved nitrogen (TDN) was digested by the macro-Kjendahl method. Total dissolved P (TDP) was also acid digested and determined by colorimetry. Total dissolved carbon (TDC) was measured with an Auto Analyzer of carbon (TOC CM 5012) module for liquids (UIC-COULOMETRICS). Inorganic dissolved carbon (IDC) was determined in an acidification module CM5130. DOC, dissolved organic nitrogen (DON) and dissolved organic phosphorous (DOP) were calculated as the difference between total dissolved forms and inorganic dissolved forms.

Microbial N (Nmic) and Cmic concentrations were determined by the chloroform fumigation extraction method (Vance et al., 1987). Fumigated and non-fumigated samples were incubated for 24 h at 25 °C and constant moisture. Cmic was extracted from fumigated and non-fumigated samples with 0.5 M K_2SO_4, filtered through Whatman No. 42 filters (Brookes et al., 1985). C concentration was measured from each extract as total and inorganic concentration by the method described before. The difference between TC and IC was used for microbial C calculation. Cmic was calculated by subtracting the extracted carbon in non-fumigated samples from that of fumigated samples and dividing it by a K_{EC} value (=extractable part of microbial biomass C) of 0.45 (Joergensen, 1996). Nmic was extracted with the same procedure used for Cmic but was filtered through a Whatman No. 1 paper. The filtrate was acid digested and determined as TN by Macro-Kjeldahl method (Brookes et al., 1985). Nmic was calculated similarly to Cmic, but divided by a K_{EN} value (=extractable part of microbial biomass N after fumigation) of 0.54 (Joergensen and Muller, 1996). Finally, the values of Cmic and Nmic were corrected to dry soil basis.

2.3. Incubations

Aerobic incubation in the laboratory was used to estimate potential carbon mineralization, net nitrogen mineralization, ammonification, nitrification, DOC and DON changes. Samples of 100 g of fresh soil were placed in a PVC (polyvinyl-chloride) tube cores with a 0.05 mm mesh at the bottom. Samples were incubated in 1 L jars for 14 days at 25 °C, each sample was wetted by capillarity to field water holding capacity with deionized water. For potential C mineralization estimates, the jars were regularly aerated and the evolved CO_2–C was collected in 10 ml traps of 0.2 M NaOH solution. Afterwards, carbonates were precipitated by adding 1.5 M $BaCl_2$ and then titrated with 0.2 M HCl. Before and after the incubation, NH_4^+, NO_3^-, DOC and DON were determined with the methods described above. Differences between post- and pre-incubation values were used to calculate N mineralization with inorganic N forms (NH_4^+ plus NO_3^-), net nitrification (with NO_3^-), and changes in DOC and DON.

2.4. Molecular analyses

Total DNA was extracted using the Soil Master DNA Extraction Kit (EPICENTRE Biotechnology) according to the manufacturer's instructions, with an additional step of bacterial isolation using the fractionation centrifugation technique described in Holben et al. (1988). This step was performed on frozen 50 g soil samples before DNA extraction as a way of reducing the concentrations of salts, polysaccharides and secondary compounds. DNA was stored at −20 °C. DNA molecular weight and quality was confirmed using agarose gel electrophoresis. The 16S rRNA gene was amplified from each sample with the polymerase chain reaction (PCR) with the universal primers F27 (5′AGAGTTTGATCMTGGCTCAG3′) and R1492 (5′TACGGYTACCTTGTTACGACTT3′). Three independent PCRs were performed for each sample. The PCR reaction consisted of 50 μl containing 4 μl of DNA, 1 μl PCR buffer 10×, 0.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.2 mM of each primer, 1 unit of platinum Taq DNA polymerase high fidelity (Invitrogen), 5% DMSO and 0.05 mg of BSA. All reactions were carried out in a thermal cycler (MJ Research, Watertown, MA) with the following program: an initial denaturation step at 95 °C for 10 min, then 30 cycles at 95 °C for 1 min, 52 °C for 2 min, and 72 °C for 3 min, a final extension step at 72 °C for 10 min and storage at 4 °C. The three reactions were pooled and purified in a 2% agarose gel by the QIAquick gel extraction kit (Qiagen). The purified fragment was cloned into the vector PCR 2.1 and transformed into *Escherichia coli* following the manufacturer's instructions (Invitrogen). Only the plasmids containing inserts were isolated for sequencing with the Montage Plasmid Miniprep

Table 1
Means (±standard error) of moisture, pH, total organic carbon (TOC), total nitrogen (TN) and total phosphorous (TP) for grassland and desert scrub soil, for two different seasons (summer and winter) in the Cuatro Ciénegas Basin, Coahuila, Mexico.

Parameters	Grassland		Desert scrub	
	Summer	Winter	Summer	Winter
Soil moisture (%)	36.71 (2.031)^{Aa}	20.75 (0.947)^{Ab}	3.05 (0.311)^{Ba}	1.10 (0.02)^{Ba}
pH	8.62 (0.063)	8.59 (0.039)	9.57 (0.092)	9.42 (0.055)
Total				
Organic carbon (mg C g^{-1})	13.42 (1.821)	12.24 (2.001)	13.46 (3.826)	6.4 (0.888)
Nitrogen (mg N g^{-1})	0.90 (0.166)^{Aa}	0.77 (0.101)^{Aa}	0.33(0.018)^{Bb}	0.64(0.051)^{Aa}
Phosphorous (mg P g^{-1})	0.09 (0.011)	0.07 (0.017)	0.22 (0.018)	0.17 (0.17)
C:N	17 (2)	16 (1)	21 (5)	10 (2)
Available				
Ammonium (NH_4^+) (μg NH_4 g^{-1})	11 (3.26)	0.88 (0.11)	1.72 (0.83)	0.47 (0.06)
Nitrate (NO_3^-) (μg NO_3 g^{-1})	0.27 (0.14)^{Aa}	0 (0.09)^{Ba}	0.5 (0.20)^{Aa}	1.35 (0.35)^{Aa}
Inorganic phosphorous (μg P g^{-1})	2.05 (0.38)	1.82 (0.35)	26.14 (1.96)	26.6 (1.6)
Dissolved				
DOC[a] (μg C g^{-1})	496 (11)^{Ab}	1045 (38)^{Aa}	90.26 (4)^{Bb}	1068 (39)^{Aa}
DON[b] (μg N g^{-1})	264 (62)	37 (7.8)	124 (18)	19.7 (3)
DOP[c] (μg P g^{-1})	12.8 (0.64)^{Aa}	2.36 (0.34)^{Ab}	8.23 (0.2)^{Ba}	0.98 (0.14)^{Bb}
Microbial				
Carbon (μg C g^{-1})	814 (98)	566 (59)	723 (42)	642 (40)
Nitrogen (μg N g^{-1})	14 (2.1)	26 (3.8)	22.8 (5)	19 (3.5)

[a] Dissolved organic carbon.
[b] Dissolved organic nitrogen.
[c] Dissolved organic phosphorous. Values followed horizontally by a different uppercase letter (A and B) indicate that means are significantly different ($P < 0.05$) between sites (grassland and desert scrub) within sampling date (summer and winter); whereas different lowercase letters (a and b) vertically indicate that means are significantly different ($P < 0.05$) among sampling dates within a site.

kit (Millipore). The insertion within the plasmids was sequenced by the Sanger method using vector-based primer 27F.

For processing and classification of the sequence data, Mothur open source software package (v 1.15.0; Schloss et al., 2009) was used. Sequences were screened for potential chimeric reads using Chimera.slayer (Haas et al., 2011) and the linked SILVA template database. High-quality sequences were compared against SILVA database to obtain their taxonomic rank.

2.5. Statistical analyses

Soil biogeochemistry data were subjected to a repeated measures analysis of variance (RMANOVA; Von Ende, 1993). The plant-soil system (grassland and scrubland) was considered as between-subject factor and year (summer and winter), and the interaction were considered as within-factors. Log-transformations were applied when data deviated from normality. When RMANOVA indicated significant factor effects, mean comparisons were performed with Tukey's multiple comparisons test (Von Ende, 1993). To explore relationships among soil parameters, Pearson correlations were used. For grouping soil samples with active nutrients forms (dissolved, available and microbial), Principal Components Analysis (PCA) was carried out. The variables used for PCA were pH, available forms, dissolved organic forms and microbial forms. Stepwise multiple-regression analysis was performed to examine the driving variables of microbial activity and N dynamics measured in laboratory incubations. To explore relationships among vegetation and the quantified soil parameters, Pearson correlations were used. All statistical analyses were performed with Statistica 7 software (StatSoft, 2000).

Table 2
F-ratios and significant levels of the repeated measures ANOVA for variables of grassland and desert scrub soils collected in the Cuatro Ciénegas Basin, Coahuila Mexico.

Parameters	Source of variation		
	Between subject	Within subjects	
	Site	Date	Date X site
Soil moisture	381 (<0.001)	114 (<0.001)	70 (<0.001)
pH	188 (<0.001)	2 (0.2)^{ns}	0.80 (0.4)^{ns}
Total			
Organic carbon	6.3 (0.02)	1.7 (0.2)^{ns}	0.6 (0.5)^{ns}
Nitrogen	14 (0.001)	5.5 (0.03)	8.6 (0.01)
Phosphorous	36 (<0.001)	4.5 (0.05)	0.105 (0.8)^{ns}
C:N	0.02 (0.90)^{ns}	3.2 (0.08)^{ns}	1.8 (0.2)^{ns}
Available			
Amonium (NH_4^+)	10.21 (0.005)	13.42 (0.002)	4 (0.06)^{ns}
Nitrate (NO_3^-)	16 (0.0008)	2 (0.19)^{ns}	6 (0.2)^{ns}
o	217 (<0.001)	0.02 (0.9)^{ns}	0.23 (0.6)^{ns}
Dissolved			
DOC[a]	2.3 (0.14)^{ns}	110 (<0.001)	11.66 (0.003)
DON[b]	7 (0.01)	34 (<0.001)	0.1(0.76)^{ns}
DOP[c]	70 (<0.001)	461 (<0.001)	15 (0.001)
Microbial			
Carbon	0.01 (0.92)^{ns}	8 (0.01)	2 (0.17)^{ns}
Nitrogen	0.16 (0.68)^{ns}	1 (0.32)^{ns}	3 (0.8)^{ns}
Incubation			
CO_2–C	4.55 (0.05)	3.62 (0.07) ^{ns}	0.39 (0.54) ^{ns}
Ammonification	7 (0.02)	12 (0.002)	6 (0.02)
Nitrification	34 (<0.0001)	23 (0.0001)	6 (0.02)
ΔDOC[d]	1.03 (0.32)^{ns}	5.9 (0.02)	1.6 (0.21)^{ns}
ΔDON[e]	4.57 (0.051)^{ns}	30 (0.0001)	1 (0.24)^{ns}

ns = not significant.
[a] Dissolved organic carbon.
[b] Dissolved organic nitrogen.
[c] Dissolved organic phosphorous.
[d] Changes in dissolved organic carbon.
[e] Changes in dissolved organic nitrogen.

3. Results

3.1. Soil nutrients

The grassland soil had greater soil moisture than the desert scrub soil (Table 1). In grassland, soil moisture was greater in samples collected in summer than those collected in winter; while in the desert scrub soil moisture did not vary between the two sampling dates (Table 1). Samples collected in the desert scrub had higher pH than samples collected in grassland (Tables 1 and 2). The grassland soil had higher TOC (12.8 mg g^{-1}) than the desert scrub soil (9.9 mg g^{-1}). The effect of date and interaction (SITEXDATE) was not statistical significant (Table 2). Grassland also had higher TN concentration than the desert scrub soil, but the difference between sites was only significant in samples collected in summer (Tables 1 and 2). In contrast, desert scrub had higher TP than grassland (0.19 and 0.08 mg g^{-1} respectively; Tables 1 and 2).

DOC concentration was higher for the winter soil samples and did not differ between both sites. In the summer soil samples, grassland had higher DOC concentration than desert scrub soil (Tables 1 and 2). The grassland soil had higher NH_4^+, DON and DOP concentration than the desert scrub soil (6, 150, 15 μg g^{-1} and 1, 72, 9 μg g^{-1}; respectively). All these concentrations decreased from the summer to the winter (Tables 1 and 2). In contrast, NO_3^- concentration was higher in the desert scrub, and this difference was only for the winter soil samples (Tables 1 and 2). Cmic concentration was higher in summer soil samples, but did not differ between sites (Tables 1 and 2). In contrast, Nmic concentration did not show any differences between study sites or seasons (Table 2).

Correlation analysis showed that soil moisture was positively correlated with DOP, NH_4^+ and DON, but negatively with pH, IP and NO_3^- (Table S1). The IP was negatively correlated with NH_4^+ and

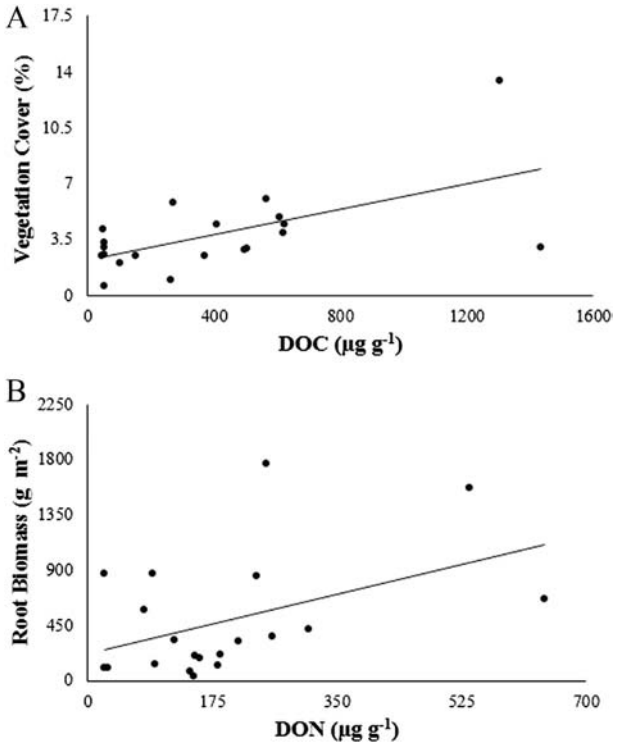

Fig. 1. Correlation Analysis between (A) vegetation cover and soil dissolved organic carbon (DOC), $r = 0.58$, $p = 0.006$ and (B) root biomass and soil dissolved organic nitrogen (DON), $r = 0.44$, $p = 0.049$ at Cuatro Ciénegas basin, Mexico.

positively with NO_3^-. Finally, Cmic was positively correlated with DOP.

The principal component analysis (PCA) indicated differences in the overall concentration of nutrients between sites. The first two principal components (PC) explained 58% of the total variance. The values of the eigenvectors showed that the higher positive loadings on the first PC (PC1 explains 36% of the total variation) corresponded to IP and −DOP (0.70 and −0.81, respectively), while on PC2 (explains 22% of the total variation) corresponded to pH and -DOC (0.69 and −0.71, respectively). The results of the PCA showed that the principal components are separated by vegetation-soil system (PC1 $t = -5.7$ and $p < 0.001$; PC2 $t = 4.98$ and $p < 0.001$).

3.2. Vegetation

Grassland had higher root biomass than the desert scrub (751 ± 170 and 224 ± 77 g m^{-2}, respectively) and also higher TN and TP concentrations in the roots (11 ± 0.8 and 7.6 ± 0.2 μg g^{-1}; 0.51 ± 0.05 and 0.38 ± 0.03 μg g^{-1}; respectively), but TC did not show any differences between sites (293 ± 7 and 306 ± 6, respectively). The C:N ratio in the belowground biomass was higher in the desert scrub than in the grassland (40 and 29, respectively). In contrast, aboveground biomass did not show biomass differences between study sites (493 and 394 g m^{-2} for grassland and desert scrub, respectively). Correlation analysis showed a positive correlation between DOC and vegetation cover, and DON with root biomass (Fig. 1).

3.3. Laboratory soil incubations

The grassland soil had higher potential C mineralization than the desert scrub soil (165 and 129 μg C g^{-1}, respectively), and there were no differences between sampling dates (Table 2). All DOC and DON values after soil incubation were lower than before incubation, thus negative delta (Δ) values were obtained, suggesting C and N mineralization during incubation. The ΔDOC differed only between seasons (Table 2). The winter soil samples had greater negative values than the summer samples (−693 and −328, respectively). Similarly, ΔDON also showed differences only between seasons (Table 2), but the greater negative values of DON were measured in the incubated samples collected in summer (−155 and −25, respectively). Net N ammonification had greater negative values in incubated soil samples collected in summer in the grassland than in the scrubland soil samples (more negative values, which represent N immobilization from microbial biomass), while there were no differences between sites in the winter samples (Fig. 2a). The scrubland soil samples had higher positive values of net nitrification (more positive values, which represent a higher nitrification than N immobilization within microbial biomass) than the grassland samples in both seasons (Fig. 2b). Only the scrubland soil samples presented differences between seasons with higher values in winter season (Fig. 2b).

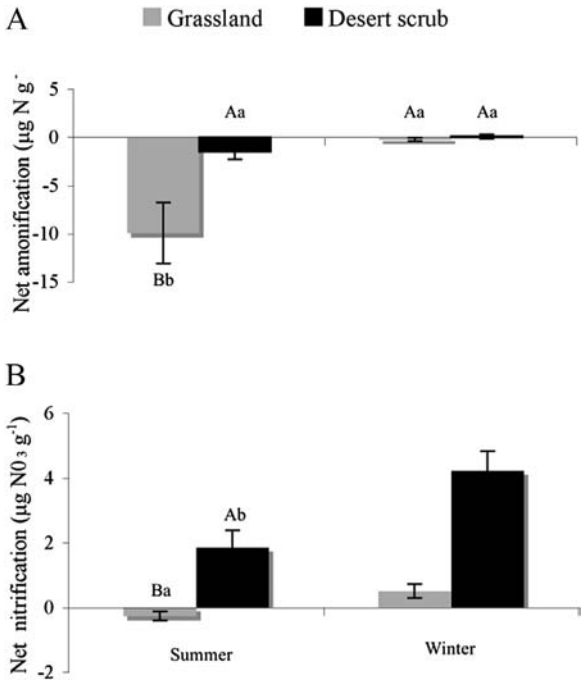

Fig. 2. Seasonal variation of (A) Net ammonification and (B) Net nitrification after a 14-days incubation of grassland and desert scrub soil at Cuatro Ciénegas basin, Mexico. Bars with different uppercase letters (A and B) indicate that means are significantly different ($P < 0.05$) between sites (grassland and desert scrub) within a sampling date. Different lowercase letters (a and b) indicate that means are significantly different ($P < 0.05$) among sampling dates within a site.

The potential C mineralization, ammonification and nitrification was analyzed by stepwise multiple regression. The analysis showed that in the summer soil samples, the ammonification had a negative correlation with NH_4^+, the nitrification had a negative relation with DOC and the CO_2–C had a positive correlation with Nmic. However, soil samples collected in winter did not show any correlation with ammonification and nitrification, but CO_2–C correlated positively with DON (Table S2).

3.4. Bacterial composition

For the grassland, we obtained a clone library with 182 sequences, 89 corresponding to summer and 93 corresponding to winter. The desert scrub soils, however had 197 sequences (93 in summer and 104 in winter). In the summer samples, the grassland sequences were distributed in 17 phyla and 25 classes, while the desert scrub sequences were distributed in 9 phyla and 13 classes. These results suggest that the bacterial community of the desert scrub soil was distributed in fewer phyla than in the grassland soil. For example, the Actinobacteria was the more abundant bacteria phyla in both sites, but it represents 68% and 30% for desert scrub and grassland, respectively (Fig. 3). Similarly, Gemmatimonadetes and Chloroflexi were more abundant in the desert scrub soil than in the grassland soil. In contrast, Proteobacteria was more abundant in grassland than in desert scrub (26 and 10%, respectively).

In the winter samples, the sequences in the grassland were distributed in 11 phyla and 16 classes, while they were distributed in 11 phyla and 19 classes in the desert scrub soil. As in the summer samples, the Actinobacteria dominated in the desert scrub soil (representing 64%), while Proteobacteria was in the grassland soil (42%). In contrast to summer samples, Gemmatimonadetes and Chloroflexi had similar proportion of sequences in both sites (10% and 5%, respectively).

4. Discussion

Our hypothesis was that the mineralization and immobilization processes would be more important in grassland than in desert scrub, consistent with studies from other deserts where vegetation type shows contrasting biomass and nutrient allocation (Goodale and Davidson, 2002; Haynes, 2005). This occurs because N would be more protected in a closed system such as grassland compared with desert scrub where nitrification would be the most important N transformation process due to fewer OM input to the soil. We also expected that this process would be more pronounced in summer

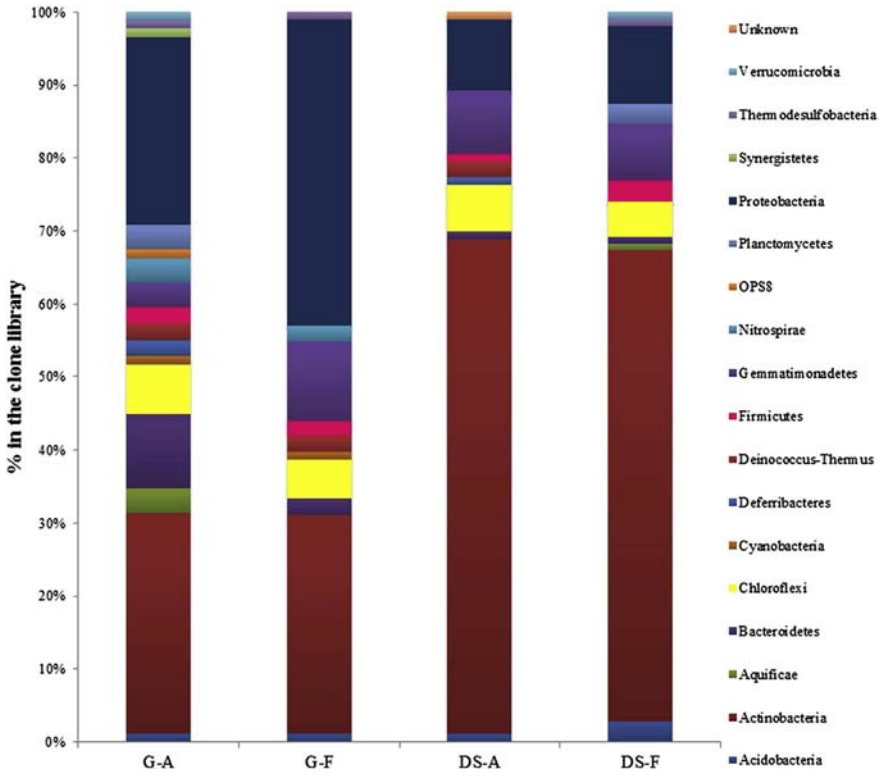

Fig. 3. Taxonomic distribution of the 16S rRNA gene sequences obtained from the clone libraries of two studied sites: grassland August (G–A), grassland February (G–F), desert scrub August (DS-A) and desert scrub February (DS-F) at Cuatro Ciénegas basin, Mexico.

than winter due to differences temperature and water availability can slow enzymatic activities.

In our study, the grassland site was located in the valley bottom over gypsisol. This soil is mainly developed in depression areas and is derived from substrates rich in calcium sulfates (WRB, 2007). In contrast, the desert scrub site was located in the foot slope of San Marcos ridge with influence of lime-stone, resulting in a thin soil over continuous rock as described for leptosols by WRB (2007). Consequently, the site with leptosols has a lower soil water holding capacity than the sites with gypsisols due to their soil physical characteristics (i.e., soil depth, texture, etc.) and topographic position. Additionally, the bottom valley has a continuous input of underground water from springs (Elser et al., 2005). Consequently, the grassland soil had higher water availability than the desert scrub soil as our data of soil humidity suggested. This higher water availability promotes higher plant productivity and therefore higher organic matter input to the soil, as root biomass data suggested.

The grassland had a higher OM input to the soil, which corresponded with a higher soil C availability in the ecosystem. Accordingly, the plant root biomass in the grassland was higher than in the desert scrub ecosystem, which is consistent with higher TOC and DOC concentrations in the grassland soils. However, a positive correlation between DOC and vegetation cover indicates that soil C availability is promoted mainly by root biomass. The role of root biomass as a main source of soil C has also been documented elsewhere in studies of desert ecosystems (Austin et al., 2004; Kögel-Knabner, 2002).

Several properties of OM composition promote higher rates of nutrient mineralization: low aromaticity, high proportions of carbohydrates (Kalbitz et al., 2003), hydrophilic compounds (Qualls and Haines, 1992) and low accumulation of molecular weight compounds. Conversely, the potential decomposition rate of OM increases when the C:N ratio decreases. At the CCB, the C:N ratio in the subterranean biomass was lower in the grassland than in the desert scrub. Furthermore, soil TN, DON, and NH_4^+ concentrations were higher in the grassland soil, which suggests that inputs of N to the grassland soil are due not only to N fixation by bacteria as reported by López-Lozano et al. (2012) but also N recycling. Additionally, the positive relationship between DON and root biomass indicates that the belowground biomass might function as the main source of soil available ON. Numerous studies have examined the composition, degradability and mineralization of OM, indicating that these processes can be different in soils under different vegetation covers (Kalbitz et al., 2003; Magill and Aber, 2000). Our results are consistent with these data. The two first principal components were explained by vegetation type; hence, vegetation-soil system can control the soil active nutrients forms and affect the pathways of soil N transformations, as explained below.

Soil nutrient availability is largely a function of soil microbial composition and vegetation cover. The grassland, which has higher SOM input as well as water content and lower root C:N ratios, promotes the activity of microorganisms from the phylum Proteobacteria. Due to the high physiological diversity that characterizes Proteobacteria (Strackebrand et al., 1988), it has been reported that this phylum is responsible for most of the N transformations occurring in aquatic and terrestrial ecosystems (Madigan et al., 2004). The higher abundance of Proteobacteria in the grassland soil, can explain the higher soil DON, and NH_4^+ concentrations than in the desert scrub soil. Subsequently, N immobilization is increased, mainly by heterotrophic bacteria, as reflected in higher potential C mineralization in the grassland than in the desert scrub. Greater negative values of net ammonification in the grassland also suggest an increase in N immobilization. These results support the hypothesis that soil C availability enhances microbial immobilization (Magill and Aber, 2000). C availability also increases both the

ON demand and the ammonification rate, which allows NH_4^+ to be released into the soil. Diverse studies conducted in different ecosystems indicate that C availability promotes N fixation by bacteria such as cyanobacteria (García-Pichel, 2006) thereby promoting microbial recycling (Montaño et al., 2007). In the grassland, this recycling can represent a closed cycle in which both the microbial community and plants mutually benefit from the increment in soil N availability.

Desert scrub, with lower SOM input, also had lower soil DOC, DON and NH_4^+ concentration, but higher NO_3^- concentration. Additionally, desert scrub soil had a different microbial composition, where Actinobacteria, which play an important role in soil organic matter decomposition (Ventura et al., 2007), dominate with above 60% in both seasons. Actinobacteria predominates specially in soil with alkaline pH (Lauber et al., 2009). The low N and C inputs in the desert scrub soil constrain microbial activity, resulting in lower N mineralization and higher soil NH_4^+ demand. This is reflected in a lower soil NH_4^+ concentration and lower N concentration in the root biomass in the desert scrub. The low C availability in this site activates microorganisms with autotrophic metabolism that use NH_4^+ as an energy source, releasing NO_3^- into the soil (Jones et al., 2004). This process is reflected in greater positive values of net nitrification and a high soil NO_3^- concentration. The results of the stepwise correlation analysis showed a negative correlation between DOC and net nitrification rate, which agrees with the results mentioned above. The nutrient dynamics in the desert scrub indicate an open nutrient cycle that can increase N loss by emissions of N oxides and nitrate leaching from the soil, as reported previously in other ecosystems (Klubek et al., 1978; Paul and Clark, 1989).

It is interesting that nitrogen fixation by cyanobacteria is rare in the desert scrub compared with the grassland, since we have no sequences of this photosynthetic bacteria in our libraries while the anoxigenic phototroph Chloroflexi was abundant in both sites and both seasons. In other arid systems, cyanobacteria are the dominant phototrophs in desert crusts and are associated with the higher C and N inputs into the system during the short pulses of rain (Gundlapally and Garcia-Pichel, 2006). In our ecosystem, cyanobacteria are rare (1.2%) while some sulfur bacteria groups of Proteobacteria, such as gammaproteobacteria and Chloroflexi, a non-sulfur phototroph, are more abundant in the grassland (17, 5% and 1%, respectively). The presence of gammaproteobacteria in the CCB grasslands-gypsisols systems with high concentrations of sulfur (López-Lozano et al., 2012), could be due not only to the capacity of this group to use sulfide as an electron donor, but also to the sharp decrease of available O_2 in microbial mats, a condition that can also explain the presence of Chloroflexi, a group of photosynthetic-anoxigenic non sulfur bacteria in the same soils. Their presence in the grassland soils suggests that these communities have a key role in both C and N soil dynamics, akin to the one they have in the microbial mats abundant in the wetlands of CCB (Peimbert et al., 2012). These groups have been isolated in microbial mats from a variety of extreme habitats (Tank et al., 2009), and both are known to perform anoxygenic photosynthesis using bacteriochlorophyll as a light-harvesting pigment (Proctor, 1997).

Our comparison of seasonal data indicate that microbial immobilization and N mineralization were higher in the summer samples than in the winter samples, as we expected, as microbial richness also diminished in winter. In contrast, the DOC concentrations were higher in the winter samples than in the summer samples, because the microbial activity is depleted in the winter due to the low temperatures. These results support the findings in other studies of arid ecosystems insofar as temperature and moisture have been found to promote soil microbial activity, mainly among heterotrophic groups (Austin et al., 2004; Burke, 1989; O'Brien, 1978).

Our results indicated different soil N transformations between the two vegetation-soil systems. The grassland soils favored N dynamics in a closed cycle. In contrast, N can be lost easily from the soil through an open cycle in the desert scrub soil. These differences were mainly associated with differences in SOC input to the soil between both sites. Additionally, it is evident that soil N transformations between different vegetation-soil systems in desert ecosystems is linked with soil bacterial composition, but the large amount of phylogenetic and physiological diversity of each bacterial phyla (Fierer et al., 2007) makes it difficult to understand clearly the relationships between soil nutrients transformation and bacterial community. Even so, we know that a more nuanced description of the bacterial community is needed to better understand this relationship and establish better hypothesis.

Acknowledgments

This work was financed by the National Autonomous University of Mexico (PAPIIT-DGAPA-UNAM grant: Análisis de la vulnerabilidad de la dinámica de nutrientes en un ecosistema árido de México, IN204013) and Fundación Carlos Slim. Constitutes a partial fulfillment of the Graduate Program in Biological Sciences of the National Autonomous University of México (UNAM). Y. Tapia-Torres acknowledges the scholarship and financial support provided by the National Council of Science and Technology (CONACyT, scholarship number 210603), and UNAM. We are grateful to two anonymous reviewers, Antonio Gonzalez Rodríguez, Christopher Geldis and Elizabeth Cash for comments on the manuscript. To Rodrigo Velázquez-Durán for assisting with soil sampling and chemical analysis, and to CONANP for permission to collect soil.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaridenv.2014.11.007.

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Capítulo IV

Relationship between soil P fraction and microbial biomass in an oligotrophic grassland-desert scrub system

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**** Publicado en: Ecological Research, 29: 463-472, 2014***

Ecol Res (2014) 29: 463–472
DOI 10.1007/s11284-014-1138-1

ORIGINAL ARTICLE

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Relationship between soil P fractions and microbial biomass in an oligotrophic grassland-desert scrub system

Received: 8 August 2013 / Accepted: 1 March 2014 / Published online: 26 March 2014

Abstract Phosphorus (P) is an essential element of the biosphere, both as a constituent of living organisms and as a regulator of biological processes. The Cuatro Ciénegas Basin in the central Chihuahuan Desert of Mexico is characterized by extreme P oligotrophy. The aim of this study was to quantify P distribution in soil P fractions, P sorption capacity, and P in microbial biomass in a desert scrub and grassland soil system in the Churince area of the Cuatro Ciénegas Basin over summer and winter seasons. Our objective, as part of an exploration of ecosystem functioning, was to ascertain the relationship between soil P fractions and P in microbial biomass. Our results demonstrate a scarcity of P, mainly in grassland, and also a higher P sorption capacity in grassland soil than in desert scrub. Desert scrub soil retained more P (228 ± 5 μg g^{-1} dry soil) than grassland soil (87 ± 10 μg g^{-1} dry soil), mainly in inorganic forms, but grassland soil retained more P in accessible organic forms. We suggest that biotic controls regulated by access to water shape the dynamics of soil P availability in the Churince grassland-desert scrub system.

Keywords P soil fractions · Microbial P · Chihuahuan Desert · Cuatro Ciénegas Basin · P sorption capacity

Electronic supplementary material The online version of this article (doi:10.1007/s11284-014-1138-1) contains supplementary material, which is available to authorized users.

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Introduction

Phosphorus (P) is an essential element for all living cells and is also one of the most limiting factors. It is therefore important to understand the distribution, availability, and retention patterns of this element in soils of different habitats. P in soil solution has several possible destinations: it may be acquired by plants and soil microbiota, or be adsorbed in different fractions and at different degrees of sequestration by soil minerals and organic compounds. Chemical soil conditions are critical for the occlusion of soluble P, as this element may eventually be bound, especially in alkaline soils, by calcium and magnesium ions (Froelich 1988; Lathja and Schlesinger 1988; Cross and Schlesinger 2001; Buckingham et al. 2010). According to the Walker and Syers (1976) model, a decrease in available soil P takes place over time along with a relative increase in organic P forms, especially when an excessively drained soil is included in the model. Furthermore, at an advanced stage in soil development, only organic forms of P and occluded P remain dominant, such that P availability then becomes dependent upon the activity of soil microorganisms and their ability to solubilize and mineralize inorganic and organic soil P fractions (Walker and Syers 1976; Stewart and Tiessen 1987; Tiessen et al. 1984; Crews et al. 1995; Richardson et al. 1994). Similarly, arbuscular mycorrhizal fungi play a critical role in plant P uptake, mainly under conditions of P limitation (Jeffries et al. 2003). The role of soil microbiota is important in the dynamics of bioavailable P, not only for their ability to access P adsorbed and occluded in the soil but also because biomass itself constitutes a reservoir of immobilized but potentially bioavailable P (Richardson et al. 1994). The relationship between the distribution of soil P and P in the biomass is important for understanding the cycling of this element in soil. Knowledge of these relationships in oligotrophic conditions could therefore contribute to understanding ecosystem functioning under limited soil P conditions.

The Churince system within the Cuatro Ciénegas Basin (CCB) features soil derived from Jurassic marine sediments. This soil is rich in calcium and sulfates but very poor in total P (McKee et al. 1990). Under these conditions, large reservoirs of P would not be expected in the primary minerals (McKee et al. 1990), and thus P has been scarce from the very outset. This natural scarcity is further exacerbated by the advanced age of the Churince site (~35 million years old; Lehmann et al. 1999).

The aim of this study was to quantify P distribution in different soil fractions and to evaluate soil P retention capacity by estimating P sorption capacity during both summer and winter in two habitats (grassland and desert scrub) within the CCB Churince system. Near-zero concentrations of dissolved inorganic P have been recorded in grassland (López-Lozano et al. 2012) and in the aquatic system of the valley (ca. 0.60 μM total dissolved P; Elser et al. 2005; Souza et al. 2006). Given the existence of organisms in grassland and desert scrub habitats of the Churince system that require P to complete their metabolism (e.g., plants and soil microorganisms), we expected that dominant forms of P (organic and inorganic) would be occluded, as suggested by the Walker and Syers (1976) model, and that the P sorption capacity of soil in both habitats would be very high because of the soil chemical characteristics. We further expected that C and P levels in microbial biomass would be positively correlated with dominant P fractions, thus serving as an indicator of mechanisms in the microbiota that enable access to P sequestrated by soil minerals.

Materials and methods

Description of the studied grassland-desert scrub system

The study was carried out in grassland-desert scrub of the central Chihuahuan Desert (26°50′41″ N and 102°8′11″ W). The study sites were located in the CCB Churince system, which is part of the Cuatro Ciénegas Flora and Fauna Protection Area in Coahuila, Mexico administered by the federal agency "Comisión Nacional de Áreas Naturales Protegidas" (CONANP). The valley occupies an area of 30 × 40 km, with an elevation of 740 m a.s.l. on the valley floor; it experiences a mean annual temperature of 21.4 °C and a mean annual rainfall (data from 1971 to 2000) of 246 mm. The hottest months are in summer (June–September), coinciding with the highest rainfall values. Winter rains also occur occasionally (García 1988; Martínez 2008; López-Lozano et al. 2012). Primarily Cretaceous carbonate rocks surround this ~35-million-year-old valley, with regional basement rocks composed of Triassic granodiorites (Lehmann et al. 1999). The mountains, which reach over 3,000 m a.s.l., are of marine sedimentary origin. A complex system of faults covers both the mountains and valley (McKee et al. 1990). Palynological records of the regional vegetation show that the grassland and desert scrub habitats at the bottom of the valley have remained largely unchanged for at least 30,000 years (Minckley and Jackson 2007).

Grassland and desert scrub are the two most common vegetation types in the basin (Pinkava 1974; Rzedowski 2006). On the flat basin floor, lagoons, pools, and rivers are surrounded by grassland, while the foothills are characterized by desert scrub vegetation (see Supplementary Material). The grassland is subjected to moderate grazing in the summer months. Dunes associated with the study area consist of more than 95 % pure gypsum (Minckley and Cole 1968). The soil type of the grassland is Gypsisol, featuring accumulations of gypsum, while the desert scrub soil is Leptosol (IUSS Working Group WRB 2007). To reduce the influence of factors unrelated to the natural establishment of the two vegetation types, we chose two adjacent sites in the Churince system on the west flank of the Sierra de San Marcos. The grassland site was located on gypsum in the valley basin, away from the influence of San Marcos ridge sedimentation. The desert scrub sample site was located on a foot slope of the Sierra de San Marcos, an area influenced by the sediment of the limestone-dominated ridge. Both sites were derived from the same parent material (gypsum from the Jurassic), had the same slope orientation, and had high soil concentrations of clays and sulfates (McKee et al. 1990). General characteristics of soil and vegetation for both sites are given in Table 1.

Soil sampling

The soils at both sites were sampled concurrently in August 2007 (summer) and February 2008 (winter). At each site, ten plots were randomly established within 50 × 100-m areas that presented the same characteristics (slope, sunlight, and topography) in each habitat and season. The total selected area occupied ca. 30 % (3.5 km^2) of the Churince area. Specific sampling sites for each plot were different from one season to the next, although they were consistently located in the same general area. In each plot, a composite soil sample was derived from five separate locations (four 2 × 2-m corners and one central point). Soil samples were taken from the top 15 cm of mineral soil with a soil core sampler (5-cm diameter), placed in black plastic bags, and stored at 4 °C until laboratory analysis.

Soil P fractionation

A modified Hedley sequential extraction procedure (Hedley et al. 1982) for soil P fractioning was used to obtain separate soil organic and inorganic P fractions from fresh soil samples (see also Tiessen and Moir 1993; Cross and Schlesinger 1995; Levy and Schlesinger 1999). To prevent overestimation of Ca-bound P in calcareous soil, a modification of the Hedley procedure—prewashing of soil with a buffered NaCl-EDTA solution—was carried

Table 1 Soil and vegetation characteristics of the Churince system of the Cuatro Ciénegas Basin, Coahuila, in the central Chihuahuan Desert of Mexico

Variable	Grassland	Desert scrub
Gravimetric soil moisture content (%)[a,*]	29 ± 2	2 ± 1
Soil pH[a,*]	8.6 ± 0.1	9.5 ± 0.1
Aboveground biomass (g m^{-2})[b]	493 ± 61	395 ± 33
Total root biomass (g m^{-2})[b,*]	752 ± 170	224 ± 77
Root C concentration (mg g^{-1})[b]	293 ± 7	306 ± 6
Root P concentration (mg g^{-1})[b,*]	0.51 ± 0.05	0.38 ± 0.03
Soil total N (mg g^{-1})[a,*]	0.84 ± 0.1	0.50 ± 0.01
Soil organic C (mg g^{-1})[a,*]	13 ± 1	10 ± 2
Dominant plant species[c]	*Sporobolus airoides, Allenrolfea occidentalis*	*Jatropha dioica, Larrea tridentata, Opuntia leptocaulis, Cordia parviflora*

Mean ± SE (n = 10 plots) of soil variables in the first 15 cm of soil and dominant plant species are given
* Differ significantly between habitats at $p < 0.05$
[a]Modified from Tapia-Torres (2010)
[b]García-Oliva et al., unpublished
[c]Nomenclature according Pinkava (1974)

out following Perrot (1992) and Lathja et al. (1999). This sequential extraction procedure has been previously demonstrated to be a valid technique for extraction of P in alkaline soils (Selmants and Hart 2010).

Five soil inorganic P fractions were determined: soluble P (water-extracted), bicarbonate-extracted P (P*i*-Bica), NaOH-extracted P (P*i*-NaOH), HCl-extracted P (P*i*-HCl), and residual P (extracted with H_2SO_4 + H_2O_2). Three soil organic P fractions were determined: P*o*-Bica, P*o*-NaOH, and P*o*-HCl. Field soil samples (1 g fresh weight) were placed in 70-cm^3 centrifuge tubes. After addition of 30 ml of deionized water, the tubes were shaken for 1 h and then centrifuged at 3,500 rpm for 20 min at ambient temperature. The supernatant was filtered through Whatman 42 filter paper to obtain soluble P. Soil remaining in the tubes was used to determine the next fraction. After addition of 30 ml of 0.5 M $NaCO_3$ (pH 8.5), the mixture was shaken for 16 h and then centrifuged at 3,500 rpm for 30 min at ambient temperature. The supernatant was again filtered through Whatman 42 filter paper, and the resulting solution was allowed to rest for 12 h. The solution was acidified with 0.55 ml of 5 M HCl and then neutralized with 5 M NaOH and 5 M HCl to obtain P*i*-Bica.

P*o*-Bica was estimated as total P-Bica minus the inorganic fraction (P*i*-Bica). To obtain total P in solution, a solution aliquot was acid digested with 50 % ammonium persulfate ($[NH_4]_2S_2O_8$) and 0.113 ml of 11 N H_2SO_4, and then neutralized with 5 M NaOH and 5 M HCl. Using different extraction solutions, we determined the other sequential fractions from the soil remaining in the tubes. An identical sequential extraction procedure using 0.1 M NaOH as an extractant was followed to obtain P*i*-NaOH and P*o*-NaOH fractions. Extractions with 1 M HCl were used to obtain P*i*-HCl and P*o*-HCl fractions, and H_2SO_4 + H_2O_2 was used to obtain residual P.

The procedure for obtaining P*o*-NaOH and P*o*-HCl fractions was the same as for the P*o*-Bica fraction, except that 10 M NaOH was used as a neutralizer in the final step. As recommended by Perrot (1992) and Lathja et al. (1999) for calcareous soils, soil pre-washing with a buffered NaCl-EDTA solution was performed before NaOH-P extraction. Total organic P (TP*o*) and total inorganic P (TP*i*) were calculated as the sum of organic P fractions and inorganic P fractions, respectively. All orthophosphate-P determinations were performed by the ascorbic acid reduction-molybdate colorimetric method (Murphy and Riley 1962) using a Braun + Luebbe Autoanalyzer III (Norderstedt, Germany). The Autoanalyzer had a minimum P detection limit of 0.04 μg ml^{-1}.

Bioavailable P corresponded to soluble P (extracted with water) plus bicarbonate extracts (Cross and Schlesinger 1995).

Non-biological P fixation (soil P sorption capacity)

Estimation of P fixation by the non-biological soil fraction was carried out using the procedure proposed by Lathja et al. (1999). This method entailed the addition of 30 ml of a known concentration of P to aliquots of fresh soil (3 g), followed by shaking in polypropylene tubes for 24 h at a constant temperature (ca. 25 °C). After shaking, samples were centrifuged at 3,500 rpm for 30 min. Known concentrations of P (working solutions) were obtained from a stock solution of 4.39 g KH_2PO_4 in 1,000 ml of deionized water containing a few drops of chloroform. The following working solutions were used: 10, 50, 100, and 150 μg ml^{-1} P in 0.01 M KCl. For analysis, each concentration of working solution was applied to aliquots of six soil samples randomly chosen from the ten plot samples collected per vegetation type and season. Sorbed P (X_s) for each concentration of working solution and season was then estimated using the formula $X_s = (s - c) \times F$, where s is the concentration of P (in μg ml^{-1}) in the original working solution, c is the concentration of P (μg ml^{-1}) in the solution at equilibrium, and F is the working solution volume (ml) divided by the mass (g) of dry soil used (Lathja et al.

1999). To allow P concentrations to be corrected for soil sample moisture content, a 100-g subsample was oven-dried at 75 °C to constant weight using the gravimetric method. The concentration of P per ml of solution at equilibrium was measured as orthophosphate-P. Sample groups for each working solution concentration and season were run simultaneously to avoid possible systematic errors associated with the operation of the Braun + Luebbe autoanalyzer.

Carbon and P in microbial biomass

Concentration of C in microbial biomass was determined using the chloroform fumigation-extraction method (Vance et al. 1987). This procedure consisted of weighing 20-g samples of fresh soil in duplicate to have both fumigated and unfumigated samples. Fumigation was performed with 80 ml of chloroform. Both fumigated and unfumigated samples were subsequently incubated at 25 °C for 24 h at a constant relative humidity. Microbial C was extracted from both sample types using 80 ml of 0.5 M K_2SO_4, with samples then filtered through Whatman 42 filters (Brookes et al. 1985). Total and inorganic C was determined for both extracts using a coulometric carbon analyzer (UIC model CM5012; Chicago, IL, USA). Organic C was calculated as the difference between total and inorganic C. Microbial C was calculated by subtracting the organic C extracted from unfumigated samples from that extracted from fumigated samples, and then dividing this figure by a recovery value of 0.45 (Joergensen 1996).

To estimate P concentration in microbial biomass, we applied the method developed by Hedley and Stewart (1982). This procedure was followed by the fumigation-extraction technique of Anderson and Domsch (1978), in which the estimate of P in the microbial biomass was based on the labile P fraction in soil recovered by extraction using 0.5 M $NaCO_3$ 0.5 M at pH 8.5 (total P-Bica). We then performed the chloroform fumigation-extraction technique (Anderson and Domsch 1978; Cole et al. 1978) with a recovery factor of 0.4 (Lathja et al. 1999; Hedley and Stewart 1982). Microbial P samples were determined colorimetrically in a Braun + Luebbe Autoanalyzer III.

Statistical analysis

Distributions of total organic P (TP*o*) and total inorganic P (TP*i*) in grassland and desert scrub habitats were statistically evaluated using the *t* test. Seasonal variations in soil P fraction variables, soil P sorption capacity, and C and P in microbial biomass were examined by split-plot ANOVA (Montgomery 1991) with habitat ($n = 10$, grassland and desert scrub) as the main plot factor and season ($n = 2$, summer and winter) as the subplot factor. Box-Cox transformations were performed to normalize data where necessary (Box and Cox 1964), and mean values and standard errors of the variables were reported. Relationships between major P fractions (available P [= soluble P + P*i*-Bica], P*o*-HCl, TP_o, and TP_i) and microbial variables (C and P in microbial biomass) were analyzed with ANCOVA models. In each ANCOVA model, C and P in microbial biomass were used as continuous predictor variables, habitat was used as a categorical predictor variable with two levels (grassland and desert scrub), and soil P fractions, TP_o, and TP_i were the response variables. In each case, four parameters were fitted (two intercepts and two slopes, one for each habitat). All analyses were conducted using the R statistical program (R Development Core Team 2007). In all cases, an α value of 0.05 was used.

Results

Distribution of total organic and inorganic P

The *t* tests showed significant differences between habitats in terms of total organic P (TP*o*, $t = 3.6$, $p = 0.001$) and total inorganic P (TP*i*, $t = 17.9$, $p < 0.0001$) concentration. Concentration of TP*o* was 2.5 times higher in basin grassland than in desert scrub (43.3 ± 5 vs. 17.3 ± 5 μg P g^{-1} dry soil, respectively). Inorganic P was five times higher in desert scrub than in basin grassland (211.1 ± 8 μg P g^{-1} vs. 44.0 ± 5 μg P g^{-1} dry soil). In basin grassland, TP*o* and TP*i* represented 49.6 and 50.4 %, respectively, of the total extracted P (Table 2). In contrast, TP*o* and TP_i in desert scrub constituted 7.5 and 92.5 %, respectively, of the total P (Table 2). Desert scrub was characterized by a higher total P than was basin grassland (228.3 ± 10 vs. 87.3 ± 5 μg P g^{-1} dry soil) (Table 2).

Soil P fractions

In both habitats, the fractions that produced the greatest P concentrations were those extracted with HCl. These fractions mainly reflected inorganic forms of P (Table 2). P distribution patterns in soils of the two habitats were as follows. Bioavailable fractions (P soluble + P*i*-Bica) were greater in desert scrub than in basin grassland (26.4 ± 1 vs. 1.9 ± 0.3 μg P g^{-1} dry soil). Unavailable organic forms (P*o*-NaOH + P*o*-HCl) were greater in basin grassland than in desert scrub (40.7 ± 5 vs. 17.0 ± 5 μg P g^{-1} dry soil), whereas unavailable inorganic forms (P*i*-NaOH + P*i*-HCl) were greater in desert scrub than in basin grassland (184.8 ± 8 vs. 42.1 ± 5 μg P g^{-1} dry soil) (Table 2).

Fixation capacity of P by the non-biological portion of soil (P sorption capacity)

Regardless of season, basin grassland soil fixed more P than that of desert scrub, as a higher amount of P was

Table 2 Distribution of soil P in different fractions of organic and inorganic P for each of two habitats in a basin grassland-desert scrub system in the Churince area of Cuatro Ciénegas Basin, Coahuila, in the central Chihuahuan Desert of Mexico ($n = 20$)

P-extract	Grassland		Desert scrub	
	Mean (μg g^{-1}) (total %)	±SE	Mean (μg g^{-1}) (total %)	±SE
Soluble P***	0.0 (0 %)	0.0	8.9 (3.9 %)	0.5
P*i*-Bica***	1.9 (2.2 %)	0.3	17.4 (7.6 %)	1.0
P*i*-bioavailable***	1.9 (2.2 %)	0.3	26.4 (11.5 %)	1.2
P*o*-Bica	1.5 (1.7 %)	0.3	2.1 (0.9 %)	0.6
P*o*-bioavailable	1.5 (1.7 %)	0.3	2.1 (0.9 %)	0.6
P*i*-NaOH	2.2 (2.5 %)	0.4	2.9 (1.3 %)	0.6
P*o*-NaOH*	3.7 (4.2 %)	0.9	1.2 (0.5 %)	0.3
P*i*-HCl***	38.5 (44.1 %)	5.3	179.3 (78.5 %)	7.4
P*o*-HCl***	38.5 (44.1 %)	4.7	14.0 (6.1 %)	5.2
P-residual*	1.4 (1.6 %)	0.3	2.5 (1.1 %)	0.4
P*i*-unavailable***	42.1 (48.2 %)	4.6	184.8 (80.9 %)	7.8
P*o*-unavailable**	40.7 (47.9 %)	4.7	17.0 (7.4 %)	5.1
Total-P*i****	44.0 (50.4 %)	4.8	211.1 (92.5 %)	7.9
Total-P*o****	43.3 (49.6 %)	4.9	17.3 (7.5 %)	5.3
Total P***	87.3 (100 %)	10.0	228.3 (100 %)	5.0

Units are μg g dry $soil^{-1}$
Po organic phosphorus fraction, *Pi* inorganic phosphorus fraction, *Bica* bicarbonate, *SE* standard error
* Means with significant differences between habitats at $p < 0.05$
** Significant differences at $p < 0.001$
*** Significant differences at $p < 0.0001$

adsorbed by the soil in the first habitat (Table 3; Fig. 1a, b). The ability of soil minerals to fix P increases with the addition of P.

Carbon and P in microbial biomass

Carbon in microbial biomass showed no significant habitat-related differences ($p = 0.6$; Table 4). C in microbial biomass exhibited a seasonal effect ($p < 0.01$), with higher concentrations observed during summer than in winter (merged habitat data: 769 ± 53 vs. 604 ± 36 μg C g^{-1} dry soil). Phosphorus in microbial biomass (Pmic) showed significant habitat-related differences ($p = 0.003$; Table 4), being higher in desert scrub than in basin grassland (merged seasonal data: 6.9 ± 1.3 vs. 3.9 ± 1.6 μg P g^{-1} dry soil). No significant differences in P were observed between seasons (Tables 4, 5).

Relationship between P fractions and microbial C and P

ANCOVA modeling revealed a significant relationship between P in microbial biomass and some fractions and forms of soil P (Fig. 2a–c). Concentration of bioavailable P (soluble P + P*i*-Bica) was positively correlated with P in the microbial biomass of desert scrub, but not with that of basin grassland (ANCOVA model: $F_{3,36} = 234.9$, $p < 0.0001$, $R^2 = 0.9$; Fig. 2a). The P*o*-HCl fraction showed a negative relationship in the case of desert scrub, but no significant correlation for grassland soil (ANCOVA model: $F_{3,36} = 6.8$, $p < 0.001$, $R^2 = 0.4$; Fig. 2b). In grassland, the concentration of P in the microbial biomass accounted for 5.8 and 12.2 % of total soil organic P in summer and winter, respectively. In desert scrub, however, these values increased to 50.9 and 28.3 %. ANCOVA analysis showed that the concentration of total organic P had a different correlation with P in microbial biomass of each habitat ($F_{3,36} = 6.7$, $p = 0.001$, $r^2 = 0.4$): the relationship was not significant in basin grassland, but was negative in desert scrub (Fig. 2c). In contrast, total inorganic P showed no relationship to P in microbial biomass. Carbon in microbial biomass was not significantly correlated with any particular fraction or form of P.

Discussion

Desert scrub in the CCB had more than twice the concentration of total P found in basin grassland (228 ± 10 vs. 87 ± 5 μg P g^{-1} dry soil). Levels of total P in desert scrub were within the range reported for soils in the northern part of the Chihuahuan Desert (192–338 μg g^{-1}) and for Aridisols in general (Cross and Schlesinger 2001). Total P in basin grassland was extremely low, even lower than that reported for sites with very weathered soils such as Ultisols (200–431 μg g^{-1}; Cross and Schlesinger 1995).

As expected based on the Walker and Syers model (1976), we found that over 80 % of P in either soil was in unavailable or occluded forms. In both basin grassland and desert scrub, the dominant P fraction in the soil was

Table 3 Fisher and probability values from a split-plot analysis of variance, with habitat (grassland and desert scrub) as the main plot factor and season (summer and winter) as the subplot factor, of soil P sorption capacity in a basin grassland-desert scrub system in the Churince area of Cuatro Ciénegas Basin, Coahuila, in the central Chihuahuan Desert of Mexico

P sorption capacity	Factors					
	Habitat		Season		Habitat × Season	
	F	*p*	*F*	*p*	*F*	*p*
10 μg P ml^{-1} water	0.1	0.828	41.5	< 0.001	10.4	0.009
50 μg P ml^{-1} water	81.6	< 0.001	1.3	0.282	0.4	0.544
100 μg P ml^{-1} water	25.0	0.001	1.3	0.277	0.1	0.811
150 μg P ml^{-1} water	129.9	< 0.001	1.0	0.337	11.1	0.008

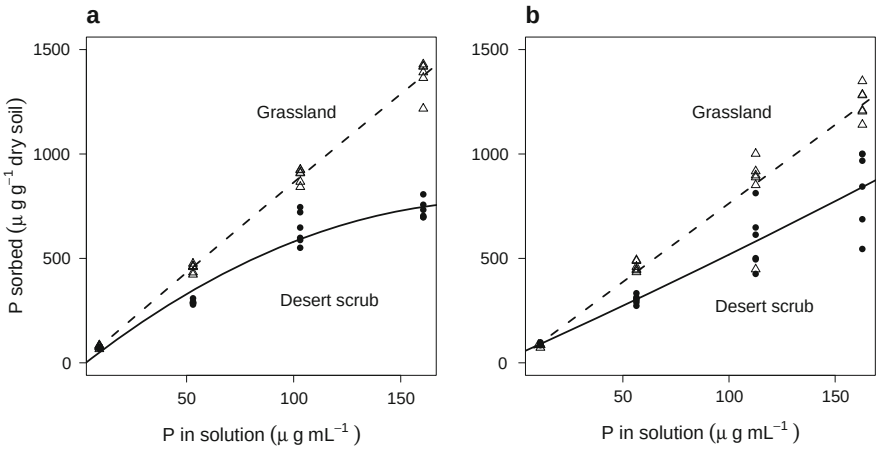

Fig. 1 Soil phosphorus (P) sorption capacity. Plots showing fixation of P by the non-biological portion of soil. *Dashed lines* represent soil P sorption isohyets per habitat (grassland and desert scrub) and season (**a** summer and **b** winter) in the grassland-desert scrub system of the Cuatro Ciénegas Basin, Coahuila, in the central Chihuahuan Desert of Mexico. *Black circles* indicate data pertaining to desert scrub ($n = 20$), and *white triangles* correspond to data from grassland ($n = 20$). *Black lines* represent model predictions of P sequestration by the non-biological portion of soil for each habitat, with artificial available P

P extracted with HCl, suggesting that only a low proportion of P was bioavailable. High concentrations of P in unavailable forms are a common phenomenon in soils similar to those found in the northernmost Chihuahuan Desert sites (Lathja and Schlesinger 1988; Cross and Schlesinger 1995, 2001). The most important consequence of long-term weathering in the Chihuahuan Desert, and in other arid areas in which P is scarce, is that any available and moderately labile P form may eventually be bound by calcium and magnesium ions (Lathja and Schlesinger 1988; Cross and Schlesinger 2001; Buckingham et al. 2010).

Although the most recalcitrant forms of P prevailed at both sites, one important difference was that inorganic forms dominated in desert scrub (92.5 %), whereas in basin grassland, inorganic and organic forms accounted for only 50.4 and 49.6 % of P, respectively. We found that soil P sorption capacity was greater in basin grassland than in desert scrub. This difference can be explained not only by intrinsic soil factors, such as pH and moisture content, but also by higher concentrations of soil organic compounds. In basin grassland, P occlusion in crystals with different ions (Ca and Mg) may occur alongside that of non-crystalline (amorphous) forms favored by the presence of organic compounds. Yuan and Lavkulich (1994) have observed that organic acids produced by biota can inhibit the crystallization of P oxides, allowing the formation of non-crystalline amorphous forms that control soil P sorption capacity. Amorphous minerals tend to adsorb P more rapidly than crystalline material (Ryan et al. 1985). In basin grassland, organic compounds likely control soil P sorption capacity at a relatively fast rate; in contrast, relatively slow linkage with Ca_2^+ ions may be the principal determinant of P sorption capacity in desert scrub, as occurs at other Chihuahuan Desert sites (Cross and Schlesinger 2001).

Although productivity data is lacking, we know that plant root biomass was higher in basin grassland than in scrub (752 vs. 224 g m^{-2}; Table 1), and that basin grassland soil contained more organic C and N than that of desert scrub (Table 1). This difference in root biomass production may be the result of continuous input of underground water in basin grassland arising from springs that are not entirely rainfall dependent (Elser et al. 2005). For this reason, grassland is a relatively productive area with a perennial water source, whereas desert scrub is a more typical rain-pulse driven desert

Table 4 Fisher and probability values from a split-plot analysis of variance, with habitat (grassland and desert scrub) as the main plot factor and season (summer and winter) as the subplot factor, of C and P in microbial biomass of a basin grassland-desert scrub system in the Churince area of Cuatro Ciénegas Basin, Coahuila, in the central Chihuahuan Desert of Mexico

Variable	Factors					
	Habitat		Season		Habitat × Season	
	F	*p*	*F*	*p*	*F*	*p*
Cmic	0.3	0.618	7.6	0.010	1.8	0.187
Pmic	10.8	0.003	0.7	0.398	3.7	0.065

Cmic C in microbial biomass, *Pmic* P in microbial biomass

Table 5 Mean (±1 SE) concentrations of C and P in microbial biomass in a basin grassland-desert scrub system in the Churince area of Cuatro Ciénegas Basin, Coahuila, in the central Chihuahuan Desert of Mexico

Variable	Habitat Season	Grassland Mean ± SE	Desert scrub Mean ± SE
Cmic (μg g^{-1} dry soil)	Summer	$814.0 \pm 98^{a,A}$	$723.0 \pm 42^{a,A}$
	Winter	$566.0 \pm 59^{b,A}$	$642.0 \pm 40^{b,A}$
Pmic (μg g^{-1} dry soil)	Summer	$2.5 \pm 1.1^{a,A}$	$8.8 \pm 1.3^{a,B}$
	Winter	$5.3 \pm 2.0^{a,A}$	$4.9 \pm 1.3^{a,A}$

For each variable, significant habitat and seasonal differences are denoted by *uppercase* and *lowercase letters*, respectively
Cmic C in microbial biomass, *Pmic* P in microbial biomass

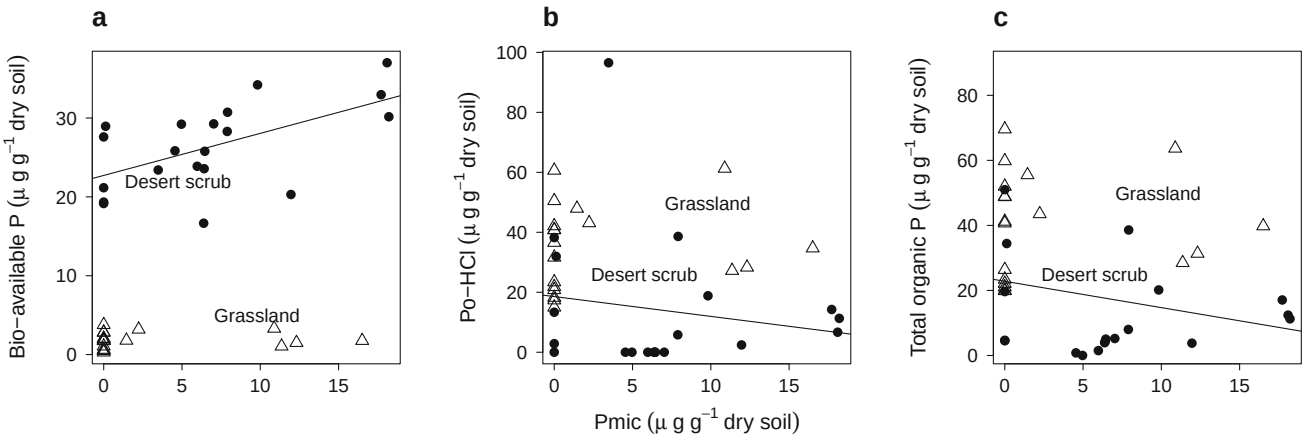

Fig. 2 Plots of analysis of covariance modeling of inter-relationships of dependent variations **a** bioavailable inorganic P (Pi), **b** HCl-extracted organic P (Po-HCl), and **c** total organic P with the P concentration in soil microbial biomass (Pmic; independent variable) in the grassland-desert scrub system of the Cuatro Ciénegas Basin, Coahuila, in the central Chihuahuan Desert of Mexico. Bioavailable Pi = soluble P + bicarbonate-extracted P*i*. *Black circles* indicate data pertaining to desert scrub ($n = 20$), and *white triangles* correspond to data from grassland ($n = 20$). *Black lines* represent model predictions significant at $p < 0.05$ for each habitat

featuring dry soil. The existence of a more active biological P cycle in grassland is plausible, as organic C contributed by its more plentiful vegetation serves as an energy source for the soil biota.

To estimate the contribution of biological processes to the distribution of P in the CCB, we applied the index proposed by Cross and Schlesinger (1995): P*o*-Bica/(soluble P + P*i*-Bica + P*o*-Bica). We found that 44.1 % of biologically available P in basin grassland was the product of biological recycling. In desert scrub, this value was only 8.3 %. Soil microbiota have been found to successfully compete with minerals for sequestration of inorganic P (Olander and Vitousek 2004; Bünemann et al. 2011). The same situation may be true for the plant rhizosphere of the Churince grassland, where the roots were found to have higher mean P concentration than those of desert scrub (0.51 ± 0.04 vs. 0.38 ± 0.03 μg g^{-1} dry root; Table 1). The percentage of biologically available P from the product of biological recycling in the basin grassland is more akin to that of other grasslands that grow on Mollisols (Cross and Schlesinger 2001). In desert scrub, however, values are closer to those of sites featuring Aridosol soils (Lathja and Schlesinger 1988; Cross and Schlesinger 1995), such as

those observed in the Chihuahuan Desert (Lathja and Schlesinger 1988; Cross and Schlesinger 1995, 2001).

Concentrations of C and P in microbial biomass showed pronounced seasonal variations, as has been reported for humid tropical soils (Cleveland et al. 2004). Positive relationships between Pmic and bioavailable P indicate that acquisition of this resource by desert scrub biota is occurring. The organic P fraction includes orthophosphate mono- and diasters (Newman and Tate 1980; Tate and Newman 1982) and phosphonates. Orthophosphates are easily mineralized by microbial phosphatases that are widely distributed in soil (Acosta-Martínez et al. 2008; Mubarak and Langer 2009; Baldrian et al. 2010). Biochemical studies of microorganism phosphonate utilization suggest that these compounds are highly stable as a result of the nature of their C–P bond. Nevertheless, this more recalcitrant organic fraction can be made accessible through the mineralization activities of microbial phosphonatases and C–P lyases (Kononova and Nesmeyanova 2002). These organic P fractions can provide a ready supply of available Pi in this fashion, but soil C availability is also required for mineralization. Basin grassland has been found to possess greater soil-dissolved organic carbon than desert scrub (Tapia-Torres 2010). Another possibility is an accumulation of P in the microbial biomass. Several studies have investigated the activity of polyphosphate-accumulating microorganisms (PAOs) that have the metabolic capacity to remove inorganic P from the environment and to accumulate it within cells in the form of polyphosphates (García et al. 2006; Fukushima et al. 2010). One group of PAO microorganisms, the *Gemmatimonadetes* (Zhang et al. 2003), have been detected through the use of 16S rDNA clone libraries (Tapia-Torres 2010) in both study sites, where they accounted for 19 % of grassland and 17 % of desert scrub microbial communities (Tapia-Torres 2010). These values are higher than those reported by DeBruyn et al. (2011), who found that this bacterial group comprises approximately 2 % of soil bacterial communities. The role of this bacterial group, although still poorly understood, may be similar to that of other microbiota found in these sites, such as Actinobacteria, Proteobacteria, Planctomycetes, Chloroflexi, Bacteroidetes, Acidobacteria, and Firmicutes (Tapia-Torres 2010).

Our results suggest a considerable scarcity of P, especially in grassland, and the probable influence of biotic controls—dictated by access to water—in the dynamics of P availability and retention in the soils of the CCB Churince area. Biological control of P dynamics is a strong possibility. Because P is an essential nutrient for living organisms, severe P limitation could select for higher efficiency of biotic mechanisms in terms of the acquisition, retention, and use of soil P. A reduction of biotic activity under severe P limitation is expected over geological time (Walker and Syers 1976; Selmants and Hart 2010). However, the point at which this biotic activity is reduced under severe P limitation may depend on the mechanisms of acquisition, retention, and use that have evolved in the biota. This point may be extremely low, but cannot be predicted based on current theory.

Acknowledgments We are grateful to Keith MacMillan for English revision, two anonymous reviewers for useful suggestions, Rodrigo Velázquez-Durán for assistance with soil sampling and chemical analysis, and CONANP for permission to collect soil. This study was funded by a CTIC-UNAM postdoctoral scholarship (to YP), by a PAPIIT-DGAPA-UNAM (grant: Análisis de la vulnerabilidad de la dinámica de nutrientes en un ecosistema árido de México, IN204013) and Fundación Carlos Slim. This work constituted a partial fulfillment of requirements of the Graduate Program in Biological Sciences of the National Autonomous University of México (UNAM). YT-T acknowledges a scholarship and financial support provided by the National Council of Science and Technology (CONACyT; scholarship number 210603) and UNAM.

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Capítulo V

Ecoenzymatic stoichiometry at the extremes: How microbes cope in an ultraoligotrophic desert soil

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**** Enviado a: Soil Biology & Biochemistry***

ABSTRACT

Arid ecosystems are characterized by stressful conditions of low energy and nutrient availability for soil microorganisms. It has been observed that the ecoenzymes needed for the transformation of organic compounds into assimilable products show similar scaling relationships in different habitats (logarithmic C:N:P scaling ratios ~1:1:1). In this study in Cuatro Ciénegas Basin (CCB) in the Chihuahuan desert of México, we report the lowest ecoenzymatic activities yet quantified in soil. Nevertheless, activities for both organic N and organic P acquisition enzymes scale with C acquisition with a slope of ~1.0, indicating that the soil microbial communities of this ultra-oligotrophic desert ecosystem follow the global ecoenzymatic stoichiometry patterns. CCB soil microbial communities were co-limited by C and either by N or P but this co-limitation played out differently in different parts of the CCB as indicated by microbial ecoenzymatic shift to allocate more resources to acquire and immobilize the scarcer nutrient. By extending ecoenzymatic analyses to these ultra-oligotrophic soils, our findings support the broad utility of the approach in illuminating how microbes acquire limiting resources in arid ecosystems.

Keywords. Chihuahuan desert, ecological stoichiometry, enzyme, México, microbial community homeostasis, threshold elemental ratio.

1. Introduction

Drylands cover more than one-third of the Earth´s continental surface and thus constitute the most extensive terrestrial biome on the planet (Pointing and Belnap, 2012). Estimates of carbon storage for dryland regions indicate that they contribute 36% of the total carbon storage worldwide (Campbell et al., 2008). A high proportion of these dryland areas is covered by grasslands, which represent an important pool (8%) of global carbon (C) reservoirs (IPCC, 2001). In desert grasslands, the main inputs of soil organic matter (SOM) are from underground biomass rather than from aerial biomass (Sims and Singh, 1978); the former also represents the principal source of soil nitrogen (N) and phosphorus (P). Due to the low water availability of desert ecosystems, SOM decomposition is slower than in more humid settings (i.e. tropical or temperate forest ecosystems; Burke et al., 1998). Thus, arid ecosystems are usually characterized by stressful conditions of low energy and nutrient availability for soil microorganisms (Schimel et al., 2007) and N and P availability often limit primary productivity as well as microbial activity (López-Lozano et al., 2012). Additionally, in soils derived from geologic substrata with low apatite content (as is the case for the soil in our study), both organic P and occluded P are the dominant forms in the soil (Walker and Syers, 1976; Perroni et al., 2014a) but these are relatively unavailable to plants. Therefore, P availability in these soils depends on mineralization of organic P fractions by soil microorganisms (Walker and Syers, 1976; Cross and Schlesinger, 2001), making microbial P limitation especially relevant for soil carbon processing in desert regions.

Most soil organic compounds are transformed or metabolized by microbes (Bradford et al., 2013), mainly by heterotrophic microorganisms that produce extracellular

enzymes (ecoenzymes) that cleave organic molecules to allow C, N, and P assimilation (Waring et al., 2014). Ecoenzyme biosynthesis responds to environmental signals such as low nutrient availability to meet microbial nutrient demands; additionally, ecoenzymes can also enter the soil after cell lysis (Sinsabaugh et al., 2009; Rilling et al., 2007). After the recognition that ecoenzymes are major drivers of C and nutrient cycling in terrestrial, freshwater, and marine ecosystems, several ecoenzymes have been identified as useful indicators of nutrient deficiency and microbial nutrient demand (Burns, 1982; Nannipieri, 1994; Olander and Vitousek, 2000; Schimel and Weintraub, 2003; Renella et al., 2006; Sinsabaugh et al., 2009; Sinsabaugh and Follstad, 2012; Sinsabaugh et al., 2012; Waring et al., 2014). These enzymes are: β-1, 4-glucosidase (BG) and cellobiohydrolase (CBH) as indicators of energy (C) demand; β-1, 4-N-acetylglucosaminidase (NAG) and leucineaminopeptidase (LAP) as indicators of N demand; and acid or alkaline phosphatase (AP), as indicator of P demand (Schimel and Weintraub, 2003). These enzymes catalyze terminal reactions that produce assimilable molecules containing C, N, and P from high molecular weight organic compounds (Sinsabaugh et al., 2009).

Soil microorganisms acclimate to stress by reassigning key resources (i.e. energy, C, N, and P) to acquisition mechanisms rather than growth (Schimel et al., 2007). While it has also been reported that the ratios of C:N:P in microbial biomass are relatively constrained across ecosystems relative to variability in environmental nutrient availability (Cleveland and Liptzin, 2007), these ratios in the soil microbial biomass could nevertheless indicate how allocation shifts alter nutrient demand. For example, higher biomass C:N ratios likely reflect a greater overall investment in C-rich structural cellular material (Paul and Clark, 1996) while lower N:P ratios may reflect the higher allocation to P-rich ribosomes (Elser et

al., 2003). Because ecoenzymatic activities reflect the microbial cell's response to meet its metabolic nutrient demands in response to environmental nutrient availability, ratios of commonly measured ecoenzymatic activities can be used to assess how the microbial community invests in energy relative to multiple nutrient acquisition under *in situ* conditions as it copes with resource limitation.

According to Sinsabaugh et al. (2009), ecoenzyme activity involves an intersection of Ecological Stoichiometry Theory (EST) with the Metabolic Theory of Ecology (MTE), offering promise to improve our understanding of energy and nutrient controls on microbial community metabolism (Sinsabaugh et al., 2012). This intersection can be understood via the Threshold Elemental Ratio (TER), that defines the element ratio at which growth shifts between nutrient limitation (represented by N and P, at high C:N or C:P) and energy (represented by C, lower C:N or C:P; Sterner and Elser, 2002; Frost et al., 2006). Additionally, under EST, organisms can be characterized with respect to their strength of stoichiometric homeostasis, e.g. the degree to which their biomass elemental composition shifts in response to the elemental composition of its diet or environmental resource supplies (Sterner and Elser, 2002). When the stoichiometric composition of the organism does not vary with changes in resource stoichiometry, the organism is considered strictly homeostatic. The growth of such strictly homeostatic organisms is strongly regulated by the most limiting nutrient and such an organism would be expected to respond with shifts in ecoenzymes that contribute to this homeostasis. In contrast, when the stoichiometry of the organism changes proportionately with the stoichiometry of the resource, the organisms are characterized as weakly or non-homeostatic; such adjustments may dampen the immediate

impacts of nutrient limitation on growth but require a capacity for extensive storage (Sterner and Elser, 2002).

Our main objective in this study was to calculate the soil ecoenzymatic stoichiometry and determine its relation with soil energy (organic carbon) and nutrient availability for the soil microbe community in an extremely oligotrophic desert ecosystem with very low soil organic matter content. For that, we measured soil organic nutrients, nutrients within the microbial biomass, soil ecoenzyme activities, and we estimated microbial homeostasis at the community level in two energy-contrasting soils within the Cuatro Ciénegas Basin, México. We sought to quantify soil and microbial C:N:P ratios together with ecoenzymatic activity to determine the roles of energy and nutrient limitation in affecting microbial metabolism under these stressful conditions. Our hypothesis is that under lower soil C availability, the microorganisms invest more energy in nutrient acquisition rather than on increasing their biomass, by producing more ecoenzymes associated with the scarcer nutrient. This mechanism allows the microbial soil community to maintain nutrient homeostasis in soils with constrained energy availability. Our data shed light on the factors controlling carbon and soil nutrient cycling within and across desert ecosystems and extend the range of our current understanding of ecoenzymatic coupling in soil ecosystems.

2. Methods

2.1. Site description and soil sampling

This study was carried out in a grassland soil in the central region of the Chihuahuan Desert in the Cuatro Ciénegas basin (26°50´N and 102°8´W) in Coahuila, Mexico (740 m. a. s.l).

The climate is hot and arid; in spite of an average annual temperature of 21°C, temperatures as high as 45°C have been reported, mainly in July, as well as temperatures below 0°C in January (SMN, CONAGUA, 2013). The mean annual precipitation is 253 mm but this is highly variable among years (Fig. 1). The majority of rainfall occurs mainly in summer. In the western side of the basin, Jurassic-era gypsum is the dominant parent material while in the eastern side Jurassic-era sandstones dominate (McKee et al., 1990). According to the world reference base for soil resources (WRB), the dominant soils are *Gypsisols* and *Calcisols* for the western and eastern sides, respectively. In both parts of the basin the grass *Sporobolus airoides* (Torr.) is the dominant plant species (Perroni et al., 2014b).

A sampling site was selected in each side of the basin: Churince (CH), in the western side; 26° 50.561´N; -102° 08.099´W; and Rancho Pozas Azules reserve (PA) in the eastern side; 26° 49.635´N; -102°01.470´W. Total aboveground biomass was 493 ± 61 and 323 ± 9 g m^{-2}; meanwhile total belowground biomass was 751±170 and 289±23 g m^{-2} for the western and the eastern sides, respectively (Tapia-Torres et al., 2015; Montiel-González unpublished data). Additionally, total C, N and P concentrations were 13.4 ± 1.8 mg g^{-1}; 0.9 ± 0.2 mg g^{-1}; 0.09 ± 0.01 mg g^{-1}, for the western side; and 5.9 ± 0.7 mg g^{-1}; 0.6 ± 0.07 mg g^{-1}; 0.09 ± 0.01 mg g^{-1} for the eastern side (Tapia-Torres et al., 2015; Montiel-González, unpublished data). Soil moisture contents were 13% and 9% for the western and the eastern side, respectively. The fine soil fraction (silt + clay) was 33% and 41% for the western and the eastern side, respectively. At each site, a 100 by 50 m plot was demarcated, which was then divided into 10 sections, separated from each other by 10 m. A random sampling transect was then selected in each section, with 15-cm top deep soil samples taken from ten sampling points (every 5 meters); these were then mixed to form one composite sample. In

total, 10 composite samples were taken at each plot in September of 2010, 2011, and 2012. Previous studies conducted on the study site have reported that soil biological activity increases mainly in the months of August and September because most of the annual precipitation is concentrated during these two months; soil moisture also increases (Tapia-Torres et al., 2015; Perroni et al., 2014a). Soil for biogeochemical and enzymatic activity analyses was stored in black plastic bags and refrigerated at 4°C for laboratory analyses.

2.2. Biogeochemical Analyses

Soil pH was measured in deionized water (1:2 w: v) with a Corning^{TM} digital pH meter. To allow nutrient concentrations and enzymatic activities to be corrected for soil sample moisture content, a 100-g subsample was oven-dried at 75°C to constant weight for soil moisture determination using the gravimetric method.

All C forms were determined with a Total Carbon Analyzer (UIC Mod. CM5012; Chicago, E.U.A), while N and P forms were determined by colorimetric analyses using a Bran-Luebbe Auto Analyzer III (Norderstedt, Germany). Microbial P was determined by colorimetric analyses using a spectrophotometer (Evolution 201, Thermo Scientific Inc.)

Available, dissolved, and microbial nutrient forms were extracted from moist soil samples. Inorganic nitrogen forms (NH_4^+ and NO_3^-) were extracted with 2 M KCl (Robertson et al., 1999) and determined colorimetrically by the phenol-hypochlorite method. Inorganic phosphorus (IP) was extracted with sodium bicarbonate (pH 8.5) and was determined colorimetrically by the molybdate-ascorbic acid method (Murphy and Riley, 1962).

Dissolved nutrients were extracted with deionized water after shaking for 45 min and then filtering through a Millipore 0.42-µm filter (Jones and Willet, 2006). Previous to acid

digestion, one aliquot of the filtrate was used to determine dissolved ammonium (DNH_4^+) and IP in deionized water extract. Total dissolved nitrogen (TDN) was digested by the macro-Kjendahl method. Total dissolved P (TDP) was also acid digested and determined by colorimetry. Total dissolved carbon (TDC) was measured with an Auto Analyzer of carbon (TOC CM 5012) module for liquids (UIC-COULOMETRICS). Inorganic dissolved carbon (IDC) was determined in an acidification module CM5130. Dissolved organic carbon (DOC), nitrogen (DON) and phosphorous (DOP) were calculated as the difference between total dissolved forms and inorganic dissolved forms.

Microbial C (C_{mic}), N (N_{mic}) and P (P_{mic}) concentrations were determined by the chloroform fumigation extraction method (Vance et al., 1987). Fumigated and non-fumigated samples were incubated for 24 h at 25°C and constant moisture. C_{mic} was extracted from fumigated and non-fumigated samples with 0.5 M K_2SO_4, filtered through Whatman No. 42 filters (Brookes et al., 1985). C concentration was measured from each extract as total and inorganic concentration by the method described before. C_{mic} was calculated by subtracting the extracted carbon in non-fumigated samples from that of fumigated samples and dividing it by a K_{EC} value (extractable part of microbial biomass C) of 0.45 (Joergensen, 1996). N_{mic} was extracted with the same procedure used for C_{mic} but the extract was filtered through a Whatman No. 1 paper. The filtrate was acid digested and determined as TN by Macro-Kjeldahl method (Brookes et al., 1985). N_{mic} was calculated as for C_{mic}, but divided by a K_{EN} value (extractable part of microbial biomass N after fumigation) of 0.54 (Joergensen and Muller, 1996). P_{mic} was extracted using $NaCO_3$ 0.5M at pH 8.5. After this, the fumigation-extraction technique involving chloroform was performed (Cole et al., 1978). P_{mic} was calculated as for C_{mic} and N_{mic} and converted using a K_P value (extractable part of

microbial biomass P after fumigation) of 0.4 (Lathja et al., 1999). P_{mic} was determined colorimetrically by the molybdate-ascorbic acid method using an Evolution 201 Thermo Scientific Inc. spectrophotometer (Murphy and Riley, 1962). Finally, C_{mic}, N_{mic} and P_{mic} values were normalized on a dry soil basis.

2.3. Ecoenzyme Activity Analyses

We measured the activities of four ecoenzymes with assay techniques reported by Tabatabai and Bremner (1969); Eivazi and Tabatabai, (1977; 1988) and Verchot and Borelli (2005). The potential activities of β-1, 4-glucosidase (BG), cellobiohydrolase (CBH), β-1, 4-N-acetylglucosaminidase (NAG), and alkaline phosphatase (AP) were quantified colorimetrically using ρ-nitrophenol (ρNP) substrates. For all enzymes, we used 2 g of fresh soil and 30 ml of modified universal buffer (MUB) pH 9 for ecoenzyme extraction. After that, three replicates and one control (sample without substrate) per sample were prepared. Additionally three substrate controls (substrate without sample) were included per assay. We centrifuged the tubes after the incubation period and then 750 µl of supernatant was diluted in 2 ml of deionized water and measured for the absorbance of ρ-nitrophenol (ρNP) at 410 nm on an Evolution 201 Thermo Scientific Inc, spectrophotometer. Ecoenzyme activities were expressed as nanomoles of ρNP formed per gram of soil dry weight per hour (nmol ρNP $[g\ SDW]^{-1}\ h^{-1}$).

2.4. Data analyses

Soil biogeochemistry and ecoenzymatic data were subjected to a repeated measures analysis of variance (RM-ANOVA; Von Ende, 1993). Site (CH and PA) was considered as between-subject factor and year (2010, 2011 and 2012), and their interaction, were

considered as within-subject factors. When RM-ANOVA indicated significant factor effects, mean comparisons were performed with Tukey's multiple comparisons test (Von Ende, 1993). Ecoenzyme activities were normalized to units per μg of available organic carbon (OC) using the DOC data corresponding to each sample. Unlike previous work in which the total organic carbon (TOC) value was used for ecoenzyme activity normalization (Sinsabaugh et al., 2009; Sinsabaugh et al., 2010), we used DOC as we consider it to be a better indicator of soil C availability. However, specifically for the purpose of comparing our results with previously reported global patterns, ecoenzyme activities were also normalized to units per mg total organic carbon (TOC) using observed correlations between DOC and TOC from Cuatro Ciénegas samples.

Data were $\log_e$-transformed prior to regression analysis to conform to the conventions of stoichiometric analyses and to normalize variance (Sterner and Elser, 2002; Sinsabaugh and Follstad, 2012). After that, relationships between ecoenzyme activities were calculated with type II regression using SMATR (SMATR, R Development Core Team, 2007).

2.5. Stoichiometric homeostasis and Threshold Elemental Ratio

We used equation (1) to calculate the degree of community-level microbial C:N and C:P homeostasis (H′) by soil microorganisms (Sterner and Elser, 2002)

$$H' = 1/m \quad (1)$$

In Equation 1, m is the slope of $\log_e$ C:N_R (resources) versus $\log_e$ C:N_B (microbial biomass) or slope of $\log_e$ C:P_R versus $\log_e$ C:P_B scatterplot. H′>>1 represents strong stoichiometric homeostasis, while H′≈1 represents weak or no homeostasis (Sterner and Elser, 2002).

To connect measured ecoenzyme activity with Ecological Stoichiometry Theory (EST) and the Metabolic Theory of Ecology (MTE), we followed Sinsabaugh et al., (2009) to calculate the TER for C:N and C:P using the following equations:

$$TER_{C:N} = ((BG/NAG)B_{C:N})/\ n_o \quad (2)$$

$$TER_{C:P} = ((BG/AP)B_{C:P})/\ p_o \quad (3)$$

where $TER_{C:N}$ and $TER_{C:P}$ are the threshold ratios (dimensionless), BG/NAG is the ecoenzymatic activity ratio for β-1,4-glucosidase and β-1,4-N-acetylglucosaminidase, BG/AP is the ecoenzymatic ratio for β-1, 4-glucosidase and alkaline phosphatase, $B_{C:N}$ and $B_{C:P}$ are the C:N or C:P ratios of the microbial biomass (respectively), and n_0 and p_0 are the dimensionless normalization constants for N and P respectively. These normalization constants p_0 and n_0 are the intercepts in the SMA regressions for $\log_e$(BG) vs $\log_e$(NAG) and $\log_e$(BG) vs $\log_e$(AP) respectively. For a more detailed analysis of the derivation of the equations, see Sinsabaugh et al. (2009).

3. Results

As expected for a desert ecosystem, annual precipitation varied considerably during the study period (2010, 2011 and 2012). The highest annual precipitation was observed in 2010 with 379 mm, following by two dry years (36 mm and 102 mm for 2011 and 2012, respectively; Fig. 1).

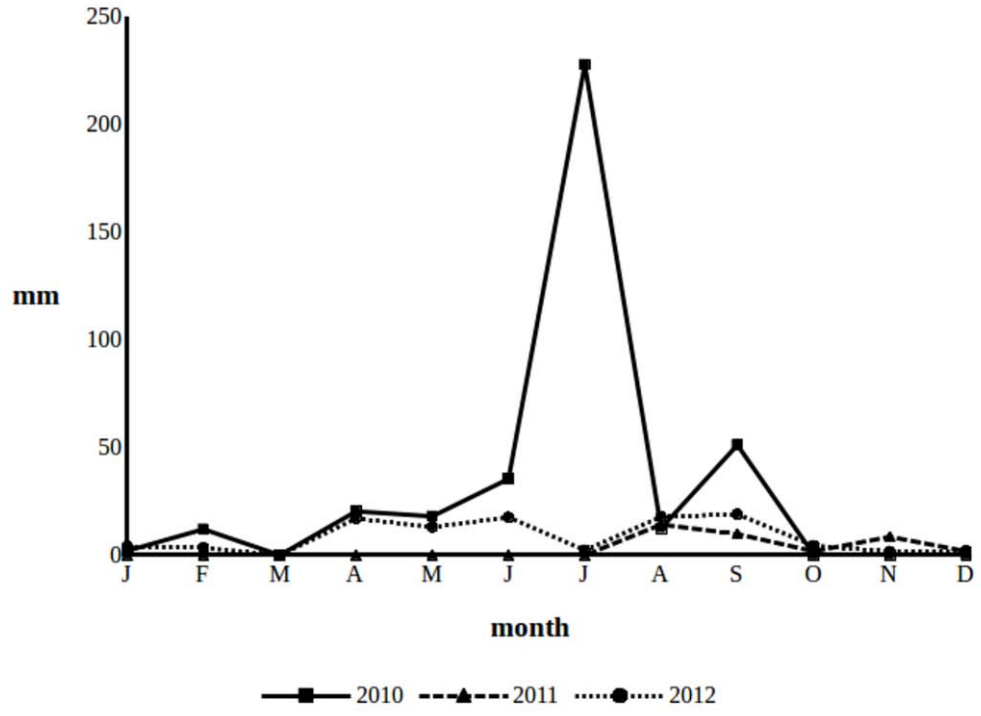

Figure 1. Monthly rainfall for 2010, 2011 and 2012 year at Cuatro Ciénegas Basin.

3.1. Dynamics of soil and microbial nutrients

During all three years, soil samples in the western, Churince end of the basin (CH) always had higher DOC concentrations than in the eastern, Pozas Azules end (PA). Among years, 2010 showed the highest DOC concentration and 2012 showed the lowest concentration (Table 1 and 2). DON concentration also varied among years but did not differ between sites; 2012 had the highest values and the lowest concentrations were observed in 2011 (Table 1 and 2). NH_4^+ concentration was only different between sites in 2010, with CH having a higher concentration than PA (Table 1 and 2). In the rainy year (2010), PA had higher DOP concentration than CH. Meanwhile, in 2011 and 2012 the patterns were inverted: CH showed higher concentration than PA (Table 1 and 2).

Meanwhile, DOC:DON, DOC:DOP, and DON:DOP ratios did not differ significantly among sites and years (Table 1 and 2).

Table 1. Means of soil nutrients and ecoenzyme activities quantified in two grasslands during three consecutive years (2010, 2011, 2012) in the Cuatro Ciénegas Basin, Coahuila, Mexico.

	Year					
	2010		2011		2012	
PARAMETERS	Churince	Pozas Azules	Churince	Pozas Azules	Churince	Pozas Azules
pH	8.5	8.4	8.6	8.6	8.8	8.9
DOC [a]	12.89	5.06	26.92	8.16	31.63	13.43
DON [b]	9.76	9.54	4.34	7.66	12.50	9.34
DOP [c]	1.69 ^{Ba}	2.56 ^{Aa}	2.04 ^{Aa}	1.13 ^{Bb}	2.43 ^{Aa}	2.09 ^{Aa}
Ammonium (NH_4^+)	5.78 ^{Aa}	1.69 ^{Ba}	3.06 ^{Ab}	1.64 ^{Aa}	1.69 ^{Ab}	1.73 ^{Aa}
C mic [d]	382	371	163	108	165	116
N mic [e]	39.73 ^{Aa}	15.36 ^{Ba}	14.33 ^{Ab}	13.8 ^{Ba}	14.34 ^{Ab}	5.88 ^{Bb}
P mic [f]	1.49	3.10	3.4	1.95	3.3	2.65
BG [g]	190 ^{Ba}	330 ^{Aa}	160 ^{Aa}	60 ^{Bb}	30 ^{Ab}	40 ^{Ab}
CBH [h]	140	130	190	200	2	2
NAG [i]	120	118	29	67	23	20
AP [j]	326 ^{Aa}	158 ^{Ba}	137 ^{Ab}	133 ^{Aa}	94 ^{Ab}	38 ^{Aa}
DOC:DON	1.5	1.01	5.9	1.22	4.0	4.1
DOC:DOP	8.6	5.6	14.3	18.7	12.2	9.1
DON:DOP	6.1	3.0	6.2	6.6	4.6	2.7
C:N mic	31	28	9	8	20	24
C:P mic	173 ^{Aa}	74 ^{Ba}	28 ^{Ab}	49 ^{Aa}	44 ^{Ab}	37 ^{Aa}
N:P mic	20.1 ^{Aa}	2.82 ^{Ba}	3.1 ^{Ab}	6.61 ^{Aa}	3.33 ^{Ab}	1.58 ^{Aa}
BG:NAG	1.74 ^{Ab}	2.92 ^{Aa}	10.58 ^{Aa}	0.98 ^{Ba}	1.51 ^{Ab}	3.8 ^{Aa}
NAG:AP	0.42	0.82	0.199	0.519	0.284	0.743
NAG:CBH	2.5	0.68	0.19	0.238	6.59	8.61
BG:AP	0.58 ^{Ba}	2.26 ^{Aa}	1.57 ^{Aa}	0.55 ^{Ab}	0.28 ^{Aa}	1.22 ^{Ab}

[a] Dissolved organic carbon; [b] Dissolved organic nitrogen; [c] Dissolved organic phosphorous, [d] Microbial carbon; [e] Microbial nitrogen; [f] Microbial phosphorus; [g] β-1, 4-Glucosidase; [h] Cellobiohydrolase; [i] β-1, 4-N-acetylglucosaminidase; [j] Alkaline phosphatase. ns= not significant. Values followed horizontally by a different uppercase letter (A and B) indicate that means are significantly different ($P < 0.05$) between sites (Churince and PozasAzules) within sampling year (2010, 2011 and 2012); whereas different lowercase letters (a, b and c) vertically indicate that means are significantly different ($P < 0.05$) among sampling dates within a site. Nutrients and C_{mic}, N_{mic}, P_{mic}, in $\mu g\ g^{-1}$, enzymes in nmol ρ-NP g^{-1} SDW h^{-1}.

For microbial nutrients, during the three years, soil C_{mic} concentrations in CH were always higher than in PA. Among years, highest concentrations occurred in 2010 while 2011 had the lowest concentrations (Table 1 and 2). However, N_{mic} concentration was higher in CH than in PA regardless of year (Table 1 and 2). $C:P_{mic}$ and $N:P_{mic}$ ratios, both only showed site differences in 2010, when CH had a higher value than PA (Table 1 and 2). In contrast, P_{mic} and $C:N_{mic}$ ratios were not different for any of the comparisons (Table 1 and 2).

Table 2. F-ratios and significant levels of the repeated measures ANOVA for soil variables quantified in two grasslands during three years (2010, 2011 and 2012) in Cuatro Ciénegas Basin, Coahuila Mexico.

PARAMETERS	**Source of variation**		
	Between subject	Within subjects	
	SITE	**DATE**	**DATE X SITE**
pH	0.1 (0.7)^{ns}	65 (< 0.000001)	2.3 (0.11)^{ns}
DOC [a]	19.89 (0.0003)	7.85 (0.001)	1.58 (0.229)^{ns}
DON [b]	0.29 (0.59)^{ns}	5.97 (0.006)	3.27 (0.051)^{ns}
DOP [c]	0.14 (0.70)^{ns}	2.7 (0.07)^{ns}	4.2 (0.02)
Amonium (NH_4^+)	17.5 (0.0005)	13.7 (0.00003)	13.9 (0.00003)
C mic [d]	4.73 (0.04)	52.9 (< 0.00001)	0.4 (0.67) ^{ns}
N mic [e]	14.9 (0.001)	13.19 (0.00005)	5.82 (0.006)
P mic [f]	0.12 (0.72) ^{ns}	0.57 (0.52) ^{ns}	3.11(0.056) ^{ns}
BG [g]	2.02 (0.17) ^{ns}	68.6 (< 0.00001)	17.8 (< 0.00001)
CBH [h]	0.03 (0.85) ^{ns}	83.8 (< 0.00001)	0.1 (0.89) ^{ns}
NAG [i]	1.68 (0.21) ^{ns}	50 (< 0.00001)	2.79 (0.07) ^{ns}
AP [j]	12.27 (0.002)	16.7 (< 0.00001)	3.79 (0.03)
DOC:DON	3.8 (0.08) ^{ns}	3.4 (0.053) ^{ns}	2.6 (0.1) ^{ns}
DOC:DOP	0.014 (0.90) ^{ns}	1.98 (0.16) ^{ns}	0.41 (0.66) ^{ns}
DON:DOP	0.79 (0.39) ^{ns}	2.14 (0.13) ^{ns}	0.93 (0.040) ^{ns}
C:N mic	0.8 (0.38) ^{ns}	1.6 (0.21) ^{ns}	0.7 (0.48) ^{ns}
C:P mic	7.21 (0.04)	24.67 (0.001)	10.16 (0.003)
N:P mic	49.3 (0.002)	14.78 (0.002)	19.8 (0.0007)
BG:NAG	4.1 (0.057) ^{ns}	3.7 (0.03)	11.1 (0.0001)
NAG:AP	8.31 (0.012)	1.32 (0.28) ^{ns}	0.09 (0.911) ^{ns}
NAG:CBH	0.003 (0.95) ^{ns}	7.36 (0.01)	0.43 (0.65) ^{ns}
BG:AP	4 (0.06) ^{ns}	1.95 (0.16) ^{ns}	8.29 (0.0016)

[a] Dissolved organic carbon; [b] Dissolved organic nitrogen; [c] Dissolved organic phosphorous, [d] Microbial carbon; [e] Microbial nitrogen; [f] Microbial phosphorus; [g] β-1, 4-Glucosidase; [h] Cellobiohydrolase; [i]β-1, 4-N-acetylglucosaminidase; [j] Alkaline phosphatase; ns= not significant.

3.2. Ecoenzymatic stoichiometry

NAG activity differed significantly among years but not between sites, decreasing over time; 2010 showed the highest activity and 2012 the lowest activity. In the same way, CBH differed significantly among years but not between sites; 2011 showed the highest activity followed by 2010 and the lowest activity was observed in 2012. In the rainy year (2010), PA had higher BG activity than CH but in 2011 the pattern was reversed, as CH showed higher activity. Meanwhile, in 2012 BG activity did not show any difference between sites (Table 1 and 2). Similarly, the rainy year also had higher AP activity than the other two years (2011 and 2012). CH had higher AP activity than PA but only in 2010 (Table 1 and 2).

CBH:NAG ratio was highest in 2010 relative to the other two years but the sites did not differ for this ratio (Table 1 and 2). However, CH had higher BG:NAG ratio than PA but only in 2011. Samples from PA had higher BG:AP ratio than those from CH, again only in 2010 (Table 1 and 2). On the other hand, PA showed higher NAG:AP ratio than CH during all three sampling years (Table 1 and 2).

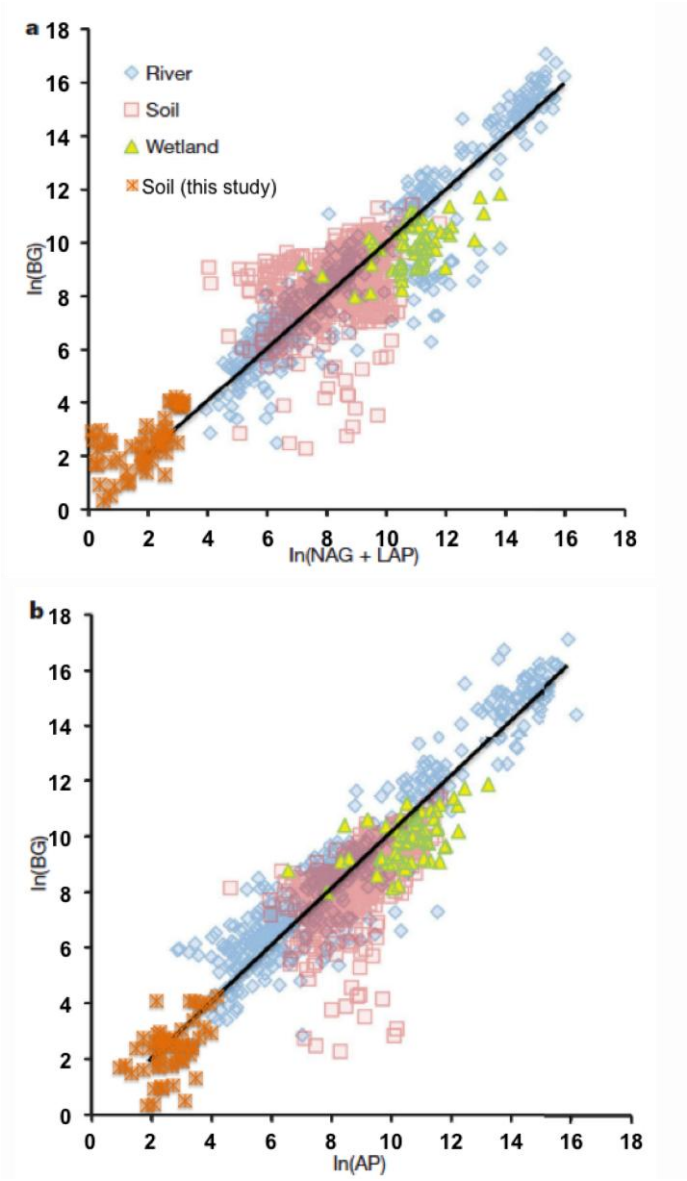

Figure 2. Global patterns of organic nitrogen acquisition activity and organic phosphorus acquisition activity in relation to carbon acquisition. The figure of Sinsabaugh et al. (2009) with data for terrestrial (red squares) and wetland (green triangles) soils and river (blue circles) sediments was modified and our data for Cuatro Cienegas soil were superimposed (orange stars). N acquisition for river, soil and wetland soils and sediments was measured by the potential activities of β-1,4-N-acetylglucosaminidase (NAG) and leucine aminopeptidase (LAP) (Sinsabaugh et al. 2009). In this study, N acquisition was measured by the potential activity of β-1,4-N-acetylglucosaminidase (NAG) (a); P acquisition was measured as phosphatase (AP) activity (b); β-1,4-glucosidase (BG) represented C acquisition. Enzyme activities for Cuatro Ciénegas (CH and PA) soil represented the lowest data yet quantified; however, they followed the same global ecoenzymatic stoichiometry patterns.

For most of the model II regressions analyzed, the results showed differences between sites (CH vs PA) in 2010, as PA had steeper slopes than CH for ln(BG) vs ln(NAG) and ln(CBH) vs ln(NAG). Meanwhile, CH had a steeper slope for ln(NAG) vs ln(AP) than PA (Table S1). In contrast, there were no differences in regression slopes between sites in 2011. Meanwhile, in 2012, a difference in slope was observed between sites only for ln(CBH) vs ln(AP), as PA showed a steeper slope than CH (Table S1). Additionally, when all the data were analyzed together, the ecoenzymatic stoichiometry of these soils for organic N and organic P acquisition both scale with C acquisition with a slope of ~1 that follows the global ecoenzymatic stoichiometry pattern but in a range that is one order of magnitude lower than previous observations (Fig. 2; Sinsabaugh *et al*. 2009).

3.3. Stoichiometric Homeostasis and Threshold Elemental Ratios

To test for the strength of stoichiometric homeostasis, we analyzed for associations between microbial biomass elemental ratios and those in soil resources. When all the data were analyzed together by site, the relationships between log $C:N_R$ and log $C:N_B$, and between log $C:P_R$ and log $C:P_B$ were not different from zero ($p > 0.05$), indicating strong community-level elemental homeostasis in both sites (CH and PA) regardless of year (Fig. 3).

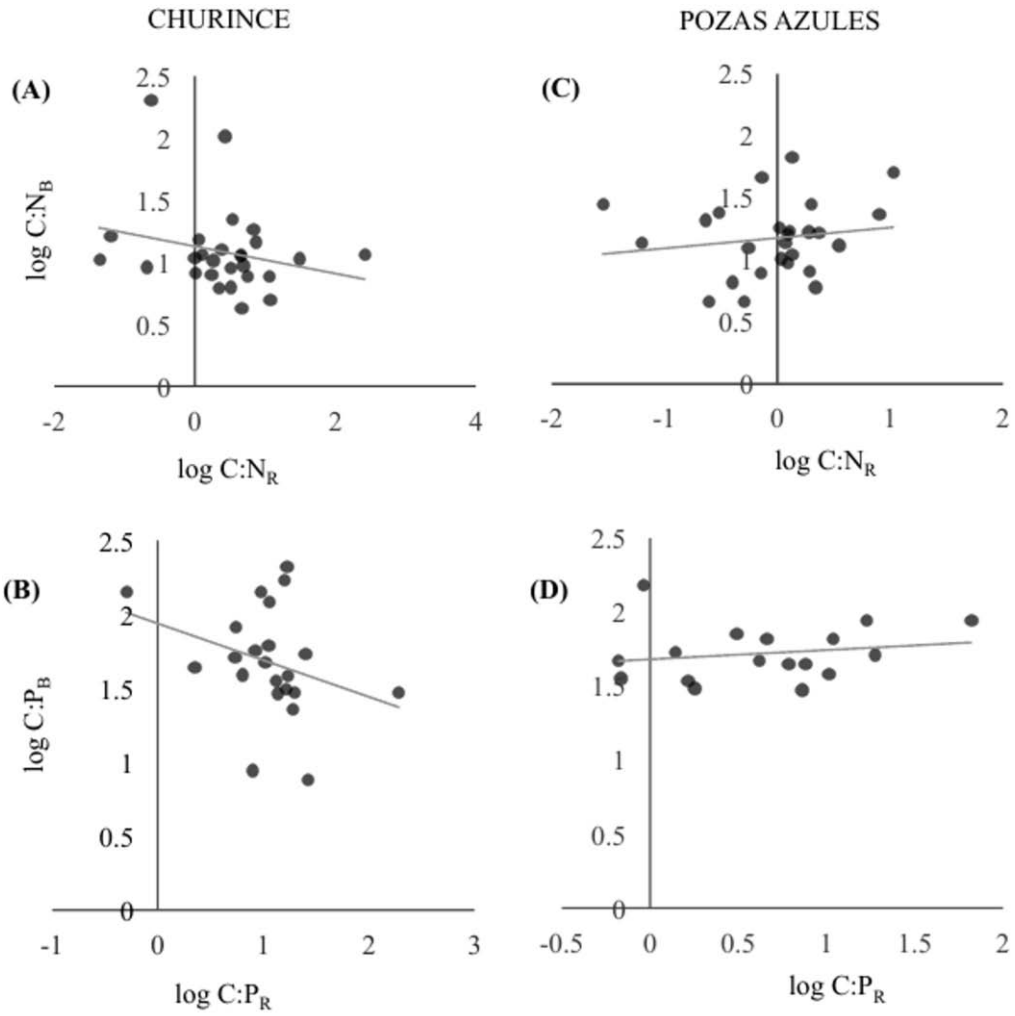

Figure 3. Soil microbial community homeostasis related with nitrogen (N; panels on top) and phosphorus (P; panels on down) acquisition. A and B) Data from Churince (western side of the valley), C and D) Data from Pozas Azules (eastern side of the valley). Indicating similar patterns of soil microbial community stoichiometry among sites in all cases "strong homeostasis" (slopes are not different to zero: A) p=0.154, B) p=0.47, C) p=0.22, D) p=0.44). The equations for each panel, is as follow: A) y= -0.1097x + 1.1272, R2= 0.06. B) y= -0.2482x + 1.9382, R2= 0.10. C) y= 0.0839x + 1.1767, R2= 0.024. D) y= 0.0626x + 1.6809, R2= 0.03

Based on the microbial C:N:P stoichiometric values and parameters generated from the enzymatic data, estimated $TER_{C:N}$ values in PA were higher than in CH in 2010 and 2012 although no differences between sites were observed in 2011 (Fig. 4A). We observed the opposite pattern for $TER_{C:P,}$ as $TER_{C:P}$ in CH exceeded that for PA but only in 2010; there were no statistically significant differences in 2011 and 2012 although in both of these years $TER_{C:P}$ values were higher in CH than in PA (Fig. 4B).

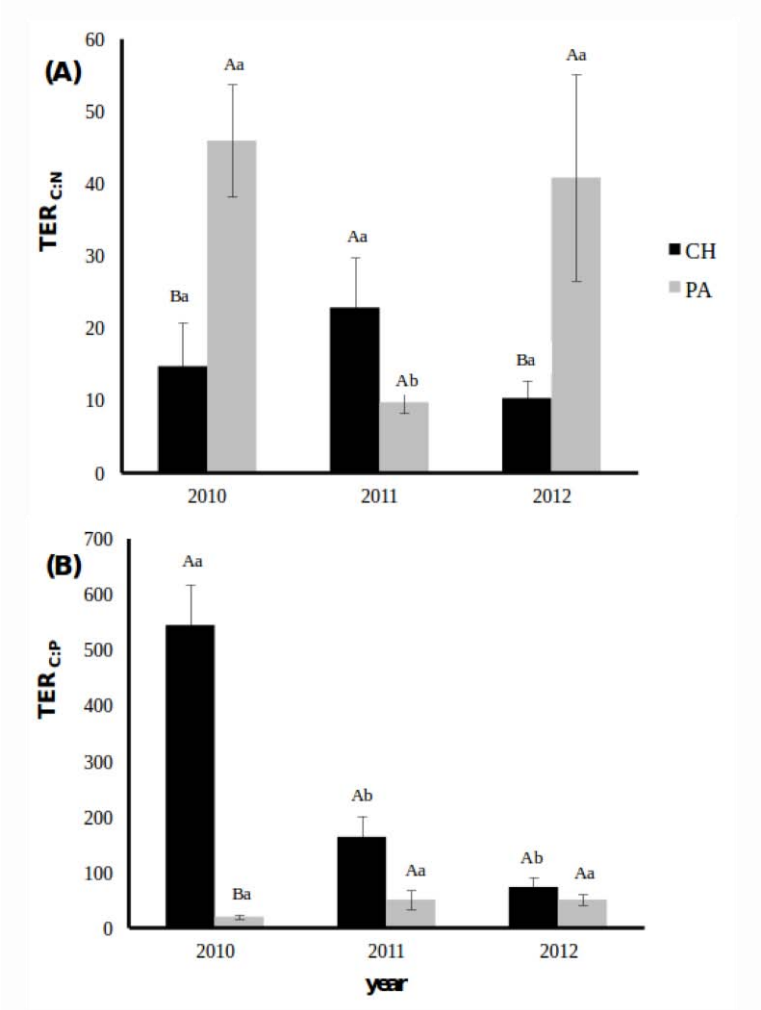

Figure 4. Threshold Elemental Ratio (TER)C:N and (TER)C:P (A and B, respectively) of soil microbial community during three consecutive years (2010, 2011 and 2012). Different

uppercase letter (A and B) indicate that means are significantly different ($P < 0.05$) between sites (Churince and Pozas Azules) within sampling year (2010, 2011 and 2012); whereas different lowercase letters (a, b and c) vertically indicate that means are significantly different ($P < 0.05$) among sampling dates within a site.

4. Discussion

The soil ecoenzymatic activities reported in this study (Fig. 2) encompass the lowest enzyme levels yet quantified in soil (Acosta-Martinez et al., 2003; Bastida et al. 2006; Sinsabaugh et al., 2008; Abdalla and Langer, 2009; Hortal et al. 2013), reflecting the aridity and ultraoligotrophic nature of the site (Elser et al., 2005). Intriguingly, the ecoenzymatic stoichiometry of these soils nevertheless follows the global ecoenzymatic stoichiometry patterns (Fig. 2). While we found differences in the slopes of the ecoenzymatic stoichiometry regressions between sites when the results were analyzed by sampling year (Table S1), the model II regression results indicated no differences between sites when all the data for the three years were analyzed together by site (Table S2). Previous studies have shown that the slopes of ecoenzymatic regressions can differ significantly by habitat (e. g. soil vs. lotic sediment vs. lentic sediment) (Sinsabaugh et al., 2009; 2012; Sinsabaugh and Follstad, 2012). However, within these habitats, C acquisition enzymes have similar scaling relationships with N and P acquisition enzymes. The N and P ecoenzyme scaling coefficient values for soils have been reported as 1.09 and 1.16 (Sinsabaugh et al., 2009); our results indicated very similar values ranging from 0.96-1.19 and 1.12-1.49, respectively. While our N acquisition values are similar to those previously reported by Sinsabaugh et al. (2009) for data dominated by temperate soils, our P acquisition values are

more similar to those reported in more weathered tropical soil (1.18; Waring et al., 2014) and consistent with the previously documented strong P limitation of the Cuatro Ciénegas ecosystem. Nevertheless, despite the low overall soil ecoenzymatic activity in this arid ecosystem, activities for organic N acquisition and organic P acquisition both scaled with C acquisition with a slope of ~1.0 (Fig. 2), indicating that the soil microbial communities exhibit similar patterns of allocation to nutrient acquisition despite the diverse community composition and conditions exhibited by the two regions of the ultra-oligotrophic Cuatro Ciénegas valley.

4.1. Microbial nutrient limitation in desert soils

Consistent with known effects of water availability on nutrient cycling in arid and semiarid ecosystems (Schimel and Parton, 1986), we observed differences in nutrient dynamics among years in concert with high interannual variation in moisture input. Due to the effects of Hurricane Alex (June 25 to July 2nd 2010), 2010 was the wettest year in the last 40 years (379 mm), followed by the two driest years in the same period (36 and 102 mm respectively; Figure 1). The low rainfall in both dry years (2011-2012) appeared to favor nutrient limitation of overall ecosystem productivity, as reflected in decreased available soil and microbial nutrient concentrations, as well as in lower absolute levels of ecoenzymatic activity (Table 1). On the other hand, increases in soil nutrient transformations and microbial activity in the year with higher water availability (2010) allowed us to observe more clearly different forms of nutrient limitation on microbial activity. Thus, in the following, we focus on soil microbial nutrient status and enzymatic allocation patterns during this highly productive year.

Despite conditions of strong nutrient and water limitation, CCB soil microbial communities adjust physiologically to process low N and P resources, an inference supported by the soil community homeostasis analyses, in which the relationships between log $C:N_R$ and log $C:N_B$, and between log $C:P_R$ and log $C:P_B$ in both regions of the valley had slopes not significantly different from zero (Fig. 3). Theses physiological adjustments can be reflected in the degree of ecoenzyme expression. Generally, ecoenzyme expression is related to the quality of available organic matter and nutrient demands of the microbial biomass (Sinsabaugh et al., 2009; Sinsabaugh and Follstad, 2012). Based on ecoenzyme stoichiometry, the microbes of the two edaphically contrasting regions of the Cuatro Ciénegas valley exhibited different patterns of nutrient limitation in 2010. Soil microbes in the western side of the Cuatro Ciénegas valley exhibited lower BG:NAG ratios (Table 1 and 2) as well as lower $B_{C:N}/R_{C:N}$ ratios (where $B_{C:N}$ is C:N ratio of microbial biomass and $R_{C:N}$ is the C:N ratio of labile organic matter; Sinsabaugh et al., 2009; Sinsabaugh and Follstad, 2012), indicative of N limitation of the soil microbial community. This interpretation of a greater role of N limitation in CH is consistent with the shallower slope for ecoenzymatic regressions in Churince than Pozas Azules for ln(BG) vs ln(NAG) (0.85 and 1.14 respectively for CH and PA) and ln(CBH) vs ln(NAG) (0.67 and 1.17 respectively; Table S1), indicative of disproportionate allocation to N-acquisition enzymes relative to energy / C enzymes in Churince. These site-specific differences in C / N enzyme coupling in the two lobes of the CCB valley point to the importance of characterizing spatial variation in soil enzyme patterns, especially where edaphic conditions influence the geochemical composition of the soils.

A converse pattern was observed for P limitation. While previous studies of P concentrations in the Cuatro Ciénegas valley have shown no differences in soil total P concentration (TP) between the western and eastern sides (Perroni et al., 2014a), P availability may nevertheless differ due to the high reactivity of PO_4^-, as P may be strongly bound by calcium and magnesium ions in alkaline soils such as those at Cuatro Ciénegas (Perroni et al., 2014a). For example, near-zero concentrations of dissolved inorganic P (the most available P fraction) have been recorded for grassland soil at Cuatro Ciénegas (López-Lozano et al., 2012; Perroni et al., 2014a), as well as very low concentrations in various aquatic systems in the valley (ca. 0.60 μM total dissolved P; Elser et al., 2005). Therefore, soil P likely represents a critical nutrient constraint within this valley but its role in limiting microbial activity may vary in different sites in the valley. The lower $B_{C:P}/R_{C:P}$ ratio (where $B_{C:P}$ is C:P ratio of microbial biomass and $R_{C:P}$ is the C:P ratio of labile organic matter) in the eastern side (Pozas Azules) of the Cuatro Ciénegas valley is consistent with enhanced P limitation in PA. Therefore soil microbes of Pozas Azules should need to invest disproportionately in P acquisition. Thus, the higher AP activity in the site with lower apparent P limitation (Churince) was unexpected (Table 1). This might be explained by differences in the substrates targeted by different phosphatase enzymes. Among the variety of phosphatase enzyme classes, phosphomonoesterases (PM; often called acid or alkaline phosphatases, AP) that mineralize P from inositol phosphates, nucleotides, phosphoproteins, and sugar phosphates (Turner et al., 2005) are the most frequently evaluated. However, phosphodiesterases (PD), that mineralize P from nucleic acids, phospholipids and other diester phosphates (Turner et al., 2002), are also relevant in sites with low P concentrations (such as Cuatro Ciénegas valley). Phosphate diesters are the main input of organic P (P_O) to soils, but typically constitute only a small fraction of soil P_O

(Turrion et al., 2010). In contrast to AP activity, the PD activity was significantly higher in Pozas Azules than in Churince (252 ±16 and 162 ±19 nmol ρNP [g SDW]$^{-1}$ h^{-1} respectively, $p<0.05$; Tapia-Torres unpublished data). These ecoenzyme activity patterns between sites suggest different preferences and capacities of the microbial community to mineralize diverse phosphorus substrates and therefore suggest different microbial strategies for P acquisition between the western and eastern sides of the valley.

We next combined our ecoenzymatic data and elemental composition data to estimated microbial TER values to better understand microbial metabolic limitation at the community level in this arid and ultra-oligotrophic site. Notably, we found pronounced site-specific contrasts. For example, estimated $TER_{C:N}$ was lower in the western side (Churince) than in eastern side (Pozas Azules) of the Cuatro Ciénegas valley, while $TER_{C:P}$ had the opposite pattern (higher in the western side than in eastern side of the valley; Fig. 4). The lower $TER_{C:N}$ and $TER_{C:P}$ observed in the sites with N or P limitation, respectively, likely reflect shifts in the soil microbial community that modulate their sensitivity to nutrient limitation. If the C:N or C:P ratio of the organic matter being consumed is greater than the TER for that element, it suggests nutrient limitation (Sterner and Elser, 2002). In the eastern side (Pozas Azules) of the valley, Montiel-González (unpublished data) observed a total organic C:P ratio of 64.7 ± 5, this value is significantly greater ($p<0.05$) than the $TER_{C:P}$ estimated in this work (19.4 ± 3.4, Fig. 4), which supports the idea of stronger microbial P limitation in the eastern side of the Cuatro Ciénegas valley, reflecting the effects of edaphic substrata.

Overall, our work helps in understanding of how resources are allocated to enzymatic activity by the microbial community depending on soil resource availability.

However, the nutrient limitation patterns at the community level are not yet clear. Liebig's Law of the Minimum, which states that only one element limits the growth of organisms at any given time, is widely used in ecology (Danger et al., 2008) but its applicability is not always clear for highly diverse microbial communities (as is the case for the microbial community in CCB, López-Lozano et al., 2012). Indeed, it has been observed in microbial communities that some organisms can adjust their effort to collect various nutrients by allocating more resources to scarce, and less effort to abundant, nutrients (Bloom et al., 1985). Therefore, at a community scale, the concept of the minimum has been replaced by the broader view that C, N and/or P co-limit community production by differentially impacting different members of the community (Sinsabaugh et al., 2010). Our data are consistent with an interpretation that the soil microbial communities of this ultra-oligotrophic desert ecosystem are co-limited by C and either by N (Churince, western side of the valley) or by P (Pozas Azules, eastern side of the valley). This C limitation is observed in a high BG/DOC ratio, indicating a high production of enzyme per unit of available carbon in both sites. Our data also suggest that members of these microbial communities allocated more for acquiring the scarcer nutrient, consistent with a variety of emerging views about how energy and material flows are coupled in soil ecosystems (Danger et al., 2008).

4.2 Conclusions

The soil ecoenzymatic activities reported in this study encompass the lowest enzyme levels yet quantified in soil. However, activities for organic N acquisition and organic P acquisition both scaled with C acquisition with a slope of ~1.0, indicating that the soil microbial communities exhibit similar patterns of allocation to nutrient acquisition despite

contrasting edaphic conditions and community composition in different regions of the Cuatro Cienegas valley. We also showed that the soil microbial communities of this ultra-oligotrophic desert ecosystem were co-limited by C and either by N (Churince, western side of the valley) or by P (Pozas Azules, eastern side of the valley). Nevertheless, our results indicated strong community-level elemental homeostasis in both sites (CH and PA) that is must be maintained by differential investment in enzymes according to the scarcer nutrient. Our findings support the broad generality of the ecoenzymatic approach in microbial resource ecology and illustrate how enzyme responses support balanced resource acquisition by microbes experiencing diverse geochemical and hydrologic conditions.

Acknowledgments

This work was financed by the National Autonomous University of Mexico (PAPIIT DGAPA-UNAM grant: Análisis de la vulnerabilidad de la dinámica de nutrientes en un ecosistema árido de México, IN204013). Constitutes a partial fulfillment of the Graduate Program in Biological Sciences of the National Autonomous University of México (UNAM). Y. Tapia-Torres acknowledges the scholarship and financial support provided by the National Council of Science and Technology (CONACyT, scholarship number 210603), and UNAM. To Gerardo Vázquez-Marrufo, Rodrigo Velázquez-Durán and Victor Rocha for assisting with soil sampling and chemical analysis, to Juan L. Peña-Mondragon for technical help, and to CONANP and Rancho Pozas Azules (PRONATURA) for permission to collect soil. J. Elser acknowledges support from a U. S. National Science Foundation grant (DEB-0950175) and a National Aeronautics and Space Administration Astrobiology grant (NAI5-0018). We thank R.L. Sinsabaugh and two anonymous reviewers for comments on a draft of the manuscript.

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Table S1. Comparison of the intercepts and slopes of ecoenzymatic activity relationships for terrestrial soil of grassland in the Cuatro Ciénegas Basin, Coahuila, Mexico during 3 consecutive years (2010, 2011 and 2012) using standardized major axis (Type II) regression.

Regression		Site	Intercept	95% CI		Slope	95% CI		R^2	p	
				Lower	Upper		Lower	Upper			
2010											
ln(BG) vs ln(AP)	C vs P	CH	-1.802	-3.362	-0.241	1.293	0.951	1.759	0.853	**0.0001**	**a**
		PA	1.72	-0.639	4.095	0.757	0.391	1.466	0.851	**0.025**	**a**
ln(BG) vs ln(NAG)	C vs N	CH	0.823	0.155	1.490	0.850	0.656	1.101	0.897	**3.23 e-5**	**b**
		PA	0.566	-0.113	1.244	1.143	0.965	1.355	0.978	**2.44e-5**	**a**
ln(NAG) vs ln(AP)	N vs P	CH	-3.089	-4.857	-1.322	1.522	1.132	2.048	0.864	**9.93e-5**	**a**
		PA	0.823	-1.507	3.153	0.700	0.348	1.406	0.831	**0.031**	**b**
ln(CBH) vs ln(AP)	C vs P	CH	-0.844	-2.313	0.624	0.984	0.678	1.430	0.817	**0.0008**	**a**
		PA	0.553	-1.346	2.453	0.808	0.484	1.348	0.915	**0.011**	**a**
ln(CBH) vs ln(NAG)	C vs N	CH	1.040	0.020	2.060	0.668	0.418	1.066	0.706	**0.004**	**b**
		PA	-0.469	-1.221	0.284	1.172	0.976	1.408	0.974	**3.63 e-5**	**a**
2011											
ln(BG) vs ln(AP)	C vs P	CH	-0.919	-3.523	1.685	1.700	0.760	3.798	0.0004	0.956	**a**
		PA	-0.436	-1.911	1.038	0.890	0.529	1.497	0.556	**0.013**	**a**
ln(BG) vs ln(NAG)	C vs N	CH	1.568	0.582	2.554	-0.932	-1.963	-0.443	0.0001	0.969	**a**
		PA	-0.193	-1.268	0.882	1.046	0.686	1.594	0.716	**0.002**	**a**
ln(NAG) vs ln(AP)	N vs P	CH	-2.288	-3.786	-0.791	1.261	0.647	2.456	0.356	0.089	**a**
		PA	-0.233	-0.963	0.497	0.851	0.644	1.125	0.880	**6.04e-5**	**a**
ln(CBH) vs ln(AP)	C vs P	CH	0.170	-1.210	1.550	1.123	0.565	2.230	0.312	0.118	**a**
		PA	0.267	-0.973	1.507	1.053	0.721	1.538	0.774	**0.0008**	**a**
ln(CBH) vs ln(NAG)	C vs N	CH	2.074	1.527	2.621	0.677	0.342	1.341	0.181	0.220	**a**
		PA	0.555	-0.522	1.632	1.238	0.863	1.775	0.796	**0.0005**	**a**

Table S1. Continued.

Regression		Site	Intercept	95% CI		Slope	95% CI		R^2	p	
				Lower	Upper		Lower	Upper			
2012											
ln(BG) vs ln(AP)	C vs P	CH	-1.649	-2.088	-1.210	1.287	1.021	1.623	0.918	**1.29e-5**	**a**
		PA	0.167	-0.437	0.771	0.931	0.691	1.255	0.910	**0.0002**	**a**
ln(BG) vs ln(NAG)	C vs N	CH	0.287	-0.413	0.987	1.746	1.005	3.034	0.492	**0.024**	**a**
		PA	0.784	-0.246	1.815	1.210	0.644	2.269	0.434	0.053	**a**
ln(NAG) vs ln(AP)	N vs P	CH	-1.109	-1.757	-0.461	0.737	0.422	1.288	0.481	**0.026**	**a**
		PA	-0.594	-2.012	0.824	0.782	0.372	1.645	0.337	0.131	**a**
ln(CBH) vs ln(AP)	C vs P	CH	7.845	-9.352	25.042	-5.153	-20.113	-1.320	0.015	0.844	**b**
		PA	-1.576	-2.998	-0.153	0.829	0.407	1.691	0.398	0.093	**a**
ln(CBH) vs ln(NAG)	C vs N	CH	-2.007	-3.655	-0.359	2.279	0.703	7.394	0.362	0.282	**a**
		PA	-1.116	-2.311	0.077	1.120	0.537	2.337	0.193	0.236	**a**

Table S2. Comparison of the intercepts and slopes of ecoenzymatic activity relationships by site (Churince and Pozas Azules) for terrestrial soil of grassland in the Cuatro Ciénegas Basin, Coahuila, Mexico, using standardized major axis (Type II) regression.

Regression		Site	Intercept	95% CI		Slope	95% CI		R^2	p
				Lower	Upper		Lower	Upper		
ln(BG) vs ln(AP)	C vs P	CH	-1.127	-1.801	-0.454	1.222	0.991	1.506	0.715	$7.61e^{-9}$
		PA	-0.331	-1.101	0.439	1.065	0.848	1.337	0.744	$1.19e^{-7}$
ln(BG) vs ln(NAG)	C vs N	CH	0.789	0.306	1.271	0.991	0.773	1.269	0.582	$9.61e^{-7}$
		PA	0.351	-0.244	0.947	1.110	0.893	1.378	0.731	$2.65e^{-8}$
ln(NAG) vs ln(AP)	N vs P	CH	-1.959	-2.448	-1.470	1.224	1.051	1.427	0.849	$1.39e^{-12}$
		PA	-0.616	-1.311	0.079	0.963	0.768	1.209	0.746	$1.12e^{-7}$
ln(CBH) vs ln(AP)	C vs P	CH	-2.089	-3.617	-0.561	1.467	1.026	2.098	0.351	0.0028
		PA	-1.564	-2.757	-0.370	1.379	1.052	1.807	0.636	$5.083e^{-6}$
ln(CBH) vs ln(NAG)	C vs N	CH	0.510	-0.356	1.376	1.061	0.740	1.521	0.306	0.005
		PA	-0.893	-1.707	-0.079	1.489	1.194	1.856	0.722	$4.04e^{-8}$

Capítulo VI

Diverse scavenging strategies for phosphorus: Lessons from phosphorus limited bacteria

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**** Para ser enviado a: Environmental Microbiology***

ABSTRACT

Phosphorus (P) is a required element for life, which plays a fundamental role in microbial cell physiology and biochemistry. In oligotrophic ecosystems, microorganisms have acquired many adaptations for optimal growth and survival, especially for P acquisition and metabolism. In these environments, the capacity for use different microbial P sources is an advantage. For example, many microorganisms can synthesize more than one type of eco-enzyme like phosphomonoesterases, phosphodiesterases, phosphonatases or C-P lyases in order to P release from organic compounds. Cuatro Ciénegas (CC), Coahuila, México is considered an oligotrophic ecosystem for their low environmental concentrations of P. The main objective of the present work was to analyze the use of different substrate by soil bacterial communities in order to cope with soil P limitation, this difference can be related with different growth and survival adaptations. We collected soil samples from two sites within CC valley, Pozas Azules (PA) and Churince (CH), and additionally sediments from the Churince stream. The soil and sediment samples were analyzed for biogeochemical variables and for eco-enzymatic activity. We observed that the microbial community of the less oligotrophic site (sediment), do not expend any energy synthesizing phosphonatase. In the laboratory experiment, we found genotypic cluster defined by habitat and within this, we found high variability in strategies for P scavenging, possibly due to high horizontal gene transfer. Eco-enzymes can be "PUBLIC GOODS" and as long as some individuals of the community have these traits, the other members can profit from it.

KEY WORDS. Ester phosphates, phosphonates, 16S rRNA, phosphatases, phosphonatases.

INTRODUCTION

Phosphorus (P) is an essential element for the synthesis of many biomolecules including DNA, RNA and ATP (Huang et al. 2005). However, P is a non-renewable resource that is becoming increasingly scarce and expensive (Cordell et al. 2009), because it cannot be manufactured and it has no substitute not only in plant growth (Johnston, 2000) but also as a building block of life. Additionally, only few countries control the remaining global phosphate reserves including Morocco (70%), Iraq, China, Algeria and Syria (Jasinski, 2012). Therefore, ensuring long-term availability and accessibility to P, mainly for food security, is a global challenge.

In soils, lakes, and oceans, prokaryotic heterotrophs can control the availability of P (Cotner and Biddanda, 2002; van der Heijden et al., 2008), through different P transformations processes (e.g. P solubilization, organic matter depolymerization and P mineralization). P forms in ecosystems include mineral P (e.g. in rocks, soil, and sediment), dissolved and particulate organic P, and dissolved inorganic phosphate (Steenbergh, 2012). The latter (inorganic phosphates, Pi) is the main P source for microorganisms and plants, but its availability in soil, sediments, and water is very low due to its high reactivity (Perroni et al. 2014). Since the primary productivity and growth rate in ecosystems are highly P dependent (Elser et al. 2006), bacteria have evolved different mechanisms for the uptake and storage of Pi in response to nutrient scarcity (Alcaraz et al. 2011; Adams et al. 2008).

It is well known that the different bacterial species have the ability to use P substrates with different oxidation states (e.g. +5, +3; White and Metcalf, 2007). In its most oxidized state (valence +5), P is found as phosphate esters in many biomolecules, including nucleic

acids (DNA, RNA), phospholipids, and phosphoproteins (Martinez et al. 2012). Most phosphate esters are transformed or metabolized by microbes that produce extracellular enzymes (ecoenzymes often called phosphatases) that cleave organic molecules to allow P assimilation, as well as C and N in some cases (Schrimer 1998; Waring*et al.* 2014).

Among the different classes of phosphatase enzyme, phosphomonoesterases (PM, often called acid or alkaline phosphatases) that mineralize P from inositol phosphates, nucleotides, phosphoproteins, and sugar phosphates (Turner *et al.* 2005) are the most frequently evaluated. However, phosphodiesterases (PD) that mineralize P from nucleic acids, phospholipids, and other diester phosphates (Turner *et al.* 2002), are also relevant for microbial P requirements. Among these, phosphate diesters are the main input of organic P (P_O) to soils, but typically constitute only a small fraction of total soil P_O (Turrion *et al.* 2010).

In a more reduced state (valence +3), P is found as organophosphonates. The organophosphonates are a class of organophosphorus molecules of both biogenic and xenobiotic origin that contain at least one direct stable, covalent, carbon to phosphorus (C-P) bond (Blackburn, 1981). In organophosphonates, this carbon to phosphorus bond replaces one of the four carbon-to-oxygen-to-phosphorus bonds of the more common phosphate ester (Wanner and Metcalf 1992). Phosphonates are found largely in more primitive life forms, and occur either as free molecules or, more usually, in peptide, glycan or lipid conjugates such as the 2-aminoethylphosphonic acid (ciliatine) and 2-amino-3-phosphonopropionic acid (phosphonoalanine) components of membrane phosphonolipids (Hilderbrand, 1983).

Four different phosphonate degradative pathways have been reported to be present in various bacterial species: phosphonopyruvate hydrolase pathway, phosphonoacetate hydrolase pathway, phosphonoacetaldehyde hydrolase (phosphonatase) pathway, and carbon-phosphorus lyase (C-P lyase) pathway (White and Metcalf, 2007).

The acquisition of adequate supplies of P is a priority for all living cells (Quinn et al. 2007). Although, microorganisms preferred Pi for growth, under conditions of Pi starvation, as is the case of many oligotrophic (low nutrient content) ecosystems, a number of gene systems are induced, whose products are involved in different ecoenzyme biosynthesis for P molecules transformation (Quinn et al. 2007).

Recent genomics studies have shown, that bacteria have different mechanisms for the uptake (can use alternative phosphorus source) and storage (use polyphosphates as storage compounds) of phosphates (Alcaraz et al. 2011). Also, it has been observed that ecoenzymes represent a "public good" for microbial communities (Folse & Allison, 2012), as they break down the complex molecules (e. g. ester phosphates and phosphonates in the case of P), into simple inorganic molecules (Pi) that microbes can take up. Eventhough, these ecoenzymes are costly to the particular producer, after their release, they benefit all cells in the local community (Hibbing et al. 2010; Cordero et al. 2012) as long as some individuals of the community have the ability of synthesize PM, PD and PN, the non-producing members can profit from it, probably in exchange for other nutrients.

So far, the existence of microorganisms capable of using organophosphonates as phosphorus, carbon and/or nitrogen source for growth has been reported. For instance, *Burkholderia cepacia* has been shown to be capable of mineralizing phosphonoalanine

(Ternan et al. 1998) and *Rhizobium huakuii*, was shown to mineralize the natural antibiotic, phosphonomycin (McGrath et al., 1998). Similarly, Hayes et al. (2000) reported a Gram-negative halophile isolate, most likely *Chromohalobacter marismortui* or *Pseudomonas beijerinckii,* that used phosphonoacetate, 2-aminoethyl-, 3-aminopropyl-, 4-aminobutyl-, methyl- and ethyl- phosphonates as phosphorus source. However, no one has studied the different strategies of bacteria for P acquisition under natural Pi starvation at community level.

Here we report for the first time a vast group of halophile bacteria, isolated from soil and sediment of an ultra-oligotrophic ecosystem, able to obtain phosphorus from substrates with different oxidation states. We expected a common strategy among these halophile bacteria living in P-limited environments for the use of diverse organic P substrates (esters phosphates and phosphonates) via secretion of ecoenzymes, which are used in the mineralization of these P substrates. Therefore, the main objective of the present study is to determine, if the bacterial isolates from an ultra-oligotrofic ecosystem, have different strategies for P acquisition. We believe that to understand how these microorganisms can naturally access to the P content in different substrates, as well as the strategies they use for this, is essential to develope management strategies that allow us to break the high dependence on phosphates fertilizer and thus migrate towards agricultural practices that can potentiate the characteristics of each natural system.

MATERIALS AND METHODS

Study site and sampling

This study was carried out in grassland soil and sediment from the Churince lagoon in the Cuatro Ciénegas basin (CCB; 26°50´N and 102°8´W), in the central region of the Chihuahuan Desert, in Coahuila, México (740 m. a. s. l). The climate is hot and arid; in spite of an average annual temperature of 21°C, temperatures as high as 45°C have been reported, mainly in July, as well as temperatures below 0°C in January (SMN, CONAGUA, 2013). The mean annual precipitation is 253 mm, but this is highly variable among years. The majority of rainfall occurs mainly in the Summer months. In the western side of the basin, Jurassic-era gypsum is the dominant parent material, while in the eastern side Jurassic-era sandstones dominate (McKee *et al.*1990). The dominant soils are *Gypsisols* and *Calcisols* for the western and eastern sides, respectively (WRB, 2007). In both parts of the basin the grass *Sporobolus airoides* (Torr.) Torr. is the dominant plant species (Perroni et al., 2014b).

Three study sites (one for grassland soil sampling and two groups for sediment sampling) were located within the Churince (CH) aquatic system on the western side of the CCB. This system consists of a water spring and two desiccation lagoons that are connected by short shallow rivers (López-Lozano et al. 2012). Additionally, one site (for grassland soil sampling) was located within the natural reserve Pozas Azules (PA) on the eastern side of the CCB. At each soil-sampling site, a 100 by 50 m plot was demarcated, which was then divided into 10 sections, separated from each other by a distance of 10 m. A random sampling transect was selected in each section with 15-cm top deep soil samples taken from ten sampling points (every 5 meters); these were then mixed to form one composite sample.

In total, 10 composite samples were taken at each plot. Soil was stored in black plastic bags and refrigerated at 4°C for laboratory analyses. At each sediment sampling sites, the points were taken from edge of intermediate lagoon and of one small fertilized pool. The distance between each sampling site, was approximately 30 m. The sediment samples were stored in falcon tubes and immediately processed for plating.

Biogeochemical Analyses

To allow nutrient concentrations and enzymatic activities to be corrected for soil sample moisture content, a 100-g subsample was oven-dried at 75°C to constant weight for soil moisture determination using the gravimetric method.

All C forms were determined with a Total Carbon Analyzer (UIC Mod. CM5012; Chicago, E.U.A), while N and P forms were determined by colorimetric analyses using a Bran-Luebbe Auto Analyzer III (Norderstedt, Germany). Microbial P was determined by colorimetric analyses using a spectrophotometer (Evolution 201, Thermo Scientific Inc.)

Dissolved, and microbial nutrient forms were extracted from moist soil samples. Inorganic phosphorus (IP) was extracted with sodium bicarbonate (pH 8.5) and was determined colorimetrically by the molybdate-ascorbic acid method (Murphy and Riley 1962).

Dissolved nutrients were extracted with deionized water after shaking for 45 min and then filtering through a Millipore 0.42-µm filter (Jones and Willet 2006). Previous to acid digestion, one aliquot of the filtrate was used to determine IP in deionized water extract. Total dissolved P (TDP) was also acid digested and determined by colorimetry. Total dissolved carbon (TDC) was measured with an Auto Analyzer of carbon (TOC CM

5012) module for liquids (UIC-COULOMETRICS). Inorganic dissolved carbon (IDC) was determined in an acidification module CM5130. DOC and dissolved organic phosphorous (DOP) were calculated as the difference between total dissolved forms and inorganic dissolved forms.

Microbial C (C_{mic}), N (N_{mic}), and P (P_{mic}) concentrations were determined by the chloroform fumigation extraction method (Vance et al. 1987; Hedley and Stewart 1982). Fumigated and non-fumigated samples were incubated for 24 h at 25°C and constant moisture. C_{mic} was extracted from fumigated and non-fumigated samples with 0.5 M K_2SO_4, filtered through Whatman No. 42 paper (Brookes et al. 1985). Organic C concentration was measured from each extract as total and inorganic concentration by the method described before. C_{mic} was calculated by subtracting the extracted organic carbon in non-fumigated samples from that of fumigated samples and dividing it by a K_{EC} value (extractable part of microbial biomass C) of 0.45 (Joergensen et al. 1996). N_{mic} was extracted with the same procedure used for C_{mic} but the extract was filtered through a Whatman No. 1 paper. The filtrate was acid digested and determined as TN by Macro-Kjeldahl method (Brookes et al. 1985). N_{mic} was calculated similarly as C_{mic}, but divided by a K_{EN} value (extractable part of microbial biomass N after fumigation) of 0.54 (Joergensen and Muller 1996). P_{mic} was extracted using $NaCO_3$ 0.5M at pH 8.5. After this, the fumigation-extraction technique involving chloroform was performed (Anderson and Domsch 1978; Cole et al. 1978). P_{mic} was calculated as for C_{mic} and N_{mic}, but divided by a K_P value (extractable part of microbial biomass P after fumigation) of 0.4 (Lathja et al. 1999; Hedley and Stewart 1982). P_{mic} was determined colorimetrically by the molybdate-ascorbic acid method using spectrophotometer (Evolution 201, Thermo Scientific Inc.; Etchevers et al., 1983). Finally,

the values of C_{mic}, N_{mic} and P_{mic} were corrected to dry soil basis.

Ecoenzyme Activity Analyses

The activities of three ecoenzymes were measured with assay techniques reported by German et al. (2011), Fioretto et al. (2009), and Verchot and Borelli (2005). The potential activity of phosphomonoesterase (PM), and phosphodiesterase (PD) was quantified colorimetrically using ρ-nitrophenol (ρNP) substrates; meanwhile, phosphonatase (PN) activity was determined colorimetrically by the molybdate-ascorbic acid method (Murphy and Riley 1962; Etchevers et al., 1983; Table 1). For all enzymes, we used 2 g of fresh soil and fresh sediment, and 30 ml of modified universal buffer (MUB) pH 9 for ecoenzyme extraction. After that, three replicates and one control (sample without substrate) per sample were prepared. Additionally three substrate controls (substrate without sample) were included per assay. We centrifuged the tubes after the incubation period and then 750 µl of supernatant was diluted in 2 ml of deionized water. For enzymes with substrates linked to ρNP, we measured the absorbance of ρNP at 410 nm on an Evolution 201 spectrophotometer (Thermo Scientific Inc.). Finally, ecoenzyme activities were expressed as nanomoles of ρNP formed per gram of soil dry weight per hour (nmolρNP [g SDW]$^{-1}$ h^{-1}). For PN, we measured the absorbance of IP at 882 nm after acid reduction. Finally PN activity was expressed as nanomoles of IP released per gram of soil dry weight per hour (nmol IP [g SDW]$^{-1}$ h^{-1}).

Isolation of microorganisms

The isolates in the collection were obtained using an inoculum of sediment and soil for each sample from each site (the Churince hydrological system and Pozas Azules). Fresh

samples where added to an eppendorf tube with 800 µl of modified universal buffer (MUB) to achieve a ratio of 1/3 (w/v); the resulting suspension was mixed continuously for 60 min. The resultant suspension was used as an inoculum, and each sample was plated on Petri dishes with modified marine agar medium (Cerritos et al., 2008) and incubated at 37°C for 2 days. Colonies with different morphotypes (i.e. size, shape and color) were selected. Purification was performed by subculturing on the same medium to ensure that the culture was axenic; all isolates were stored at -80°C in marine medium with 15% (w/v) glycerol.

Evaluation of isolates for growth in different phosphorus sources

A total of 1163 isolates were obtained (250 from CH soil, 250 from PA soil, 141 from first group of CH sediment and 512 from second group of CH sediment). All isolates were growth in the following 6 different phosphorus sources (1 mM): 1) potassium phosphate, 2) calcium phosphate, 3) phosphite, 4) 2-aminoetil phosphonic acid, 5) 2-phosphonoacetaldehyde, 6) ADN, and 7) control without P (Table 1). Each isolate were passed on Petri dishes with low phosphate defined agar medium (LPDM) containing (per l L): Tris-base, 6.057 g adjusted to pH 8.0; NH_4NO_3, 0.26 g; $MgSO_4$, 0.48 g; disodium citrate, 1.99 g; $ZnCL_2$, 0.000136 g; NaCl, 5g; $FeCl_3$, 0.27 g, KCl, 0.1 g; $MnCl_2$, 0.2 g; CaCl, 0.4 g; glucose, 9 g; amino acids mixture, 0.93 g; phosphorus source, 1mM. Heat labile substrates (B complex, biotin, nicotinic acid) were filter sterilized and added aseptically after autoclaving.

Isolates were grown at 37 ºC during 72 h in LPDM without P to ensure isolates P starvation. After that, each isolate was passed to LPDM with each one of the 6 different P sources and in a addition to a control plate lacking P, and were grown at 37 ºC during 72 h. For the isolates that grew further test its ability to use such the P source. Only the isolates

with the ability to grow in the medium after the 2nd pass were selected and used for DNA extraction and 16S rRNA identification.

DNA extraction and 16S rRNA gene amplification by PCR

Total genomic DNA was extracted from isolates using the method of phenol-chloroform (Hoffman and Winston, 1987). The 16S rRNA genes were amplified from the DNA templates by PCR with the universal primers 16S 27F (5´AGA GTT TGA TCM TGG CTC AG 3´) and 1492R (5´TAC GGY TAC CTT GTT ACG ACT T 3´) obtained from Sigma (St. Louis MO, USA). The 16S rRNA sequences were qualified with Phred (Ewing et al., 1998), and sequences of at least 500 bp were used for the analysis. The phylogenetic reconstruction was constructed using the Maximum likelihood method in phyML. The DNA sequences were retrieved from the data set and classified using Ribosomal Data base Project (RDP) Classifier tool (Wang et al., 2007). The sequences were clustered as OTU's with Mothur software (Scholoss *et al.,* 2009). The nucleotide sequences are deposited in genbank server with accession numbers of Table S1 in Supporting Information.

We analized the sequences with AdaptML (Hunt et al., 2008) to define ecologically differentiated populations; the sequences were assigned to the Churince soil, Pozas Azules Soil, Sediment and Sediment Fertilized habitats and to identify populations as groups of related strains sharing a common projected habitat.

Statistics analysis

To compare the nutrient concentration and ecoenzyme activity between sites, a one way ANOVA was used (Sokal and Rohlf 1987). Therefore, to compare the ability of isolates to grow in the different P sources, a logistic regression model (LRM) with two factors (site and P sources) was performed by using a generalized linear model with STASTISTICA package (StatSoft, 2000). This model is adequate to analyze data with

binomial distribution (Aitkin et al. 1989).

Table 1. Substrates utilized as sole phosphorus source at a final concentration of 0.1 mM.

Substrate	Abbreviation
Potassium phosphate	PP
Calcium phosphate	CP
2-aminoethyl phosphonic acid	2AEP
2- Phosphonoacetaldehyde	2PA
Phosphite	PI
DNA	DNA
P-free control	PFree

RESULTS

Nutrients and ecoenzymes distribution across sites

Since we wanted to determine, what were the different P utilization stratiges of bacteria from the microbial communities from an ultra-oligotrofic ecosystem, we first measured the soil and sediment free and micobial associated nutrients, as well as the enzymatic activities associated to the different ecosystems. Our results showed that the sediment samples are characterized by higher concentrations of DOC, DON, DOP than the samples from soil. Microbial N (N_{mic}) is also higher in the sediment samples. Additionally, $C:P_{mic}$ and $N:P_{mic}$ ratios were higher in sediment samples (Table 2). Meanwhile, soil samples showed higher microbial P (P_{mic}) concentration, DOC:DON, DOC:DOP ratio and $C:N_{mic}$ ratio (Table 2).

Table 2. Nutrients (means and standard error) quantified in grassland soil and sediment in the Cuatro Ciénegas Basin, Coahuila, Mexico.

	SOIL		SEDIMENT	
PARAMETERS	**Churince**	**Pozas Azules**	**Churince**	**Fertilized**
DOC[a]	172 (9.6)	99 (5.4)	333 (160)	342
DON[b]	10.39 (1.4)	5.59 (0.3)	50.52 (27.8)	70.24
DOP[c]	1.54 (0.32)	0.89 (0.11)	6.68 (0.25)	8.35
C_{mic}[d]	211 (52.23)	135 (16.7)	184 (23.7)	134
N_{mic}[e]	15.48 (3.6)	11.05 (2.6)	23.77 (3.4)	22.54
P_{mic}[f]	4.021 (0.03)	7.769 (0.03)	2.864 (0.5)	2.731
DOC:DON	16.55	17.74	6.59	4.86
DOC:DOP	111.5	110.5	49.82	40.93
DON:DOP	6.73	6.23	7.56	8.42
$C:N_{mic}$	13.26	12.19	7.75	5.95
$C:P_{mic}$	52.37	17.34	64.38	49.13
$N:P_{mic}$	3.85	1.42	8.29	8.25

[a]Dissolved organic carbon; [b] Dissolved organic nitrogen; [c] Dissolved organic phosphorous, [d] Microbial carbon; [e] Microbial nitrogen; [f] Microbial phosphorus. Nutrients in μg g^{-1}.

We next evaluated whether there was actualy enzymatic expression of different ecoenzymes known to be involved in phosphorus scavenging. The presence of these activities would on the one hand reveal the importance of such activity for the microbial communiy and the type of P containing resources in the environment. Table 3 shows that there is a differential ecoenzyme biosynthesis by the microbial communities among the study sites, as we observed higher values of Phosphodiesterase (PD) in soil than in sediment samples, whereas the oposite was true for Phosphomonoestherases PM, for which we observed higher values in sediment. Noteworthy, we did not detect phosphonatase PN activity in sediment samples, but we did in soil samples (Table 3). It seems then that soil microbes require to spend more energy on enzymes to retrieve P from the more recalcitrans

forms, P- diesters and phosphonates. This is consitent with the observation that soil possessed less DOP than sediment.

Table 3. Means (standard error), of ecoenzyme activities quantified in grassland soil and sediment

	SOIL		SEDIMENT	
PARAMETERS	**Churince**	**Pozas Azules**	**Churince**	**Fertilized**
PM[a]	105 (19.8)	24.06 (2.9)	557 (190)	597
PD[b]	181 (12.2)	371 (9.8)	286 (68)	176
PN[c]	63.22 (13.3)	76.23 (9.76)	0	0

in the Cuatro Ciénegas Basin, Coahuila, Mexico.

[a] Phosphomonoesterase; [b] Phosphodiesterase; [c] Phosphonatase. In nmol ρ-NP g^{-1} SDW h^{-1}.

Evaluation of Isolates' ability to grow on different P sources

A total of 1163 isolates were isolated and evaluated for thir ability to grow using different sourves of P (250 from CH soil, 250 from PA soil, 141 from first group of CH sediment and 512 from second group of CH sediment).

The three factors analyzed in the experiment (site, P substrate and site*P substrate) were significant at $p< 0.0001$, therefore, the differences observed in the isolates growth depended on P substrates and the site (interaction is interpreted in the following section,).

Aproximately, 80% of the isolates from sediments (both intermediate and the fertilized small pond) were capable of growth on medium with potassium phosphate as a sole source of P. In contrast, only 60% of the isolates from soil (Churince and Pozas Azules) were capable of growth using only this P source (Figure 1 and 2). Using calcium phosphate as a sole phosphorus sources, we observed differences in isolates growth

between the four sites; the higher percentage of growth was observed for isolates from CH-sediment, followed by isolates from soil (CH and PA) and finally isolates from fertilized-sediment (Figure 1 and 2).

Remarkably, 50% of the isolates from CH-sediment were capable of growth with phosphite as a sole source of P. However, less than 20% of the isolates from fertilized-sediment, and less than 10% of the isolates from soil (CH and PA) were capable of growth under this P source (Figure 1 and 2).

Testing the capacity of the isolates to growth in phosphonates as a sole P source, we observed that less than 10% of the isolates from the four sites had the capacity to growth in 2-PA; meanwhile we detected that ~ 20% of the isolates can growth using the P from 2-AEP, the most abundant biogenic phosphonates (Figure 1 and 2).

Noteworthy, 90% of the isolates from CH-soil, PA-soil and CH-sediment could grow with DNA as a P source. However, the isolates from fertilized-sediment had less members with this capacity, as we observed less than 30% of the isolates growing under this P source (Figure 1 and 2).

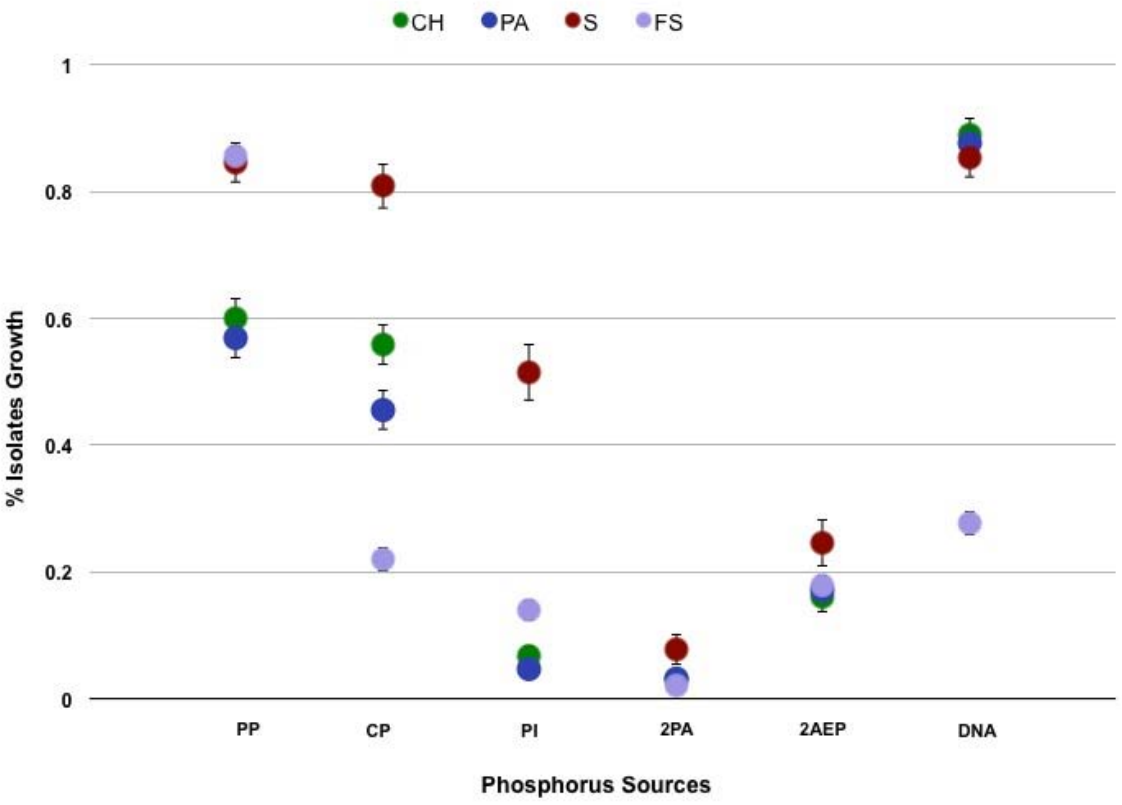

Figure 1. Isolates growth in six different phosphorus sources as a sole phosphorus source.

Phylogenetic analysis of bacteria, based on16S rRNA gene, across all sites and samples.

A total of 594 sequences were analyzed using Mothur. This program yield 210 operational taxonomic units (OTU's) grouped at 99% similarity. The OTU's were distributed as follows: 29 OTU´s from the Churince-sediment, 60 OTU´s from the fertilized-sediment, 67-OTU´s from the Churince soil, and 75 OTU´s from the Pozas Azules soil. The dominant OTU´s were classified as Firmicutes (Bacillus and Staphylococcus), followed by Proteobacteria (Aeromonas and Paracoccus) and Actinobacteria. (Although, as expected, the selection medium enriched for Bacillus, bacterial isolars from many other genera were isolated). Some bacterial groups were revovered only from some sites, but most are indistinly recovered from soil of sediment.

Noteworthy, the distribution of the ability to use different sources of phosphorus is not associated to particular taxonomic groups. All clades had members that could use PP, calcium phosphate, and/or DNA. There are, however, some clades that lack member that can grow on phosphite, 2PA or AP.

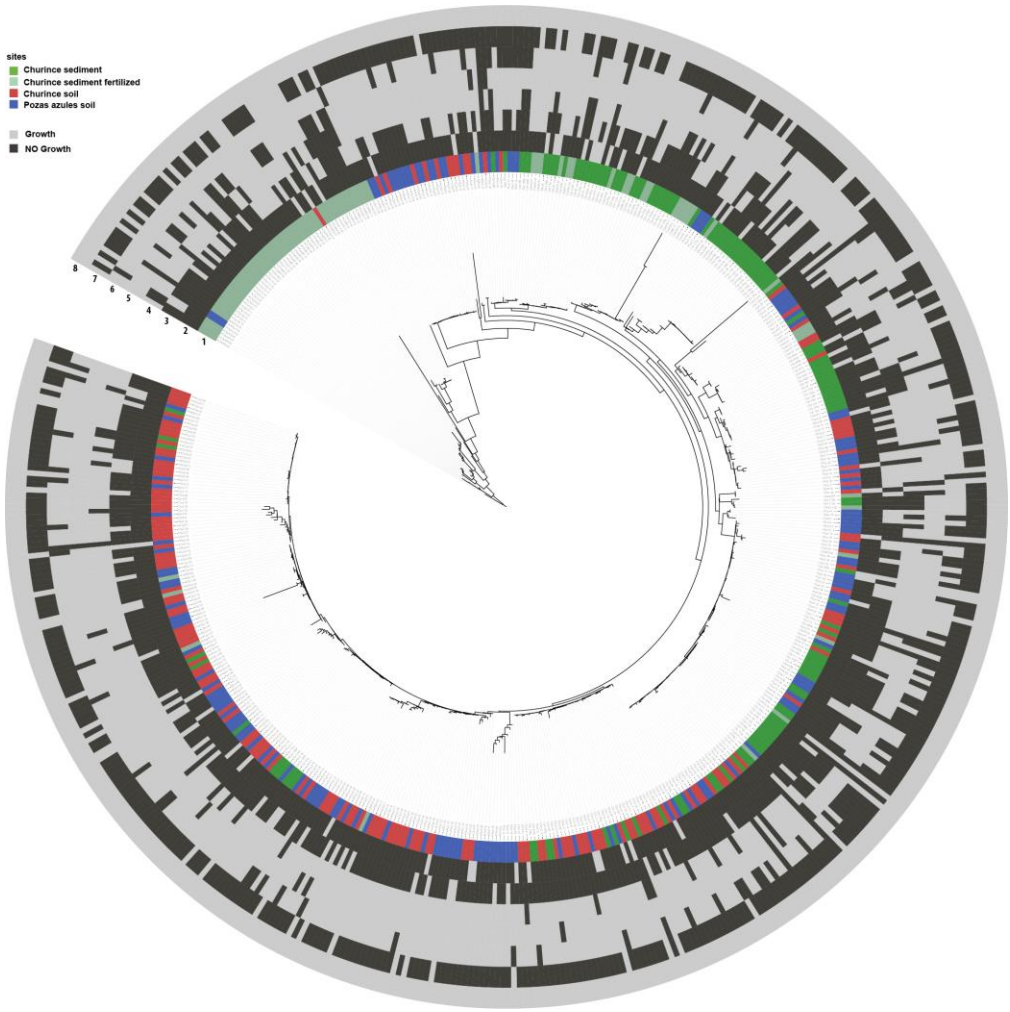

Figure 2. Ability to utilize different phosphorus sources by bacteria from sediment and soil communities. Phylogeny, based on the 16s rRNA gene. Tthe site of isolation is shown in the inner circle. In blue (Pozas Azules soil), red (Churince soil) and dark green (Churince sediment) and light green (fertilized sediment). The isolates ability to grow with a given P source is represented in dark gray; meanwhile light gray indicates lack of growth of the isolate.

Additionally, the analysis of AdaptML projected two habitats; the habitat 1 containing sequences obtained predominantly from soil and fertilized-sediment and are present in both the base and the most recent phylogeny branches; while that habitat 2 mostly contains sequences obtained from sediment. This result shows that bacteria have different niche adaptations that are influencing patterns of genotypic differentiation (Fig. 3).

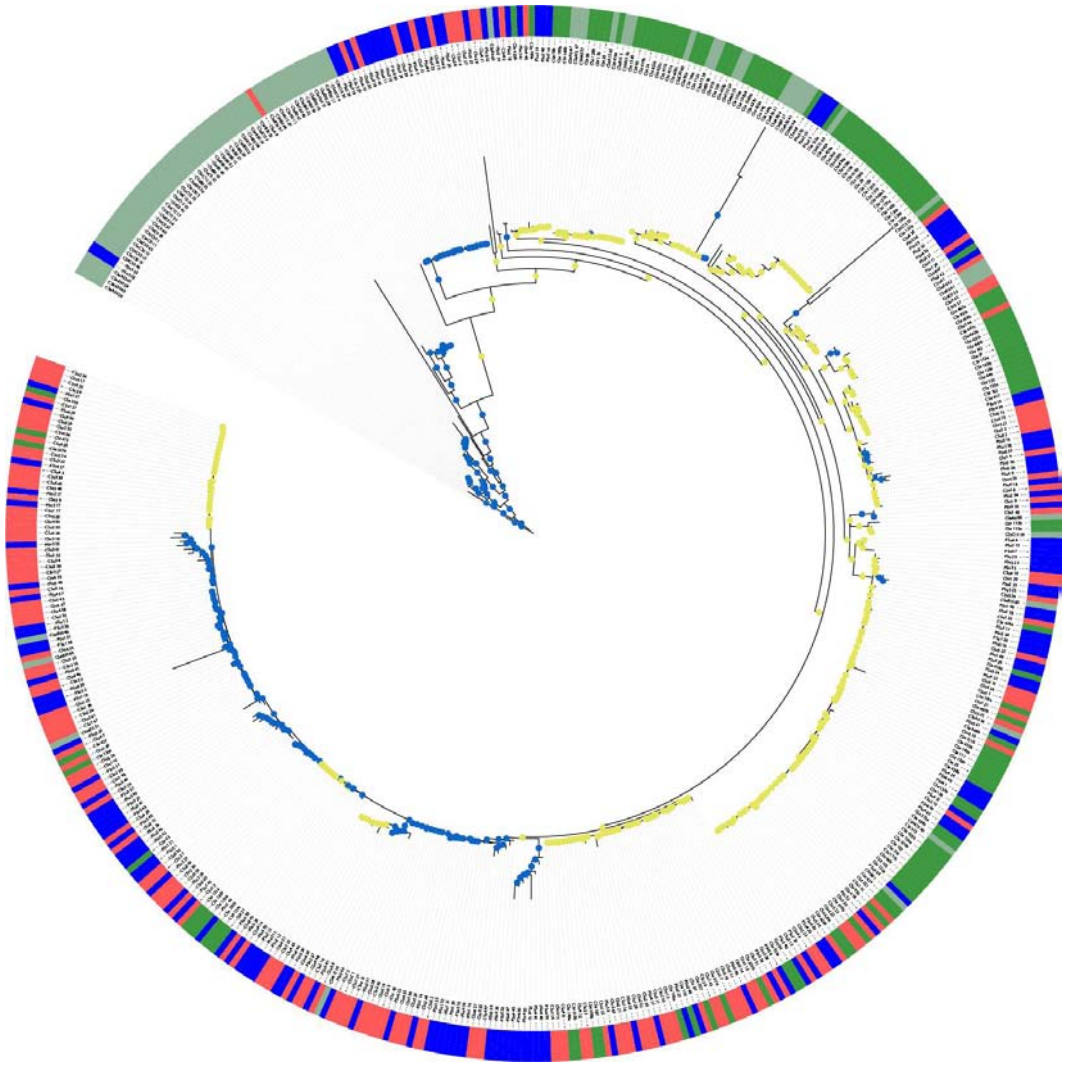

Figure 3. Analysis with AdaptML to assort the genotipes according to ecological similarity. The sequences were classified in relation with their isolation sites (outer ring). Projected habitats are showed with inner circles on the phylogenetic branches.

DISCUSSION

We found that the soil as well as sediment bacterial community of CCV has different strategies for P scavenging. These strategies included the mineralization of ester phosphates and phosphonates, via biosynthesis of different ecoenzymes, as well as the ability for inorganic P solubilization.

Our results suggest that the bacterial community of the soil and sediment from an ultra-oligotrophic site, share common strategies for P scavenging. However, within each genotypic cluster defined in this study, there are a vast diversity in strategies between individuals; suggesting that ecoenzyme biosynthesis for P acquisition is a "public good" and as long as some individuals of the CCV soil and sediment microbial community have these traits, the non-producer members can profit for it.

Among the P substrates tested in our experiment, potassium phosphate and DNA were the substrates in which it was observed a higher percentage of isolates growth. It was surprising to note that DNA was the preferred P source for isolates from soil and sediment but not for the isolates from fertilized sediment. This trend may be due to a difference in the bacterial diversity among sites, as we observed an over representation of Firmicutes in soil and sediment isolates. Meanwhile, in fertilized sediment we observed a lower proportion of Firmicutes and the presence of Actinobacteria and Proteobacteria.

The affinity to use DNA as a P source may reflect the behavior of microorganisms from a community perspective, rather than from an autonomous cell perspective. It has been proposed that nutrient limitation modifies the microorganism's behavior (e.g. spore forming microorganisms; González-Pastor 2010). Spore forming microorganisms have a cannibalistic behavior of competition for the resources (González-Pastor 2010). These microorganisms secrete extracellular killing factors that lyse non-sporulating cells. After

that, the dead cells release nutrients into the starved medium that the surviving cells can feed on; therefore, this cannibalistic behavior might be beneficial to the community (González-Pastor et al. 2003).

In ultra-oligotrophic environments, the DNA released by cannibalistic behavior, represents a P-rich source. However, the bacterial P acquisition from DNA requires phosphodiesterase biosynthesis for DNA mineralization. The ability of the microbial community in natural systems to synthesize phosphodiesterase was observed in the four sites within the CCV (Table 2). Our results suggest that this mechanism for P acquisition might be the main way by which microorganisms acquire and recycle the P in this ultra-oligotrophic system.

On the other hand, a low percentage of isolates growth was observed in media with phosphonates substrates (2-PA and 2-AEP). However, we observed isolates with the ability to grow either in 2-PA or 2-AEP in each genotypic cluster. For microorganisms from severe P-starvation systems (as is the case of CCV), the ability to obtain P from a variety of P-substrates, ensures its survival and fitness, and therefore, ensures the successful nutrient recycling.

Phosphonate degradation has been reported to occur via the phosphonatase pathway in marine bacteria (Martinez et al. 2010) as well as in soil bacteria (Panas et al. 2006). This pathway is present in diverse bacteria including representatives of Proteobacteria, Planctomicetes, Cyanobacteria (Martinez et al. 2010), and Firmicutes (Alcaraz et al. 2011). In natural conditions, we observed phosphonatase activity in soil but we cannot detect activity in sediments. These results are consistent with the idea that microbial degradation of phosphonates occurs under conditions of phosphate limitation, as we also observed lower P (DOP) concentration in soil than in sediment (Table 2).

However, there are three other degradative phosphonate pathways reported in various bacterial species: phosphonopyruvate hydrolase pathway, phosphonoacetate hydrolase pathway, and carbon-phosphorus lyase (C-P lyase) pathway (White and Metcalf, 2007). The first two pathways are highly substrate dependent and the latter is non-substrate dependent. Our experiment results suggest that the microbial pathway degradation of phosphonates, play out differently in the soils (via phosphonatase) and sediments (via C-P lyase). These results demonstrate that different bacteria found in the soil and sediment of CCV can grow well under conditions in which the only source of P is 2-PA or 2-AEP. Nevertheless, this does not provide compelling evidence that Phn (phosphonatase) biosynthetic pathways are present in the microbial community, for this, further experiments are needed.

The phylogenetic analysis shows that ecoenzymes producing microorganisms and cheater microorganisms (non-producers) coexist in a dynamic equilibrium whereby public goods are stable at the population level, but not at the individual level, this trend has already been reported (Cordero et al. 2012).

Acknowledgments

This work was financed by the National Autonomous University of Mexico (PAPIIT DGAPA-UNAM grant: Análisis de la vulnerabilidad de la dinámica de nutrientes en un ecosistema árido de México, IN204013). Constitutes a partial fulfillment of the Graduate Program in Biological Sciences of the National Autonomous University of México (UNAM). Y. Tapia-Torres acknowledges the scholarship and financial support provided by the National Council of Science and Technology (CONACyT, scholarship number

210603), and UNAM. To Pamela Chavez, Alberto Morón and Rodrigo Velázquez-Durán for assisting with soil sampling and chemical analysis, and to CONANP and Rancho Pozas Azules (PRONATURA) for permission to collect soil. V. Souza and Y. Tapia-Torres acknowledges support from Alianza FCS-WWF.

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Capítulo VII

Conclusiones

Esta tesis aporta información sobre los procesos que determinan la dinámica de C, N y P así como la relación estequiométrica C:N:P en un ecosistema desértico oligotrófico.

Las conclusiones generales de este trabajo se mencionan a continuación:

- En los ecosistemas desérticos oligotróficos (principalmente en P), además de que los ésteres de fosfato son la principal fuente de P; se observó que los fosfonatos pueden ser una fuente alternativa de P cuando la comunidad microbiana tiene la capacidad genética para degradarlos.
- La relación que se observa entre la biomasa radical, los nutrientes del suelo y la composición de la comunidad de bacterias, depende en gran medida del tipo de vegetación que domina. Por ejemplo, en los pastizales se promueve un ciclo cerrado del N, lo que favorece la conservación de este nutriente; mientras que en los matorrales se presentó un ciclo abierto donde el N es vulnerable a la pérdida del sistema.
- Así mismo, se observó que el P se encuentra en diferentes fracciones en el suelo del VCC. El tamaño de las fracciones y la disponibilidad del nutriente, sugieren que la microbiota es el factor principal en la regulación de la disponibilidad de este nutriente en los pastizales, mientras que su importancia relativa se reduce en el suelo de los matorrales.
- Se observó que en las comunidades microbianas altamente diversas como es el caso de las comunidades del suelo del VCC, la ley del mínimo de Liebig, que establece que sólo un elemento limita el crecimiento de los organismos en un tiempo determinado, no es aplicable.
- En la ecología microbiana a nivel de comunidad; el concepto del mínimo se ha

reemplazado por el concepto de la co-limitación. Donde el C, N y/o P co-limitan el desarrollo de la comunidad impactando de manera diferencial a los distintos miembros de la comunidad.

- A nivel de comunidades microbianas, observamos que los pastizales naturales del VCC están co-limitados de distinta manera en el Este y el Oeste. Las comunidades microbianas edáficas del Oeste están co-limitadas por C y N; mientras que las del Este están co-limitadas por C y P. Esta co-limitación de energía y nutrientes de los microorganismos, se ve reflejada a nivel de ecosistema; donde las plantas también se ven limitadas por el nutriente que está presente en menor concentración.
- Nuestros resultados sugieren que los miembros de las comunidades microbianas invierten un mayor esfuerzo en la obtención y acumulación del nutriente limitante, lo cual favorece la dinámica y conservación de dicho nutriente en el suelo.
- Se pudo observar que todas las estrategias para obtener el P tanto de ésteres de fosfatos como de fosfonatos, están distribuidas en las comunidades microbianas provenientes tanto de suelos como de sedimentos del VCC.
- En ecosistemas desérticos la síntesis enzimática puede considerarse como un bien público y si algún individuo de la comunidad posee esta estrategia, los demás individuos pueden beneficiarse de ella.

APÉNDICES

Descripción de técnicas estandarizadas para suelos halófilos oligotróficos

APÉNDICE 1

Determinación de Fósforo en la Biomasa Microbiana de Suelos y Sedimentos en Sistemas Halófilos Oligotróficos

Yunuen Tapia-Torres, Cristina Montiel-González y Felipe García-Oliva

Introducción

El procedimiento para estimar el P en biomasa microbiana tiene un procedimiento similar al fraccionamiento de P mediante el método de Hedley et al. (1982). Sin embargo aquí, se realiza el fraccionamiento por duplicado hasta la fracción de $NaHCO_3$. Una repetición se trata con $CHCl_3$ (fumigada) y la otra se mantiene sin tratar (no fumigada). La diferencia en P total extraído con $NaHCO_3$ entre la muestra tratada y la no tratada se atribuye al P liberado a partir de la lisis de células microbianas (Hedley y Stewart 1982). Se ha encontrado que esta liberación de P constituye aproximadamente el 40 % del P total microbiano en el suelo (Hedley y Stewart 1982). Es por esto que es necesario estimar el factor de recuperación (*Kp*) para calcular el P total microbiano en del suelo. El factor *Kp* varía con el tipo de suelo porque los efectos de la retención geoquímica pueden alterar la recuperación de P liberado de las células microbianas en el suelo (Hedley et al. 1982). Aunque el *Kp* no tiene una relación directa con la retención geoquímica de P en el suelo, el *Kp* comúnmente es pequeño en suelos con altas capacidades de retención geoquímica. Estudios de suelos dentro de un rango de pH de 6.2-8.2 sugieren que un factor *Kp* de 0.4 (38%) provee un estimador cercano de P microbiano para suelos de este rango de pH (Hedley y Stewart 1982; Lajtha et al. 1999).

A1.1. Protocolo para estimar P microbiano de suelos halófilos oligotróficos:

1. Pesar por duplicado 5 gramos de suelo fresco (para hacer dos tratamientos: fumigado y no fumigado)
2. Humedecer el suelo aproximadamente a capacidad de campo e introducir las muestras a un desecador (se tendrán dos desecadores, uno para cada tratamiento). En cada desecador colocar una caja de petri con agua desionizada y sobre ella un papel filtro, para mantener la humedad durante la incubación.
3. En el desecador con el tratamiento a fumigar introducir un vaso de precipitado con 80 ml de cloroformo ($CHCl_3$).
4. Conectar el desecador con el tratamiento a fumigar a vacío con una trampa de aire. Aplicar el vacío por 10 minutos, cerrar la llave y dejar de hacer vacío, dejar que la cámara se sature por 10 minutos. Posteriormente aplicar el vacío nuevamente por 30 minutos. Finalmente cerrar la llave de paso del desecador y retirar del vacío. Sellar esta cámara con parafilm para evitar fugas de $CHCl_3$.
5. Colocar ambos desecadores (con muestras fumigadas y no fumigadas) en una incubadora por 24 horas a temperatura de 26 a 29 °C.
6. Después de 24 horas, retirar los desecadores de la incubadora, al lote no fumigado retirarle la tapa y colocarlo en una campana de extracción mientras se manipula el lote fumigado.
7. Del desecador con el tratamiento fumigado abrir la llave de paso dentro de la campana de extracción (prendida) y permitir que salga el cloroformo. Posteriormente conectar el desecador con una trampa de aire y aplicar el vacío por 30 minutos. Por último, retirar la tapa del desecador y dejar en la campana de extracción prendida por 30 minutos.

8. Sacar las muestras de los desecadores y colocarlas en tubos falcón de 45 ml. Adicionar 30 ml de $NaHCO_3$ 0.5 M a pH 8.5 (ajustar pH con NaOH 5M). Para muestras con altos contenidos de carbonatos, es necesario agitar las muestras una vez agregado el $NaHCO_3$ y destapar para permitir la liberación de CO_2, repetir este procedimiento varias veces (las que sean necesarias para evitar la presión por el gas). Agitar a baja velocidad (150 rpm) por 16 horas (de preferencia con los tubos acostados).
9. Posterior a las 16 horas centrifugar las muestras a 6000 rpm por 25 minutos. Filtrar el sobrenadante por papel filtro Whatman No. 42 y recoger el filtrado en viales.
10. Tapar el vial y agitar con la mano, tomar una alícuota de 5 ml del filtrado y colocarla en un vial de boca ancha. Adicionar 0.11 ml de H_2SO_4 *11N*; después de la reacción adicionar 1ml más de H_2SO_4 *11N*; posteriormente adicionar 0.7 ml de Persulfato de amonio ($(NH_4)_2S_2O_8$) al 50%. Semi-tapar los viales y colocarlos dentro de un vaso de precipitado de 4L . Por último digerir en una en una autoclave a 121º C (1 a 4 Kg cm^{-2}) durante 1 hora.
11. Posterior a la digestión, retirar las muestras de la autoclave y dejar enfriar.
12. Neutralizar los extractos con ayuda de un potenciómetro calibrado. Adicionar una gota de P-nitrofenol a las muestras (aproximadamente tendrán pH 1), subir el pH con NaOH 5 M hasta pH 6 (vira a amarillo) y posteriormente con NaOH 1M o 0.5M. Dejar el pH entre 6.5 y 7 para leer las muestras. Si el pH es mayor a 7, bajarlo con HCl 1M. NOTA. El uso de P-nitrofenol puede evitarse si se tiene cuidado al momento de neutralizar utilizando el potenciómetro. Esto garantiza menores fuentes de contaminación de las muestras.

13. Colocar las muestras ya neutralizadas en un matraz volumétrico de 25ml y aforar con agua desionizada.

Preparación de Soluciones

- $NaHCO_3$ O.5M: para $NaHCO_3$ a 100% de pureza, pesar 42g de NaHCO3 y aforar a 1 L con agua desionizada.
- NaOH 5M: para NaOH al 97% de pureza, pesar 206g de NaOH y aforar a 1L (se recomienda preparar menos de 250 ml).
- NaOH 1M: para NaOH al 97% de pureza, pesar 41.23g de NaOH y aforar a 1L (se recomienda preparar menos de 200 ml).
- NaOH 0.5M: para NaOH al 97% de pureza, pesar 20.6 de NaOH y aforar a 1L (se recomienda preparar menos de 200 ml).
- H_2SO_4 11N: para H_2SO_4 al 98% de pureza y densidad de 1.8 g/cm^3, tomar 305.2 ml de H_2SO_4 y aforar a 1L (100 ml de esta solución alcanza para 50 muestras).
- $(NH_4)_2S_2O_8$ al 50%: pesar 25 g de persulfato y aforar a 50 ml.
- P-nitrofenol: 0.2g de P- nitrofenol aforar a 100 ml con alcohol
- HCl 1M: para HCl al 37.3% de pureza y densidad de 1.8 g/cm^3, tomar 82.7ml de HCl y aforar a 1L. (se recomienda preparar menos de 200 ml).

A.1.2. Determinación de P microbiano por colorimetría utilizando el sistema reductor con ácido ascórbico y antimonio como catalizador.

Basado en: Etchevers G.G., Etchevers J.D., Montes I., Gil S.I. (1983) y Cajuste L.J. (1986)

Con este procedimiento se busca determinar el P contenido en la biomasa microbiana de suelos oligotróficos que no es posible determinar con el uso de un autoanalizador debido a la baja concentración de P. Para este procedimiento, se requieren muestras tratadas anteriormente con el método de Hedley et al. (1982) (A.1.1.). Actualmente existen en el mercado, autoanalizadores con la capacidad de cuantificar P en concentraciones que van desde (0.2 ppm). Pero el uso de un espectrofotómetro es una alternativa viable para hacer estas determinaciones de P en la biomasa microbiana, siguiendo el procedimiento descrito a continuación:

1. Pipetear 5 ml del extracto previamente digerido y neutralizado.
2. Añadir 5 ml de la solución para desarrollo de color (Ac. Ascórbico y Molibdato de amonio). Esto se hace con cuidado para evitar pérdida de la muestra debido a la efervescencia excesiva.
3. Aforar con agua a 50 ml
4. Dejar reposar a 25°C durante 15-30 min
5. Leer absorbancia a una λ=882 nm

REACTIVOS

Solución de Molibdato de Amonio (basado en Etchevers et al. 1978)

Cantidad	Reactivo
20 g	Molibdato de amonio
300 ml	Agua desionizada
450 ml	H_2SO_4 14 N
100 ml	Solución al 0.5% (p/v) tartrato de antimonio y potasio
AFORAR a 1 litro	Agua desionizada

H_2SO_4 14 N

Cantidad	Reactivo
196 ml	H_2SO_4
500 ml	Agua desionizada (aforar)

Solución para desarrollo de color

Cantidad	Reactivo
0.5 g	Acido ascórbico
100 ml	Solución de Molibdato de amonio (aforar)

Tartrato de antimonio y potasio 0.5 % (p/v)

Cantidad	Reactivo
0.5 g	$C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$
100 ml	Agua desionizada (aforar)

CURVA DE CALIBRACIÓN O CURVA ESTÁNDAR

Solución A (stock estandar) 100 mg/l P

Cantidad	Reactivo
0.4394 g	Fosfato de potasio monobásico
1.0 ml	Cloroformo
1000 ml	Agua desionizada (aforar)

Solución B (stock estandar) 20 mg/l P

Cantidad	Reactivo
20 ml	Solución A
100 ml	Agua desionizada (aforar)

CURVA

Concentración (ppm) mg/l P	Stock B (ml)	Aforar con agua (ml)
0.025	0.125	100
0.05	0.25	100
0.1	0.5	100
0.2	1.0	100
0.4	2.0	100

APÉNDICE 2

Actividad de la Fosfonoacetaldehído Hidrolasa (Fosfonatasa) en Suelos y Sedimentos de Sistemas Oligotróficos

Yunuen Tapia-Torres y Felipe García-Oliva

Los fosfonatos son una clase de compuestos organofosforados que contienen un enlace directo carbono-fósforo (C-P), en lugar del enlace éster más común C-O-P (Martínez et al., 2010; Sviridov et al., 2012). Debido a la estabilidad del enlace, estos compuestos son altamente resistentes a la biodegradación por factores químicos, térmicos, fotolíticos y enzimáticos (Hayes et al., 2000; Sviridov et al., 2012). Entre los fosfonatos conocidos se incluyen compuestos biogénicos y xenobióticos (Ternan et al., 2000), ambos de gran importancia en los ecosistemas. Los organofosfonatos sintéticos son ampliamente utilizados en la industria química como detergentes, anticongelantes y pesticidas, entre otros (Hayes et al., 2000). Por otro lado, dentro de los organofosfonatos naturales se incluyen una gran variedad de antibióticos de origen microbiano (Hayes et al., 2000; White y Metcalf, 2007).

La degradación de los organofosfonatos, es mediada casi exclusivamente por acción de enzimas de origen microbiano necesarias para romper el enlace C-P. Hasta hoy se conocen 3 diferentes hidrolasas que pueden ser las encargadas de romper el enlace C-P: fosfonoacetaldehído hidrolasa conocida como fosfonatasa (Morais et al., 2004), fosfonoacetato hidrolasa y fosfonopiruvato hidrolasa (Ternan et al., 2000).

La acción de la fosfonatasa libera acetaldehído y fósforo inorgánico (Figura A2-1), por lo que para cuantificar la actividad de la enzima, cualquiera de los dos productos puede ser cuantificado mediante métodos colorimétricos.

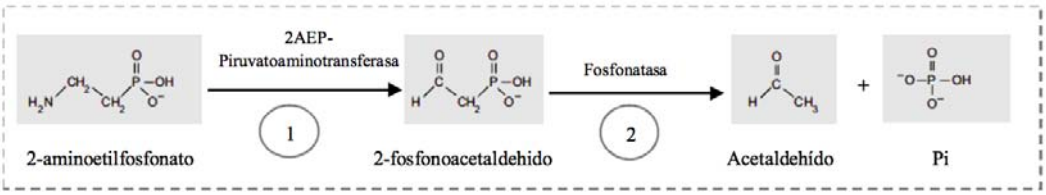

Figura A2-1. Ruta degradativa del 2-Aminoetilfosfonato (2AEP). Modificado de White y Metcalf (2007).

Muchos sustratos y los productos de las reacciones enzimáticas absorben luz, ya sea en la región visible o en la región ultravioleta del espectro. Algunas veces, los cambios en la concentración del sustrato o del producto se lee por colorimetría después de la extracción a partir de muestras de suelo incubadas con el sustrato a temperatura, tiempo y pH específicos (Tabatabai and Dick en Burns and Dick 2002). La metodología usada en este trabajo para medir la actividad de la fosfonatasa presente en el suelo, se basa en métodos espectrofotométricos, los cuales se describen a continuación.

NOTA: Para cada ensayo, es necesario hacer un control del sustrato a utilizar y un control para cada muestra que se tenga. La preparación inicial de estos controles es diferente, posteriormente deben de recibir el mismo tratamiento al igual que todas las muestras. Es necesario tener 3 o 4 réplicas por muestra.

A.2.1. Actividad de la fosfonoacetaldehído hidrolasa (Fosfonatasa, Ensayo enzimático)

1.- Extracción de la enzima de una muestra de suelo

1.1. Pesar 2 g de suelo fresco y colocarlos en tubos falcon de 45 ml

1.2. Adicionar 30 ml de buffer MUB (Modiffied Universal Buffer) a pH 9 (ajustar pH dependiendo del pH del suelo)

1.3 Agitar durante 20 min en posición horizontal

2.- Solución de trabajo

NOTA: A partir de este punto, es importante hacer las réplicas de cada muestra

2.1. Tomar 670 µl del homogeneizado (1.3) y colocarlos en tubos eppendorf de 1.5 ml
2.2. Agregar 670 µl del sustrato (depende de la enzima a la cual se le va a determinar la actividad)

CONTROL DE LA MUESTRA

- Tomar 670 µl del homogeneizado y colocarlos en tubos eppendorf de 1.5 ml
- Agregar 670 µl de MUB pH 9

CONTROL DEL SUSTRATO

- Tomar 670 µl del sustrato y colocarlos en tubos eppendorf de 1.5 ml
- Agregar 670 µl de MUB pH 9

2.3. Agitar con ayuda del vortex

2.4. Incubar a 40°C (importante determinar la temperatura óptima de la enzima) con agitación constante durante 2 horas.

2.5. Una vez terminado el tiempo de incubación, centrifugar las muestras a 10, 000 rpm durante 2 min.

2.6. Sacar las muestras con mucho cuidado, para evitar contaminar el sobrenadante.

3.- Preparación de la muestra de lectura

3.1. Tomar 750 µl del sobrenadante y colocarlos en tubos falcon de 45 ml

3.2. Adicionar 75 µl de NaOH 1 N (para parar la reacción)

3.3 Adicionar 1675 µl de agua desionizada

3.4. Agitar

4.- Determinar la concentración de Pi liberado (para esto se pueden emplear diferentes técnicas, en este caso en particular se utilizará la determinación de Pi por colorimetría utilizando el sistema reductor con ácido ascórbico y antimonio como catalizador).

A.2.2. Determinación de P inorgánico por colorimetría utilizando el sistema reductor con ácido ascórbico y antimonio como catalizador.

Basado en: Etchevers G.G., Etchevers J.D., Montes I., Gil S.I. (1983) y Cajuste L.J. (1986)

Con este procedimiento se busca determinar el Pi liberado durante el ensayo de actividad de la fosfonoacetaldehído hidrolasa (fosfonatasa) de suelos oligotróficos.

NOTA: Los reactivos necesarios para llevar a cabo esta metodología se encuentran en el apéndice I sección A1.2.

1. Pipetear 2.5 ml del extracto que resulta del ensayo de la fosfonatasa (3.4) (en este caso, el volumen final del ensayo anterior es de 2.5 ml por lo que se sugiere seguir con el paso 2.
2. Añadir 2.5 ml de la solución para desarrollo de color (Ac. Ascórbico y Molibdato de amonio). Esto se hace con cuidado para evitar pérdida de la muestra debido a la efervescencia excesiva.
3. Aforar con agua a 25 ml.
4. Dejar reposar a 25°C durante 15 min.
5. Leer absorbancia a una λ=882 nm

Calibrar el espectrofotómetro

Dependiendo del espectro que se tenga, en algunos casos (espectrofotómetros no tan nuevos) se recomienda prender el espectrofotómetro media hora antes de usarlo y calibrarlo a una **λ de 882 nm**

Una vez prendido el espectrofotómetro y la computadora, seguir el siguiente procedimiento.

-Abrir el programa "Thermo Insight"
- **Fixed** (ajustar λ de 882 nm)
- **Samples** (poner el número y nombre de las muestras)
- **Measured fixed** (revisar la lista de muestras que te despliega el programa)

En este punto hay que meter la celda con agua desionizada, al espectro

- **ZERO**
- **Measure**

Tomar 1 ml de muestra y colocarlo en la celda perfectamente lavada
Colocar la celda dentro del espectro
Leer absorbancia
Sacar la celda, desechar la muestra y lavar perfectamente con agua desionizada
Realizar la lectura de la siguiente muestra

Una vez terminadas las lecturas, generar el reporte de la corrida de muestras como se explica a continuación.
-REPORTS (seleccionar todas las muestras)
- EXPORT (Guardar archivo como documento de excel)

NOTA: El tiempo máximo que se puede emplear desde el paso 2 hasta terminar el 5 del apartado A2.2, no puede exceder los 45 minutos. Debido a que se corre el riesgo de que se pierda el color obtenido en la reacción.

Reactivos (Ensayo enzimático)

Modified universal buffer (MUB)

Reactivo	Cantidad	
Tris-hidrochloric aminomethane	6.05 g	Para 500 ml de agua desionizada
Acido maléico	5.8 g	
Ácido cítrico	7.0 g	
Ácido Bórico	3.15 g	
agua desionizada	244 ml	

NOTA: La solución de MUB esta 5X y queda con un pH final de aproximadamente 2. La solución de trabajo requiere el MUB 1X a pH = 5 o pH = 9 dependiendo del pH de la muestra a analizar.

MUB pH= 5

Reactivo	Cantidad
MUB (5 x)	20 ml
Ajustar pH con NaOH (1M)	Aprox. 2.8 ml

Para 100 ml de agua desionizada

MUB pH= 9

Reactivo	Cantidad
MUB (5 x)	20 ml
Ajustar pH con NaOH (1M)	Aprox. 6.0 ml

Para 100 ml de agua desionizada

NaOH 1N

Reactivo	Cantidad
NaOH	10 g
Agua desionizada	250 ml

Para 250 ml de agua desionizada

Buffer de acetatos

Para preparar el buffer de acetatos se requiere de una solución stock que contiene ácido acético y acetato de sodio tri-hidratado.

Solución stock

(A) Ácido acético 0.2 M

Reactivo	Cantidad
Ácido acético	1.155 ml

Para 100 ml de agua desionizada

agua desionizada	100 ml

(B) acetato de sodio tri-hidratado 0.2 M

Reactivo	Cantidad
Acetato de sodio	6.8 g
agua desionizada	250 ml

Para 250 ml de agua desionizada

Para prepara la solución final del buffer de acetatos a pH= 5

Buffer de acetatos a pH= 5

Solución A	Solución B	pH
14.8 ml	35.2 ml	5.0
10.5 ml	39.5 ml	5.5

Para 100 ml

Solución A	Solución B	pH
74 ml	176 ml	5.0

Para 500 ml

NOTA: EL VOLUMEN FINAL ES DE 250 ml, POSTERIORMENTE AFORAR A 500 ml.
Poner los 176 ml de acetato de sodio y 60 ml de ácido acético. Los 14 ml que restan de ácido agregarlos poco a poco midiendo el pH en el potenciómetro.

Preparación de sustratos

FOSFONATASA 5 mM

Reactivo	Cantidad
2-aminoetilfosfonato	0.007476 g
Aforar con Buffer de acetatos pH 5	**12 ml**