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MECANISMOS DE OSMORREGULACIÓN QUE MODULAN LA ACUMULACIÓN DE CADMIO EN *Euglena gracilis*.

TESIS

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Mecanismos de osmorregulación que modulan la acumulación de cadmio en *Euglena gracilis*.

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ABREVIATURAS

ACN	acetonitrilo
Ala	alanina
βAla	β-alanina
APx	ascorbato peroxidasa
AQP	acuaporina
CAT	catalasa
Cys	cisteína
DW	peso seco
*Eg*FQS	fitoquelatina sintasa de *Euglena gracilis*
FQ	fitoquelatinas
Gli	glicina
GSH	glutatión
hFQ	homo-fitoquelatinas
hmFQ	hidroximetíl-fitoquelatinas
hGSH	homo-glutatión
hmGSH	hidroximetíl-glutatión
isoFQ	iso-fitoquelatinas
isoGSH	iso-glutatión
EAA	espectrofotometría de absorción atómica
γ-EC	gamma-glutamíl cisteína
ERO	especies reactivas del oxígeno
GPx	glutatión peroxidasa
GR	glutatión reductasa
His	histidina
IARC	Agencia Internacional para la Investigación del Cáncer
MeOH	metanol
mOsM	miliosmolar
OPA	*o*-phtalaldehído
OMS	Organización Mundial de la Salud
PO_4^{2-}	fosfato libre
polyP	polifosfatos
SOD	superóxidodismutasa
SEMARNAT	Secretaría de Medio Ambiente y Recursos Naturales
TCA	ácido tricloroacético
Trp	tripanotión

INDICE

Resumen

Euglena gracilis es un microorganismo fotosintético que posee un gran potencial biotecnológicopara usarse en procesos de biorremediación de sistemas acuosos contaminados con metales pesados, ya que es capaz de tolerar y acumular diferentes metales pesados.

Se han descrito con amplitud los mecanismos asociados con el aumento de la síntesis de los metabolitos quelantes, que tienen grupos tiol en su estructura (Cys, γEC, GSH y FQ), durante la acumulación de Cd^{2+} en *E. gracilis*. La caracterización cinética de la enzima que sintetiza a las FQ, la *Eg*FQS (fitoquelatina sintasa de *E. gracilis*), presenta su mayor actividad en presencia de Zn^{2+} y no de Cd^{2+}, como ocurre con la mayoria de las FQSs de otros organismos. Por esta razón, en la primer parte de este proyecto se determinó la capacidad de acumulación del Cd^{2+}, la toxicidad por el Cd^{2+} y la cantidad de metabolitos quelantes (tioles y polifosfatos) en células de *E. gracilis* pre-acondicionadas con Zn^{2+}(200-1000 μM Zn^{2+}), planteando como hipótesis que el aumento en el contenido intracelular del Zn^{2+} favorecería la síntesis de FQ y aumentaría la acumulación del Cd^{2+}. La proliferación de las células pre-acondicionadas con 400 μM de Zn^{2+} fue menos susceptible al Cd^{2+} con respecto al control, mientras que la toxicidad del Cd^{2+} sobre la fotosíntesis y la respiración fue similar a las células control. La acumulación del Cd^{2+} aumentó 2 veces en células pre-acondicionadas con 400 μM Zn^{2+} y cultivadas en 20 μM Zn^{2+}. Sin embargo, la cantidad de moléculas con grupos tiol no se modificó en las células pre-acondicionadas con Zn^{2+}. La conclusión de este primer trabajo fue que el Zn^{2+} promovía una protección sobre la toxicidad del Cd^{2+} y favorece su acumulación en *E. gracilis*, aunque esta protección no está asociada con un aumento en la biosíntesis de FQ. Los datos de esta primera parte permitieron establecer que la cantidad de FQ sintetizadas durante la exposición a 200 μM de Cd^{2+} durante 8 días (≈16 nmol/10^7 células) son bajas y no son suficientes para inactivar a todo el Cd^{2+} acumulado. Esto sugirió que había otros mecanismos involucrados en la acumulación del Cd^{2+}, los cuales no habían sido caracterizados previamente en el grupo de investigación.

El análisis de la literatura sobre los organismos hiperacumuladores de metales pesados nos indicó que aquellas plantas que habitan en ambientes con un estrés salino y/o hídrico (halófitas) se podrían utilizar como modelos para la fitorremediación de metales pesados, debido a su innata capacidad de osmorregulación. Con el objetivo de describir y

entender la resistencia a metales pesados en *E. gracilis*, se analizaron las respuestas de tipo osmótico durante la acumulación del Cd^{2+} en este organismo. Se determinaron diferentes parámetros fisiológicos y bioquímicos en *E. gracilis* expuesta a 50 y 200 μM de Cd^{2+} durante 8 días. Cabe señalar que estos niveles de Cd^{2+} están dentro del intervalo de concentraciones encontrado en los cuerpos acuosos contaminados con metales pesados. La acumulación del Cd^{2+} indujo un aumento significativo en el volumen y tamaño celular después de solo 24 h de exposición, el cual se mantuvo por varios días. Los cambios en el volumen intracelular de agua correlacionaron con (i) un aumento en la osmolaridad intracelular (*i.e.* presión osmótica) debido a un incremento generalizado de diferentes osmolitos compatibles-metabolitos como son la trehalosa, los polifosfatos, los aminoácidos, las betaínas y las poliaminas, así como moléculas con grupos tiol y (ii) la activación de la protección antioxidante al incrementar la relación del GSH/GSSG y un aumento en la actividad de enzimas como la GPx, la GR y la APx. Además, la acumulación del Cd^{2+} y el volumen intracelular aumentaron en *E. gracilis* cultivada en un medio hipo-osmótico, comparado con un medio hiperosmótico. Estos cambios en el volumen intracellular disminuyeron en presencia de inhibidores de las acuaporinas (proteínas membranales que favorecen los flujos de agua), como el Hg^{2+} y la pentamidina. Se evaluó también la posibilidad de que el Zn^{2+} tuvieran efectos similares. Sin embargo, no se observaron cambios en el volumen intracelular, tamaño celular e inducción del estrés oxidante durante la acumulación del Zn^{2+}. Para el caso de otros metales pesados se observó un ligero aumento del volumen intracelular en presencia de Cu^{2+}, Ni^{2+} y Co^{2+}, y que la presencia de pentamidina aumenta la acumulación de Pb^{2+}, pero disminuye la de Ni^{2+} y Co^{2+}.

Estos resultados indicaron que las respuestas celulares al Cd^{2+} y al estrés osmótico comparten mecanismos bioquímicos en este protista, en el cual la acumulación del Cd^{2+} correlaciona con los cambios en el volumen intracelular por un proceso que parece estar mediado por la generación de especies reactivas del oxígeno. Los datos generados en el presente proyecto de doctorado lograron establecer que existen varios mecanismos de respuesta al estrés ambiental que trabajan en conjunto, ya que a pesar de que la exposición al Cd^{2+} induce la síntesis de moléculas con grupos tiol para unir y neutralizar el Cd^{2+} acumulado, este proceso también puede estar regulado por los mecanismos asociados con la presencia del Zn^{2+} y que también dependen de un mecanismo que regula el contenido de agua intracelular, el cual no había sido descrito previamente y que

abre la oportunidad de estudiar proteínas como las acuaporinas en la acumulación de los metales pesados en microorganismos.

Abstract

Euglena gracilis is a photosynthetic microorganism which have a great biotechnological potential for bioremediation purposes, due to its elevated resistance and accumulation capacity of different heavy metals. The mechanisms associated to chelating thiol molecules synthesis (Cys, γ–EC, GSH and PCs) have been characterized during Cd^{2+} accumulation in *E. gracilis*. The kinetic characterization of the enzyme that synthesizes phytochelatins (PCs), *Eg*PCS (phytochelatin synthase from *E. gracilis*), showed that it has its main activity with Zn^{2+} and not with Cd^{2+} like most PCSs of other organisms. First, we determine the Cd^{2+} accumulation, toxicity and the amount of chelating metabolites (thiols and polyphosphates) in *E. gracilis* cells pre-conditioned with Zn^{2+} (200-1000 μM Zn^{2+}), under the hypothesis that the increase in intracellular content of Zn^{2+} could stimulate PCs synthesis. Cells pre-conditioned with 400 μM Zn^{2+} were less growth susceptible to Cd^{2+} with respect to control cells, although no changes in Cd^{2+} toxicity on photosynthesis and respiration were observed. The accumulation of Cd^{2+} was greater with 20 μM Zn^{2+}, but the amount of thiol molecules was not modified. Therefore, we conclude in the first work that Zn^{2+} promotes a protection against Cd^{2+} toxicity and favors its accumulation in *E. gracilis*; however this protection is not associated with an increase on PCs biosynthesis. These results established that the amount of PCs synthesized during exposure to 200 μM Cd^{2+} for 8 days (≈16 nmol/10^7 cells) was low and was not necessary to inactivate the Cd^{2+} accumulated, which suggests the presence of other mechanisms involved in the accumulation of Cd^{2+} that have not been previously characterized.

Literature analysis on heavy metal hyperaccumulator organisms suggested that some plants that are able of living under saline and/or water stress environments (halophytes) were proposed as phytoremediation models, due to their innate osmoregulation capacity. In order to describe and understand a relatively novel mechanism associated to heavy metal resistance in *E. gracilis*, the osmotic responses during cadmium accumulation were analyzed. Several physiological/biochemical parameters were assessed in *E. gracilis* exposed to 50 and 200 μM Cd^{2+} during 8 days, concentrations well within the range found in polluted aquatic environments. The Cd^{2+} accumulation attained after only 24 h exposure induced marked increases in both the intracellular water volume and cellular size, which were maintained for several days. These changes correlated with (i) an increased intracellular osmolarity (*i.e.* osmotic pressure) driven by a generalized increase in osmo-metabolites such as trehalose,

phosphate-molecules, amino acids, thiol-molecules, betaines and polyamines content; and (ii) the triggering of antioxidant defenses by increasing GSH/GSSG ratios and GPx, GR and APx activities. In contrast, no changes in intracellular volume, cellular size and antioxidant status were observed under hyperaccumulation of Zn^{2+}. Also, a slight increase in intracellular volume was observed under Cu^{2+}, Ni^{2+} and Co^{2+}; in adittion pentamidine increases the accumulation of Pb^{2+}, but decreases Ni^{2+} and Co^{2+} accumulation.

Furthermore, *E. gracilis* cultured in a hypoosmotic medium promoted greater Cd^{2+} accumulation and water volume, compared to a hyperosmotic medium. These changes were significantly decreased by the aquaporin inhibitors Hg^{2+} and pentamidine. The results suggested that the cell responses to Cd^{2+} and osmotic stress share biochemical mechanisms in this algae-like protist, in which Cd^{2+} accumulation closely correlates with the intracellular water volume changes in an oxidative stress-mediated process. Therefore, it appears that there are several mechanisms that work simultaneously for heavy metal accumulation, one inducing thiol molecules synthesis to chelate Cd^{2+}, and other that regulates the intracellular water content and which had not been previously described, opening the opportunity to study proteins such as aquaporins on heavy metals accumulation in microorganisms.

1. INTRODUCCIÓN

Los metales pesados son elementos cómo el mercurio, el cadmio, el plomo, el cobre, el zinc, o el níquel que en términos gravitatorios poseen una densidad alta, ≥4-6 g/cm^3 (Duffus, 2002). Estos elementos se pueden encontrar en niveles elevados en el ambiente como consecuencia de las actividades humanas. Esto ha provocado un problema de contaminación a nivel mundial, que se ha incrementado gradualmente en paralelo con el desarrollo de la economía global (Su *et al*., 2014; Rai *et al*., 2019).

La dispersión de los metales pesados en el medio ambiente puede ser a través del aire, el suelo y el agua (Rai *et al*., 2019). Los metales pesados provienen de una fuente natural y/o antropogénica (Fig. 1). Las rocas primarias (magmáticas ó ígneas) son la principal fuente natural de estos elementos en el ambiente, ya que con el tiempo la meteorización química disuelve a estas rocas en iones formando complejos con otros elementos (rocas sedimentarias). Posteriormente, hay un rearreglo de los elementos por cambios de temperatura y presión (rocas metamórficas). Por último, las rocas consolidadas se desintegran por factores climáticos como el agua, la temperatura, el viento, etc., dando cómo resultado la formación del suelo (Bradl, 2005), el cual interactua directamente con los organismos. En este sentido, el tipo de suelo influye en la composición de los cuerpos de agua, principalmente el agua subterránea que puede ser utilizada para consumo humano o riego.

Además, los metales pesados también se encuentran presentes en la atmósfera en forma de gases, aerosoles y partículas que puede provenir de la actividad volcánica, aunque principalmente son emanados de la superficie terrestre de manera antropogénica (Bradl, 2005). La presencia de altas concentraciones de metales pesados en el ambiente deriva principalmente de la actividad humana, ya que los metales pesados son utilizados en la fabricación de una gran cantidad de artículos cotideanos (*e.g.* aparatos electrónicos, baterías, pigmentos, etc.), en donde la liberación de los metales se lleva a cabo durante la extracción de los mismos (actividad minera), la producción y su desecho (Bradl, 2005), aunado al manejo inadecuado de desechos industriales, alimenticios, agrícolas, ganaderos y forestales; y también al riego de cultivos con aguas residuales y al uso de pesticidas y/o fertilizantes (Rai *et al*., 2019).

Además, el confinamiento y el desecho indiscriminado de residuos industriales y, en menor medida, la composición de los suelos facilita el transporte de los metales

pesados hacia cuerpos de agua, que al entrar en contacto con la lluvia forman lixiviados que pueden llegar hasta los mantos acuíferos subterráneos. Este proceso favorece el contacto de los seres humanos con metales pesados por el consumo de agua contaminada y con la ingesta de alimentos contaminados por contacto con suelo y agua de riego contaminada, lo cual propicia un proceso de biomagnificación que se refiere al aumento de la concentración de un contaminante dentro de la cadena trófica (Hazrat y Ezza *et al*., 2018).

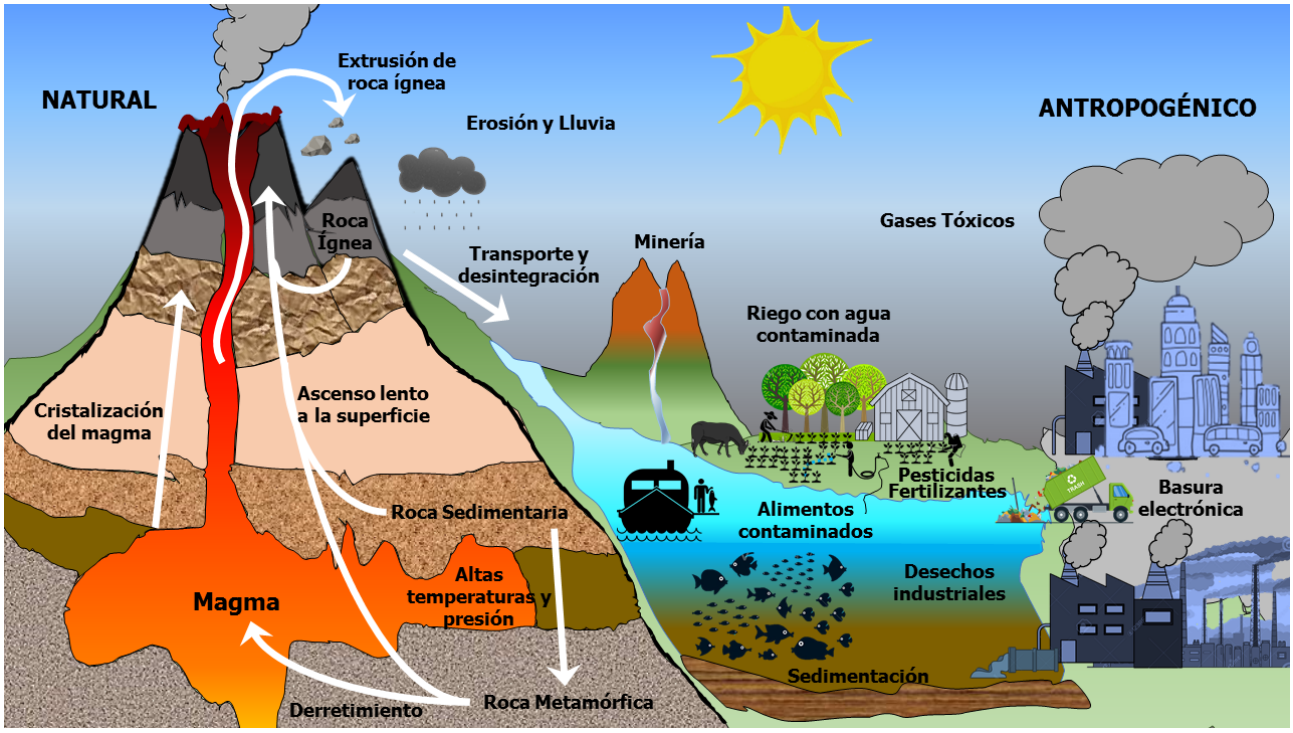

Figura 1. Fuentes de contaminación por metales pesados.

Natural: La actividad volcánica y el ciclo de las rocas contribuye de manera natural a modificar la disposición y la concentración de los metales pesados en la superficie terrestre. La lluvia y la erosión permiten la lenta desintegración de las rocas, formando lixiviados que entran en contacto con los mantos acuíferos. **Antropogénica**: está representado principalmente por industrias de extracción minera, galvanoplastia, curtidoras, pinturas, producción y desecho de materiales electrónicos, así cómo el uso indiscriminado de detergentes, pesticidas y/o fertilizantes. Estos desechos provocan que los seres humanos entren en contacto con los metales pesados mediante el consumo de frutas y verduras de cultivos regados con agua contaminada o residual, la ingesta de alimentos como la carne y el pescado que habitan en contacto con estos contaminantes y/o que consumen plantas contaminadas, y por el consumo directo de agua. Elaboración propia (RST), 2020.

1.1 La contaminación por metales pesados en México

El *"Programa Nacional de Remediación de Sitios Contaminados"* del 2018 es un

documento publicado por la Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT) dentro del marco normativo relacionado con sitios contaminados, su remediación y el diagnóstico de la situación en México. En este documento se puntualiza lo que es necesario actualizar, atender, promover y fortalecer para la remediación de sitios contaminados por metales y metaloides o hidrocarburos. También muestra que en el periodo de 2006 a 2016, la Dirección General de Gestión Integral de Materiales y Actividades Riesgosas (DGGIMAR) de la SEMARNAT recopiló la información de campo en todas las entidades federativas de nuestro país con respecto a sitios contaminados en México.

Los resultados del análisis indican que existe un total de 354 sitios potencialmente contaminados en nuestro país. Guanajuato, Colima, Chihuahua, Veracruz, Querétaro y San Luis Potosí fueron los estados más contaminados en México por tener más de 20 sitios potencialmente contaminados. A partir de estos estudios, la SEMARNAT estableció que la contaminación por metales pesados en nuestro país ocupa el segundo lugar de contaminantes más importantes debido a la cantidad de sitios que presentan este problema. Los sitios contaminados por metales pesados y metaloides se asocian principalmente con actividades mineras, en los cuales se generan residuosy sitios altamente tóxicos como jales mineros, escorias de fundición (masivas y granulares), patios de lixiviación, polvos, cenizas y calcinas, entre otros (SEMARNAT, 2018). Algunas de las empresas mexicanas que contribuyen principalmente a la generación de sitios contaminados con metales pesados y metaloides son “Metales y Derivados” en Tijuana (Baja California), el “ex-confinamiento de la Pedrera” en Guadalcazar (San Luis Potosí), “Cromatos” de Tultitlán (Estado de México), “presa de jales la Zacatecana” para la contención de metales pesados (Zacatecas) y en “Jales de Nacozari” (Sonora).

Entonces, en México la exposición humana a metales pesados se deriva principalmente de la actividad industrial y minera. Además, está documentado que la contaminación por metales pone en riesgo la salud de la población mexicana. Por ejemplo, en Tamaulipas se encontró que en el río Tigre, que desemboca en la laguna de San Andrés, las concentraciones de Cd (0.45 mg L^{-1}; 4 μM) y Pb (3.94 mg L^{-1}; 19 μM) en agua superan el límite máximo permisible para consumo humano (0.005 mg Cd L^{-1}; 0.01 mg Pb L^{-1}), uso agrícola y vida acuática establecidos por la NOM-127-SSA1-1994 y la NOM-001-SEMARNAT-1996 (Vázquez-Sauceda *et al*., 2012). Esta contaminación afecta a la población porque algunos de estos cuerpos de agua son empleados directamente en

la agricultura, con lo cual provocan que no sólo exista exposición a estos contaminantes por el consumo de agua, sino también por alimentos contaminados (Tchounwou *et al*., 2012; Vardhan *et al*., 2019). Se ha determinado en alimentos de uso común (leche y queso de Puebla), que se producen en áreas que son regadas con agua residual o tratada, que los contenidos de Pb (0.048 mg Pb Kg^{-1}) y As (0.038 mg As Kg^{-1}) rebasan los niveles (0.02 mg Pb Kg^{-1}; 0.002 mg As Kg^{-1}) establecidos en las recomendaciones internacionalmente reconocidas en relación con los alimentos (CODEX Alimentarius) de la Organización Mundial de la Salud (OMS), representando un riesgo alto a la salud humana (Castro-González *et al*., 2017).

Esta problemática ha conducido a la actualización de diferentes normas mexicanas que regulan la contaminación por metales pesados, cómo la NOM-147-SEMARNAT/SS-2004, que establece el uso de métodos fisicoquímicos para la remediación de sitios contaminados por metales pesados y metaloides. Sin embargo, es evidente que esto no ha sido suficiente para que las empresas contaminantes se responsabilicen y resuelvan los problemas ambientales que han generado. La persistencia de esta problemática también puede estar relacionada con cuestiones culturales, económicas y por la complejidad de los métodos fisicoquímicos, por lo que ha surgido la necesidad de buscar soluciones alternativas cómo la biorremediación, que es el uso de organismos con capacidades para remover contaminantes del medio circundante (Azubuike *et al*., 2016).

En este sentido, el "*Programa Nacional de Remediación de Sitios Contaminados*" de la SEMARNAT promueve la investigación en instituciones de educación superior y de investigación en temas de tecnología para la identificación, gestión y remediación de suelos y cuerpos de agua superficiales o subterráneos contaminados. Por lo tanto, el presente proyecto de doctorado está enfocado en el análisis de los mecanismos a nivel bioquímico y molecular de organismos con potencial biotecnológico, para que como meta a largo plazo este conocimiento pueda ser utilizado en procesos de biorremediación de los cuerpos de agua con altos contenidos de metales pesados.

1.2 Toxicidad por Cd^{2+}

La contaminación ambiental por metales pesados ya es un problema de salud pública que requiere ser atendido, principalmente porque estos elementos resultan ser

altamente tóxicos para los seres vivos y el humano (Tchounwou *et al*., 2012; Jaishankar *et al*., 2014; Vardhan *et al*., 2019).

El cadmio es un elemento que puede ser clasificado como un metal pesado debido a que posee una densidad mayor a 5 g/cm^3 (Ali y Khan, 2018). El cadmio se utiliza en la fabricación de baterías, cerámicas, industria electrónica, pigmentos, productos del petróleo, textiles, insecticidas, soldaduras, metalurgia, químicos sintéticos y fotografía (Vardhan *et al*., 2019). En el año 2000, se estimó que en el mundo se extrajeron y se esparcieron 19,700 toneladas de cadmio, y que el 55-73% de la emisión total de cadmio hacia el ambiente provino de la industria de las baterías de Ni-Cd, como consecuencia del aumento de las herramientas para la telecomunicación (WHO, 2003).

Los suelos, los sedimentos y los cuerpos de agua dulce representan un entorno complejo donde se llevan a cabo reacciones que modulan el flujo de cadmio desde la tierra, a través de ríos y aguas subterráneas, hasta el océano. La movilización del cadmio en suelo y agua depende de la especiación química, que a su vez está en función del potencial de oxido-reducción, la presencia de aniones y principalmente un pH ácido, el cual predomina en aguas intersticiales o ambientes donde la acidificación antropogénica es significativa (Cullen y Maldonado, 2012). Además, la especiación química del cadmio (estados de oxidación), y de otros metales pesados, es importante debido a que de esta depende la biodisponibilidad y la toxicidad en los organismos. Algunos metales pesados como el zinc, el cobre y el cobalto son esenciales en la fisiología de los seres vivos, mientras que el cadmio es un metal no esencial. El cadmio presenta como número de oxidación más común al 2+, y estas propiedades químicas son las que le confieren una alta afinidad por grupos funcionales que poseen azufre, oxígeno y nitrógeno, por lo que es capaz de interactuar con una gran cantidad de biomoléculas (Nieboer y Richardson, 1980).

1.2.1 Plantas

El Cd^{2+} es un metal muy tóxico pues se puede acumular en el interior de los organismos, causando daños irreversibles a muy bajas concentraciones.

Las plantas poseen un grado de tolerancia basal que supera el que poseen otros organismos como son los mamíferos, permitiendo que algunas plantas acumulen niveles

altos de Cd^{2+} (100 mg Cd/Kg_{DW}; Ali *et al*., 2013), lo cual posteriormente puede generar toxicidad en el ser humano y otros organismos por consumo de estas plantas (Wagner, 1993). El órgano que primero se enfrenta a la exposición a Cd^{2+} es la raíz, lo cual repercute en el metabolismo de toda la planta. Las células de la raíz no poseen transportadores específicos para el Cd^{2+}, y este metal es transportado a través de acarreadoresy/o canales de Zn^{2+}, Ca^{2+} y Fe^{2+}(Clemens, 2006).

El fenotipo de una planta intoxicada con Cd^{2+} muestra una inhibición general en el crecimiento, clorosis (coloración café-amarillenta anormal; pérdida de clorofila) y marchitamiento (pérdida de agua) (Clemens, 2006). A nivel bioquímico y genético en plantas el Cd^{2+}(1) genera daños al DNA; (2) inhibe la fotosíntesis porque interactúa principalmente con el fotosistema II, aunque también puede tener otros sitios de unión ya que es capaz de desplazar al Mg^{2+} de la clorofila; (3) induce estrés oxidante de manera indirecta cómo consecuencia de la inhibición del proceso fotosintético en donde los electrones pueden ser transferidos directamente al oxígeno generando especies reactivas del oxígeno (ERO), por lo que modifica la expresión y la actividad de enzimas antioxidantes como la superóxidodismutasa (SOD), la catalasa (CAT) o la ascorbato peroxidasa (APx), y el contenido de moléculas antioxidantes cómo el glutatión y el ascorbato; y (4) modifica el intercambio de agua (transpiración) posiblemente por un mal funcionamiento de los estomas, aunque este efecto no se ha entendido por completo (Andresen y Küpper, 2012; Ismael *et al*., 2019).

Por otro lado, la tolerancia de las plantas al Cd^{2+} y otros metales pesados puede estar relacionado con que el metal no pueda internalizarse o con la síntesis de metabolitos quelantes principalmente fitoquelatinas (FQ) y la compartimentación de los complejos metal-FQ en la vacuola, y en menor medida, con la posible expulsión de estos (Clemens, 2006; Ismael *et al*., 2018).

1.2.2 Animales (mamíferos)

Los efectos tóxicos del Cd^{2+} a nivel celular en mamíferos están relacionados principalmente con la generación indirecta de las ERO, ya que se propone que el Cd^{2+} puede desplazar al Fe^{2+} y al Cu^{2+} de ciertas proteínas, y estos iones directamente generar el estrés oxidante al participar en la reacción de Fenton (Rani *et al*., 2014). El Cd^{2+}

también puede interactuar y secuestrar directamente a el glutatión (GSH), generando estrés oxidante al disminuir la capacidad reductiva de la célula. Por otro lado, el Cd^{2+} tiene efectos genotóxicos, ya que puede afectar la proliferación y la diferenciación celular, el ciclo celular, el proceso de apoptosis, la síntesis y reparación del DNA, lo cual interrumpe o altera la síntesis de los ácidos nucleicos y de las proteínas (Tchounwou *et al*., 2012; Rani *et al*., 2014). Además, la Agencia Internacional para la Investigación del Cáncer (IARC, por sus siglas en Inglés) ha clasificado al Cd^{2+} como un carcinogénico, debido a que es capaz de activar oncogenes, alterar la metilación del DNA y desrregular la expresión génica (Bertin*et al*., 2006); sin embargo, el Cd^{2+} es un mutagénico débil comparado con otros metales (IARC, 1993).

Las principales formas de exposición de los seres humanos al Cd^{2+} es a través de la ingesta de agua, alimentos y consumo del tabaco, lo cual puede generar daño a nivel respiratorio, urinario, cardiovascular, gastrointestinal, sistemas nervioso y oseo, aunque los efectos clásicos y más comunes de intoxicación por Cd^{2+} son nefrotoxicidad, hepatotoxicidad, daño pulmonar y problemas óseos (Rani *et al*., 2014; Vardhan *et al*., 2019). El Cd^{2+} es capaz de modificar mecanismos de adhesión celular, cascadas de señalización y respuestas de autofagia en el túbulo proximal del riñón (Prozialeck y Edwards, 2012), lo cual repercute en el sistema de filtración y absorción de nutrientes en la sangre. Por otro lado, la exposición prolongada al Cd^{2+} en humanos aumenta la fragilidad ósea por una disminución en la densidad mineral de los huesos, ya que se sabe que el Cd^{2+} inhibe la actividad de la colecalciferolhidroxilasa responsable del metabolismo de la vitamina D3, que a su vez es esencial para la absorción de Ca^{2+} (Rani *et al*., 2014). Con respecto al daño pulmonar, el Cd^{2+} induce apoptosis en las células del epitelio pulmonar mediante un mecanismo posiblemente asociado con la inducción de ERO, disminuyendo drásticamente el contenido de GSH (Nair *et al*., 2013), similar a lo que sucede en el hígado donde el Cd^{2+} interactua directamente con el GSH y proteínas de unión de metal (Arroyo *et al*., 2012).

La Organización Mundial de la Salud (OMS) publicó en el 2010 los lineamientos con respecto al consumo de Cd^{2+} en seres humanos, donde se implementó que la ingesta mensual tolerable de Cd^{2+} en alimentos debe ser máximo de 25 μg Cd/Kg de peso corporal, y en agua el consumo máximo mensual debe ser menor a 3 μg L^{-1} (0.026 μM) (WHO, 2010). En un estudio con personas hispanas entre los 40 y 58 años de la región de Doña Ana, Nuevo México, USA se observó que había una correlación entre altas

concentraciones de Cd en orina con una edad avanzada, con el sexo femenino, con el trabajo como agricultor y en menor medida con el consumo de cigarros (Adams *et al*., 2016). Como se mencionó anteriormente, en varios ríos de México se han encontrado concentraciones de Cd^{2+} en agua que rebasan ≈ 90 veces el límite máximo permitido por la NOM-127-SSA1-1994 de 0.005 mg L^{-1} (0.044 μM). Sin embargo, se estima que los lugares con mayor concentración de Cd^{2+} se encuentran cerca de sitios y complejos industriales, y aunque es difícil encontrar datos de este tema tan sensible sobre México, en otros países se han determinado concentraciones de Cd^{2+} en agua de hasta 97-711 μM Cd^{2+} (Zhai *et al*., 2008; Abdul-Wahab y Marikar, 2012; Casado *et al*., 2008).

1.3 *Euglena gracilis*

El protista fotosintético flagelado unicelular de vida libre *Euglena gracilis* ha servido como un modelo por más de 50 años para entenderel funcionamiento de las células eucariontes a nivel bioquímico y molecular. En los años 60, se caracterizó por primera vez el contenido de aminoácidos en *Euglena* sp. para determinar su valor nutricional y se sugirió que este microorganismo se puede utilizar como un suplemento alimenticio (Kott y Wachs, 1964). Existe una gran cantidad de información que propone a este microorganismo como un modelo idóneo para su uso en la biotecnología, ya que posee una gran versatilidad metabólica al crecer de forma fotosintética (en aerobiosis y anaerobiosis), heterotrófica o foto-heterotrófica (Hasan *et al*., 2019). Además, es un microorganismo que puede ser utilizado para la producción de una gran cantidad de metabolitos de interés biotecnológico como proteínas, pro-vitaminas (β-caroteno, biotina), lípidos esenciales tales como los ácidos grasos poli-insaturados, ésteres de cera, inmuno-moduladores (β-1,3- glucano; paramilo), vitaminas o antioxidantes (α-tocoferol, vitamina C), azúcares fermentables, biogás, bioplásticos, nanofibras y biocombustibles (Rodríguez-Zavala *et al*., 2010; Krajčovič *et al*., 2015; Kottuparambil *et al*., 2019; Gissibl *et al*., 2019).

Con respecto al uso de microorganismos para la remoción de contaminantes, un primer problema que se tiene es que los microorganismos deben tener la capacidad para crecer en los ambientes contaminados, los cuales presentan limitaciones nutricionales, y diferentes pHs y temperaturas; un segundo problema es que se requiere que generen biomasa a una velocidad aceptable (García- García *et al.,* 2016). *E. gracilis* es un microorganismo que posee un proteoma plastídico (cloroplasto, mitocondria y

peroxisoma) muy similar en tamaño al de *Arabidopsis thaliana* pero mayor al de *Chlamydomonas reinhardtii,* y se propone que posee una capacidad funcional mayor que otros microorganismos que también obtienen energía a partir de la luz y que esto podría ser la razón de la versatilidad metabólica que posee el género *Euglena* (NovákVanclová *et al*., 2020). *E. gracilis* es un micoororganismo que puede crecer de manera autótrofa con luz (fototropía) produciendo oxígeno a partir de la fotosíntesis; también puede crecer en ausencia de luz (heterotrofía), consumiendo oxígeno a través de la respiración mitocondrial; o mediante el uso de ambas formas de obtención de energía (mixotropía) e incluso en condiciones anaerobias con o sin luz (Suzuki *et al*., 2017; Zimorski *et al*., 2017; Nakazawa *et al*., 2017). Además, este protista puede ser fácilmente cultivable a gran escala utilizando como fuente de carbono ácidos orgánicos como glutamato, malato, succinato, DL-lactato y acetato, o alternativamente glucosa, etanol, materia orgánica de las aguas residuales o compuestos de desecho (Moreno-Sánchez *et al*, 2000; Jasso-Chávez y Moreno-Sánchez, 2003, Rodríguez-Zavala *et al*., 2006, 2010), en valores de pH ácidos hasta neutros (2.5-8) y a temperaturas de 20-35ºC (Buetow, 1962; Olaveson y Nalewajko, 2000; Jasso-Chávez *et al*., 2010; Rodríguez-Zavala *et al*., 2010; Suzuki *et al*., 2017; Zimorski *et al*., 2017; Nakazawa *et al*., 2017).

1.4 Mecanismos de resistencia y acumulación de metales pesados en *E. gracilis.*

E. gracilis es capaz de acumular metales pesados tales como Cu^{2+}, Hg^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Tc^{7+}, As^{5+}, Ni^{2+} y Cr^{6+} (Devars *et al*., 2000; Einicker-Lamas *et al*., 2002; Mendoza-Cózatl *et al*., 2002; Mendoza-Cózatl *et al.,*2006a; Ishii y Uchida, 2006; Miot *et al*., 2009; García-García *et al*., 2009; Jasso-Chávez *et al*., 2010; Lira-Silva *et al*., 2011; Sánchez-Thomas *et al*., 2016; Moreno-Sánchez *et al*., 2016; Moreno-Sánchez *et al*., 2017; García-García *et al*., 2018). Los diferentes mecanismos de resistencia y acumulación de metales pesados descritos en *E. gracilis* (Fig. 2) son los siguientes:

(1) La adsorción, es decir la unión del metal a los componentes de la membrana plasmática y/o a la película, es una propiedad intrínseca de las células la cual se revela al ser expuestas a los metales pesados. En *E. gracilis* la adsorción de Cd^{2+} y otros metales como el Ni^{2+} suele ser menor al 5% del total de metal removido (Mendoza-Cózatl *et al*.,

2002; García-García *et al.,* 2018), aunque este porcentaje puede aumentar en células cultivadas en anaerobiosis (Santiago-Martínez *et al*., 2015).

(2) Los mecanismos de quelación o unión externa de iones metálicos permiten inactivar al metal y disminuir su efecto tóxico. Por ejemplo, se ha observado la expulsión de malato para secuestrar extracelularmente al Cr^{6+} y Cr^{3+} (Lira-Silva *et al.,* 2011).

(3) La biotransformación involucra el cambio en el estado de oxidación del metal, lo que puede resultar en la disminución de su toxicidad. Para este caso se ha determinado que *E. gracilis* puede transformar Hg^{2+} a Hg^{0} y volatilizarlo (Devars *et al.,* 2000), así como el Cr^{6+} a Cr^{3+} por una cromato reductasa (García-García *et al.,* 2009).

(4) En *E. gracilis* el mecanismo que mejor se ha descrito es la síntesis de moléculas que unen con gran afinidad a los iones metálicos en el interior de la célula. Por ejemplo, está documentado que durante la exposición a Cd^{2+} se induce la síntesis de moléculas con grupos tiol, como son la cisteína (Cys), la γ-glutamilcisteína (γ-EC), el glutatión (GSH) y los polímeros de glutatión o fitoquelatinas (FQ) (García-García *et al*., 2012), así como el sulfuro (Santiago-Martínez *et al*., 2015). Los compuestos formados entre el Cd^{2+} y las moléculas con grupos tiol pueden formar complejos de alto peso molecular (HMWCs) que son compartamentalizados dentro de los cloroplastos y las mitocondrias (Mendoza-Cózatl *et al.,* 2002; Mendoza-Cózatl *et al*., 2005). La composición de los HMWCs es 57-75% de moléculas de mono-tiol (Cys, γ-EC, GSH) y 25-43% de FQ, con trazas de aspartato o asparagina (Mendoza-Cózatl *et al*., 2006b). Por otro lado, la síntesis de moléculas fosfatadas (PPi y polifosfatos-polyP) y el fosfato libre (PO_4^{2-}) también participan en la resistencia y acumulación de Cd^{2+} (Santiago-Martínez *et al*., 2015; Sánchez-Thomas *et al*., 2016).

(5) La expulsión del metal es otro mecanismo que recientemente se ha descrito para *Euglena*, pero el cual no sería adecuado para fines de biorremediación. El mecanismo de expulsión se reportó en *E. gracilis* expuesta a Ni^{2+}, donde se observó que después de las primeras 24 horas de cultivo, las células disminuían drásticamente la concentración intracelular de dicho metal (García-García *et al.,* 2018). Se propuso que la expulsión del Ni^{2+} estaba mediada por algún anión desconocido (García-García *et al.* 2018), debido a que se observó una disminución simultánea de las pozas de moléculas con grupos tiol y polyP, y de la cantidad intracelular de Ni^{2+} en *E. gracilis*. Relacionado con lo anterior, también se ha descrito en este microorganismo (Einicker-Lamas *et al*., 2003) un sistema similar a una P-glicoproteína (MDR1) semejante a la de mamífero, el cual expulsa

conjugados-S de glutatión al exterior. En levaduras (*Saccharomyces cerevisiae*) y nematodos de suelo (*Caenorhabditis elegans*), la MDR se asocia a la desintoxicación de Cd^{2+} y As^{3+} (Keppler, 1999).

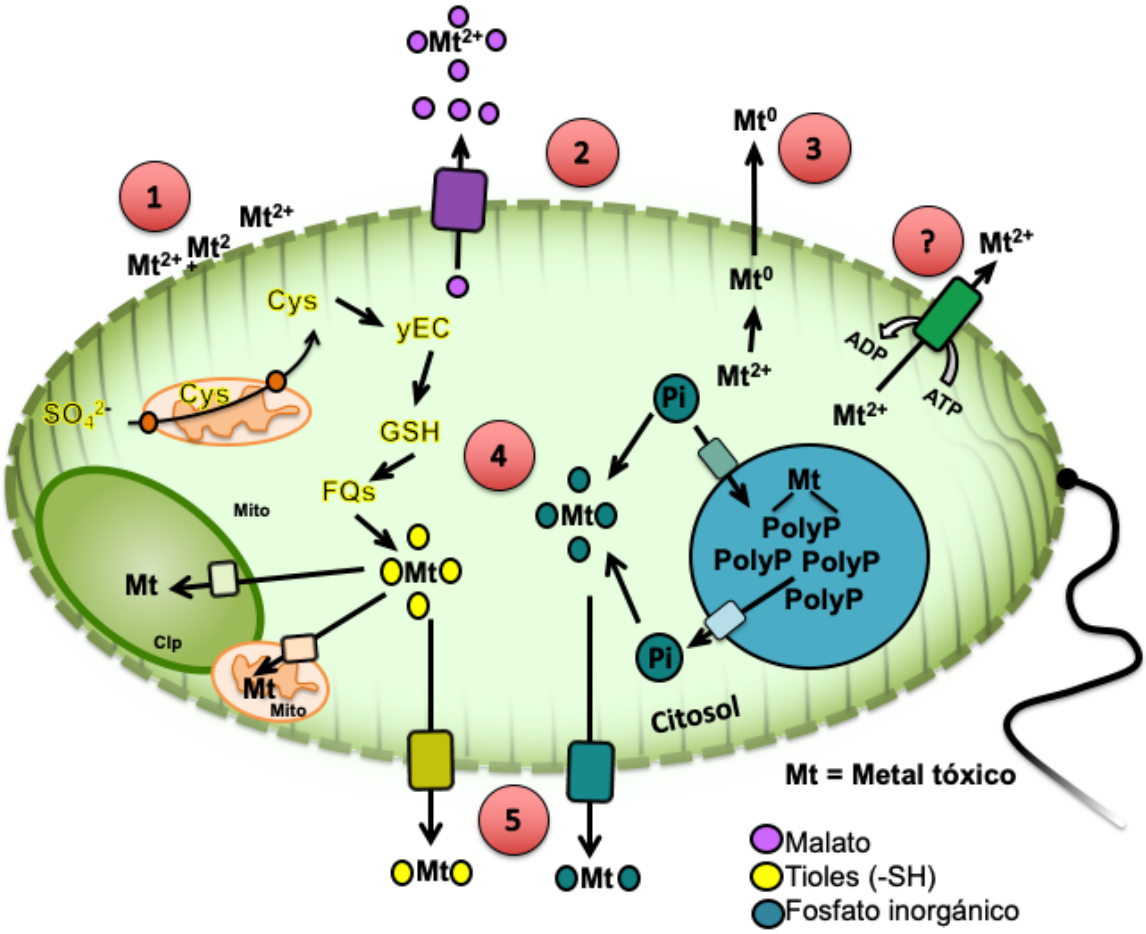

Figura 2. Mecanismos de resistencia y acumulación de metales pesados en *E. gracilis*.

(1) Adsorción del metal tóxico (Mt^{2+}) a la membrana extracelular y/o a la película (Cd^{2+}, Ni^{2+}, Zn^{2+}); (2) quelación extracelular (Cr^{6+}); (3) biotransformación del Mt^{2+} por procesos de oxido-reducción (Hg^{2+} y Cr^{6+}); (4) unión intracelular con moléculas con grupos tiol o fosfato, junto con la compartamentalización de los Mt en el cloroplasto, la mitocondria y posiblemente en los acidocalcisomas (Cd^{2+}); (5) Excreción de los complejos metálicos asociados a moléculas quelantes (Ni^{2+}). Elaboración propia (RST), 2020.

2. ANTECEDENTES

2.1 Estrategias para favorecer la acumulación de Cd^{2+} en *E. gracilis*

La toxicidad del Cd^{2+} puede estar relacionada con la cantidad de iones esenciales en el medio de exposición, ya sea en el medio de cultivo o en el medio ambiente. Existen antecedentes que indican que el Zn^{2+} es un metal que puede proteger de la toxicidad del

Cd^{2+}, ya que el Zn^{2+} es un metal esencial que está involucrado en la regulación del estrés oxidante, la síntesis de proteínas y la homeostasis de otros iones esenciales (Pan *et al*., 2017). Por ejemplo, en la microalga verde *C. reinhardtii* y la planta acuática *Ceratophyllum demersum* L. la exposición a 10 μM Cd^{2+} en presencia de 200 μM Zn^{2+}, disminuye la acumulación del Cd^{2+}, preserva la fotosíntesis y aumenta la actividad de las enzimas antioxidantes (SOD, CAT y APx) y del metabolismo del GSH (Aravind y Prasad, 2004, 2005; Aravind *et al*., 2009; Lavoie *et al*., 2012). De manera similar, otros estudios indican que el pre-acondicionamiento con 100 μM de Zn^{2+} disminuye la toxicidad por 200-400 μM de Cd^{2+} en la microalga verde *Dunaliella tertiolecta*, mediante la inducción de la síntesis de las FQ, las cuáles son indetectables en las células no pre-acondicionadas al Zn^{2+} (Tsuji *et al*., 2002; Tsuji *et al*., 2003).

En este sentido, el metabolismo de las moléculas con grupos tioles es uno de los mecanismos más explorados en el estudio de la acumulación de metales pesados en *E. gracilis* y otros organismos. Específicamente, se ha buscado aumentar la síntesis de las FQ para favorecer la acumulación del Cd^{2+}. Sin embargo, la sobre-expresión de la fitoquelatina sintasa (FQS) en plantas ha tenido resultados contrastantes y poco exitosos (Lee *et al*., 2003; Li *et al*., 2004; Gasic *et al*., 2007; Wojas *et al*., 2008), ya que a pesar de que se obtiene un aumento de hasta 3 veces en la cantidad de FQ, la acumulación del Cd^{2+} no se ve favorecida y sólo algunas plantas resultan ser más tolerantes a la exposición al Cd^{2+},mientras que otras presentan hipersensibilidad al mismo. Cabe resaltar que en estos trabajos basados en metodologías de la ingeniería genética se consiguió aumentar la cantidad de enzima en las plantas, al igual que su actividad (≈5 veces). Sin embargo, para favorecer la acumulación del Cd^{2+} tal vez sea necesario incrementar simultáneamente otras enzimas implicadas en esta vía metabólica, cómo la γ-glutamilcisteína sintetasa y/o glutatión sintetasa, y así evitar la disminución de los niveles de GSH. El aumento en la actividad de la FQS también podría lograrse favoreciendo la disponibilidad de su sustrato, el GSH, con el fin de promover la síntesis de los compuestos quelantes y así obtener una mayor eficiencia y un aumento en la acumulación del Cd^{2+}.

Los estudios más recientes *in vitro* de la FQS de *E. gracilis* (*Eg*FQS) han mostrado que esta enzima es 2.6 veces más activa con Zn^{2+}que con Cd^{2+}, lo que significa que el bis-glutationato de Zn^{2+} (Zn-GS_2) es mejor sustrato que el bis-glutationato de Cd^{2+} (Cd-GS_2) (García-García *et al*., 2014). Esto sugiere que el Zn^{2+} y el Cd^{2+} son capaces de

activar mecanismos moleculares similares en *E. gracilis,* que están relacionados con la acumulación de los metales pesados. Sin embargo, hasta el momento no se había intentado usar al Zn^{2+}como parte de una estrategia metabólica para aumentar la capacidad de acumulación del Cd^{2+} en *E. gracilis*. Debido a esto, al inicio del proyecto se realizó un pre-acondicionamiento de las células de *E. gracilis* a distintas concentraciones de Zn^{2+}, con el objetivo de aumentar las concentraciones intracelulares de este metal esencial y favorecer la biosíntesis de las FQ, de tal forma que se indujera un fenotipo que aumentara la capacidad de acumulación del Cd^{2+}.

2.2 Principales resultados asociados a la Publicación 1.

El pre-acondicionamiento con 200, 300, 400, 500 y 1000 μM de Zn^{2+} en *E. gracilis* aumentó la acumulación de este metal, *e.g.* hasta 15 veces (~ 300 nmol/10^7 células en 5 días) en la condición de 400 μM de Zn^{2+} (*Eg*Zn_{400}) con respecto a las células control que son cultivadas en 20 μM de Zn^{2+}(*Eg*Zn_{20}) (Fig. 1A). A partir de estos resultados, se eligió a las células *Eg*Zn_{400} para evaluar la posible inducción en la síntesis de FQ y el aumento en la acumulación del Cd^{2+}. El Zn^{2+} generó protección contra la toxicidad del Cd^{2+}, *i.e.* favoreciendo su crecimiento en presencia del Cd^{2+}, al disminuir 4 veces la IC_{50} por este último (Fig. 1B). Además, el pre-acondicionamiento con Zn^{2+} aumentó significativamente la acumulación del Cd^{2+} (Fig. 1C), aunque esto no correlacionó con un aumento sustancial en la biosíntesis de polímeros de GSH totales (Fig.1D). A pesar de esto, el pre-acondicionamiento con Zn^{2+} si aumentó el contenido intracelular de Zn^{2+} y Ca^{2+} (datos manuscrito), lo cual pudo haber atenuado la toxicidad del Cd^{2+} al sustituir posibles sitios de unión susceptibles al Cd^{2+}.

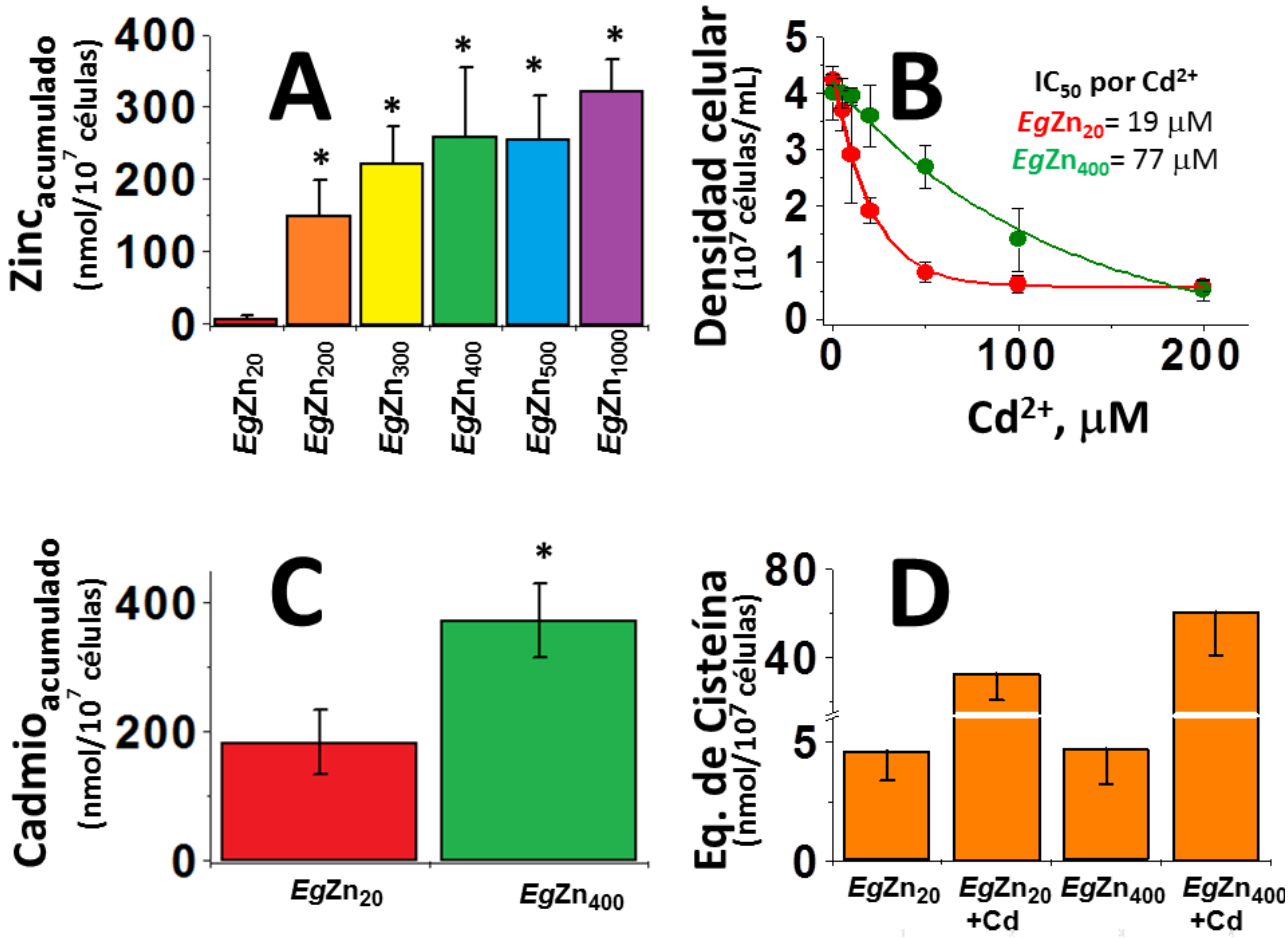

Figura 3. El pre-acondicionamiento con Zn^{2+} sobre la resistencia y la acumulación del Cd^{2+}.

(A) Acumulación del Zn^{2+} en células control (*Eg*Zn_{20}) y pre-acondicionadas con 200, 300, 400, 500 y 1000 μM de Zn^{2+} al final de la fase exponencial de crecimiento (5 días). (B) Densidad celular obtenida al 5° día de crecimiento en las células *Eg*Zn_{20} y *Eg*Zn_{400} expuestas a 5, 10, 20, 50 y 100 μM de Cd^{2+} para obtener una IC_{50} de este metal. (C) Acumulación del Cd^{2+}en las células *Eg*Zn_{20} y *Eg*Zn_{400} expuestas a 200 μM de Cd^{2+} durante 5 días. (D) Contenido intracelular de polímeros de glutatión totales en *Eg*Zn_{20} y *Eg*Zn_{400} expuestas a 200 μM de Cd^{2+} durante 5 días. Los valores mostrados representan el total de los polímeros de GSH detectados por HPLC en equivalentes de cisteína. Las barras verticales corresponden a la desviación estándar obtenida de al menos 5 determinaciones independientes. *$P< 0.05$ *versus* *Eg*Zn_{20} (ANOVA/*post hoc* Scheffé).

Dentro de los polímeros de GSH descritos, las "FQ canónicas" son metabolitos que pueden representarse con la fórmula $(\gamma\text{-Glu-Cys})_n$-Gly en donde n= 2-11, y que son las FQ más abundantes en plantas y tal vez en algunas microalgas. Sin embargo, en las plantas pertenecientes a la familia *Fabaceae* y *Poaceae* se han descrito otros polímeros de GSH que poseen una composición química diferente. En la Fig. 4A podemos observar que las "FQ no-canónicas" en lugar de presentar a la glicina en su estructura, presentan a la β-alanina (homo-fitoquelatinas; hFQ), la serina (hidroximetíl-fitoquelatinas; hmFQ) y al glutámico ó a la glutamina (isofitoquelatinas; isoFQ), para las cuales en vez de utilizar

GSH para su síntesis, se usa a el homo-glutatión (hGSH), a el hidroximetíl-glutatión (hmGSH) o a el iso-glutatión (isoGSH) (Meinhart, 1996; Oven *et al*., 2002; Sarry *et al*., 2006). En este trabajo se determinó que *E. gracilis* expuesta a 50- 200 μM de Cd^{2+} durante 8 días es capaz de sintetizar FQ canónicas que poseen de 2-4 motivos de glutatión, FQ_2, FQ_3 y FQ_4 (Fig. 4B). La detección y cuantificación de estos metabolitos permitió determinar que únicamente el 19-33% del total de polímeros de GSH sintetizados durante la exposición al Cd^{2+} correspondían a FQ canónicas, y que el otro 67-81% correspondía a otros polímeros de glutatión, posiblemente FQ no canónicas (hFQs, hmFQs e isoFQs).

Derivado de estos últimos resultados, al inicio del proyecto se decidió analizar el metabolismo de las FQ no cánónicas en *E. gracilis*. En este sentido, en extractos ácidos de *E. gracilis* expuesta al Cd^{2+}, se logró detectar la presencia de hGSH, hFQ_2, $hmFQ_2$ e $isoFQ_2$ (Fig. 4B), aunque no se observó la formación de polímeros más largos (*i.e.*, hFQ_3 y hFQ_4; $hmFQ_3$ e $hmFQ_4$; iFQ_3 e iFQ_4). Aquí cabe señalar que *E. gracilis* en condiciones control puede acumular hasta ≈300 nmol de $Cd^{2+}/10^7$ células después de 8 días de cultivo con 200 μM de Cd^{2+}, y que de los ≈240 nmol de moléculas con grupos tiol/10^7 células que sintetiza, únicamente ≈20 nmol/10^7 células corresponden a FQ canónicas y no-canónicas, lo restante corresponde a Cys, γ-EC, GSH y tripanotión (Trp) un polímero de GSH también presente en protistas que causan la tripanosomiasis (Fig. 4C). Belcastro *et al*., 2009 estimaron que para unir e inactivar por completo a un ión de Cd^{2+} eran necesarios cuatro grupos electronegativos. En consecuencia, una molécula de FQ_2 sólo puede unir un ión de Cd^{2+} pues se utilizan ambos grupos tiol de las cisteínas y los grupos carboxilo. Estos resultados, poco alentadores sobre la estequiometría entre la cantidad de las FQ sintetizadas y la cantidad de Cd^{2+} acumulado motivaron a un replantamiento del proyecto, pues aunque la síntesis de las FQ es uno de los mecanismos importantes para la acumulación del Cd^{2+}, este mecanismo solo inactiva una fracción pequeña del Cd^{2+} acumulado, lo cual denota la presencia de otros mecanismos cuantitativamente más importantes que contribuyen a la acumulación de este metal.

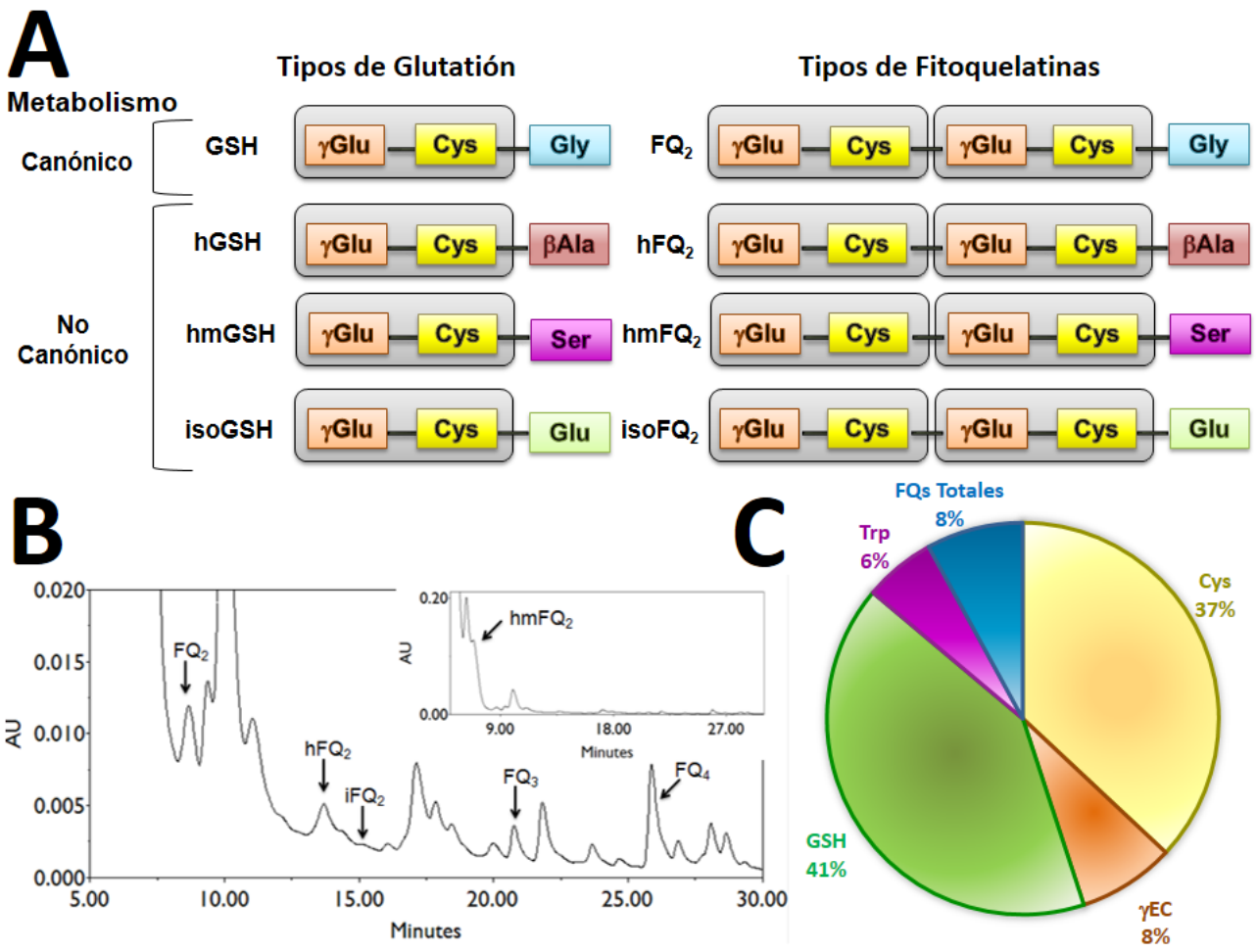

Figura 4. Caracterización de los polímeros de GSH sintetizados durante la acumulación del Cd^{2+} en *E. gracilis*.

(A) Esquema de la composición química de los diferentes tipos de GSH y FQ que se sintetizan durante la exposición al Cd^{2+} en plantas y algas. (B) Cromatograma de HPLC de la identificación de las FQ canónicas (FQ_2, FQ_3 y FQ_4) y de las no-canónicas (hFQ_2, iFQ_2 e $hmFQ_2$) en extractos ácidos de células de *E. gracilis* expuestas a 200 μM de Cd^{2+} durante 8 días. (C) Distribución porcentual del contenido de moléculas con grupos tiol en dichas condiciones.

E. gracilis resultó ser un microorganismo muy resistente al Zn^{2+}, además fue capaz de hiperacumular al Zn^{2+} y al Cd^{2+} (3000 mg Zn/Kg_{DW} y 100 mg Cd/Kg_{DW}; valores establecidos en Alí *et al*., 2003 para clasificar a un organismo como hipercumulador de dicho metal), ya que acumula 3,017 mg Zn/Kg_{DW} y 1,435 mg Cd/Kg_{DW}. Por otro lado, si se toma en cuenta los ≈200-300 nmoles acumulados de cada metal y el volumen intracelular de las células control de 2-4 μL/10^7cél y de las células expuestas a 100 μM de Cd^{2+} de 6 μL/10^7cél previamente reportado en *E. gracilis* (Avilés *et al.,* 2003), se puede calcular que la concentración intracelular que se alcanza en estas condiciones es ≈50-150 mM de zinc sin generar efectos tóxicos aparentes, y ≈35-50 mM de cadmio, lo cual denota la

participación de mecanismos asociados a la regulación osmótica y la importancia de determinar cuáles son los mecanismos asociados con este fenotipo. Asimismo, este trabajo permitió observar que la cantidad de polyP en *E. gracilis* alcanza altas concentraciones (2,500 nmol eq. $PO_4^{2-}/10^7$ células), el cual es un metabolito que se ha relacionado con la quelación de metales pesados, con la homeostasis de iones esenciales y con mecanismos de osmoregulación en otros protistas (Moreno y Docampo, 2009). En este sentido, los datos sugieren la participación de otros mecanismos que aún no han sido descritos como importantes para la resistencia y acumulación del Cd^{2+} y otros metales. Por lo tanto, se decidió evaluar posibles mecanismos relacionados a la osmoregulación que no habían sido descritos anteriormente en este microorganismo.

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Accumulation of zinc protects against cadmium stress in photosynthetic *Euglena gracilis*

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ABSTRACT

To determine the interplay between zinc and cadmium on the heavy metal accumulation capacity of *Euglena gracilis*, the effects of increasing Zn^{2+} concentrations (13–65.4 ppm or 200–1,000 μM) were analyzed on growth; O_2 consumption; photosynthesis; ascorbate (APX) and glutathione peroxidase (GPX) activities; chlorophyll a and b (Chl a+b) content; essential metals, thiol-metabolites and polyphosphates (polyPs) levels; as well as on zinc and cadmium accumulation capacities. Control cells ($EgZn_{20}$; grown with 20 μM Zn^{2+}) showed a half-maximal inhibition of growth (IC_{50}) of 1,700 μM by external Zn^{2+}. O_2 consumption, and APX and GPX activities were unaltered by Zn^{2+} treatments. Cells cultured with 500 or 1,000 μM Zn^{2+} showed photosynthesis impairment but normal Chl a+b contents. Zn^{2+} preconditioning increased the intracellular contents of zinc (25–54 times) and calcium (2–27 times); thiol-metabolites and polyPs were only marginally altered. The growth of cells preconditioned to 400 μM Zn^{2+} ($EgZn_{400}$ cells) was less susceptible to Cd^{2+} than that of $EgZn_{20}$ cells, although no differences in photosynthesis and respiration were observed. In cells chronically grown with Zn^{2+}, the cadmium accumulation capacity was unchanged or slightly increased in the same culture media with high Zn^{2+}, and increased by 42–90% in media with 20 μM Zn^{2+}. The thiol-metabolites increased at similar levels in both $EgZn_{20}$ and $EgZn_{400}$ cells when further exposed to 200 μM Cd^{2+} and polyPs were at high levels independently of Zn^{2+} or Cd^{2+} treatments. It was concluded that chronic exposure to high Zn^{2+} (1) was innocuous for *E. gracilis* at concentrations lower than 0.5 mM and (2) promoted protection against Cd^{2+} toxicity and increased cadmium accumulation; and (3) these zinc effects involved GSH and polyPs metabolism and were associated with high intracellular zinc contents.

1. Introduction

Zinc is an essential heavy metal for humans although at high intracellular concentrations is linked to formation of β-amyloid plaques associated with Alzheimer's disease, neuronal death, and degeneration of pancreatic cells (Maret, 2013; Sekler et al., 2007). On the other hand, in water bodies polluted with metals, zinc concentrations are 2.1–134 ppm or 32–2,050 μM (Bervoets and Blust, 2003; Luís et al., 2011; Sarmiento et al., 2011), while maximal concentration ranges of some toxic metals are 0.2–1.76 ppm of cadmium [1.8–16 μM]; 0.01–2.1 ppm of chromium [0.2–40 μM]; 0.2–2.2 ppm of nickel [3–37 μM]; and 0.7–4 ppm of lead [3–19 μM] (Bervoets and Blust, 2003; Luís et al., 2011; Rehman et al., 2007; Sarmiento et al., 2011; Vázquez-Suaceda et al., 2012). In comparison, maximal reported metal concentrations in polluted soils are 13,231 ppm for zinc, 1,896 ppm for cadmium, 1,028 ppm for chromium, 13,267 ppm for lead, and 4,710 ppm for nickel (Broadhurst and Chaney, 2016; Özkul, 2016; Yanqun et al., 2005).

The simultaneous exposure to Zn^{2+} (>13 ppm or 200 μM) and Cd^{2+} (0.0079–1.1 ppm or 0.007–10 μM) induces in the green alga *Chlamydomonas reinhardtii* and free-floating aquatic plant *Ceratophyllum demersum* L. lower cadmium accumulation (up to 85%), preservation of photosynthesis, and enhanced activities of antioxidant (SOD, catalase, ascorbate peroxidase) and GSH metabolism (GSH-S-transferase and GSH peroxidase) enzymes (Aravind et al., 2009; Aravind and Prasad, 2004, 2005; Lavoie et al., 2012b). Wheat (*Triticum aestivum* L.) seedlings acclimated with

Abbreviations: APX, ascorbate peroxidase; Chl a+b, chlorophyll a and chlorophyll b; DW, dry weight; *Eg*PCS, phytochelatin synthase from *E. gracilis*; GPX, glutathione peroxidase; GSH, glutathione; γ-EC, γ-glutamylcysteine; IC_{50}, half maximal inhibitory concentration for cell growth; PolyGSH, polymers of glutathione; PCs, phytochelatins; PolyPs, polyphosphates; ppm, parts *per* million.

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0.32 ppm (5 μM) zinc for 6 days also show decreased cadmium accumulation and increased catalase and SOD activities (Li and Zhou, 2012).

In contrast, the marine green alga *Dunalliela tertiolecta* preconditioned with 6.5 ppm (100 μM) zinc for 12 h was more resistant than control cells to 22.4–45 ppm (200–400 μM) cadmium, 2–5 ppm (10–25 μM) mercury, 0.37–3.74 ppm (5–50 μM) arsenate, 3.2–6.3 ppm (50–100 μM) copper, and 207.2 ppm (1,000 μM) lead (Tsuji et al., 2002). Protection against metals toxicity by zinc was attributed to increased biosynthesis of phytochelatins (PCs), which were undetectable in non-preconditioned cells (Tsuji et al., 2002). PCs are glutathione polymers that bind and inactivate heavy metals intracellularly, and were firstly described in yeast (Kondo et al., 1983; Murasugi et al., 1981), although nowadays it is known that are biosynthesized in plants, worms, yeasts, algae and protists exposed to cadmium, zinc or other essential and non-essential heavy metals and metalloids (Bräutigam et al., 2011; Brunetti et al., 2011; Clemens, 2006; Cobbett, 2000; García-García et al., 2014; Grill et al., 1985; Heiss et al., 2002; Hirata et al., 2001; Huang et al., 2012; Li et al., 2004, 2006; Oven et al., 2002; Ramos et al., 2007; Ray and Williams, 2011; Rea, 2012; Sarry et al., 2006; Tennstedt et al., 2009; Tsuji et al., 2003).

The high cadmium accumulation capacity described for the protist *E. gracilis* (4–9 mg/g dry weight) depends on an active PCs synthesis, in which Cys and GSH are key precursors and phytochelatin synthase (*Eg*PCS) is one of the key enzymes (García-García et al., 2012; Mendoza-Cózatl et al., 2002; Mendoza-Cózatl and Moreno-Sánchez, 2006a). Exposure of *E. gracilis* to Cd^{2+} (5.6 ppm or 50 μM) and Zn^{2+} (0.32–19.6 ppm or 5–300 μM) simultaneously for 8 days shows that the cadmium accumulation capacity is not affected (83–125 nmol Cd/10^7cells) by external Zn^{2+} (Mendoza-Cózatl et al., 2006b), suggesting that Zn^{2+} does not interfere with Cd^{2+} uptake and accumulation in this freshwater unicellular microorganism. In this regard, it has recently been described that the zinc-*bis*-glutathionate (Zn-GS_2) complex is the best co-substrate of *Eg*PCS over Cd-GS_2 (García-García et al., 2014).

Thus, as Zn^{2+} and Cd^{2+} are able to activate the same molecular mechanisms in *E. gracilis*, it seems plausible that chronic exposure (by more than 10 cell generations) to high Zn^{2+} concentrations could enhance both the cadmium accumulation and resistance in a process mediated by increased biosynthesis of PCs, which is a biotechnologically relevant feature of this microorganism for bioremediation of Cd^{2+} polluted aquatic systems. This hypothesis was assessed by determining the chronic effects of 13–65.4 ppm (200–1,000 μM) Zn^{2+} on several physiological parameters and zinc and cadmium accumulation capacities of *E. gracilis*.

2. Materials and methods

2.1. Growth conditions

Axenic photo-heterotrophic cultures of *Euglena gracilis* Klebs (a Z-like strain) were grown in acidic (initial pH 3.5) Hutner medium, as previously reported (García-García et al., 2012). This medium contained 34 mM glutamic acid, 15 mM malic acid, 2 mM $CaCO_3$, 0.007 mM $CoCl_2$, 0.003 mM $CuSO_4{\cdot}5H_2O$, 2 mM $MgSO_4{\cdot}7H_2O$, 0.01 mM $MnSO_4{\cdot}4H_2O$, and 0.02 mM $ZnSO_4{\cdot}7H_2O$ among other components. Inocula of 0.2×10^6 cells/mL from cultures of 5 days old (at the end of the exponential growth phase) were used to initiate a new culture. Cells grown in this culture medium (with 20 μM Zn^{2+}) were considering control cells and labeled as $EgZn_{20}$. The laboratory *E. gracilis* strain used in the present study proceeded from the collection of the Parasitology Department, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. This strain was grown in culture media containing 300 μM $ZnCl_2$ for over 20 years. These cells, further cultured with 20 μM $ZnCl_2$ for over 8 years, were used in the experiments described in this work.

The growth medium with 20 μM Zn^{2+} (control medium) was further supplemented with different aliquots from $ZnCl_2$ stock solutions (163–272 mM), calibrated by atomic absorption spectrophotometry (AAS) and sterilized by ultra-filtration through 0.22 μm pore diameter sterile cellulose esters membranes (Millipore, Bedford, MA, USA), to achieve 200–3,000 μM Zn^{2+}. Cell inocula were added after fixing the indicated final Zn^{2+} concentrations. For Zn^{2+} preconditioning, cells were cultured at the indicated Zn^{2+} concentration and a cell aliquot transferred to fresh medium with the same Zn^{2+} concentration every 5 days for at least 2 subsequent sub-cultures. *E. gracilis* cells preconditioned to 200, 300, 400, 500 and 1,000 μM Zn^{2+} were labeled as $EgZn_{200}$, $EgZn_{300}$, $EgZn_{400}$, $EgZn_{500}$, and $EgZn_{1000}$, respectively.

Cell cultures with 1–500 μM $CdCl_2$ were carried out as described above. The $CdCl_2$ stock solutions were also calibrated by AAS, sterilized by filtration, and added to the culture medium before the cell inoculum. It should be noted that the $ZnCl_2$ and $CdCl_2$ concentrations used in the present study yield fully ionized solutions with negligible binding by the culture medium components because the culture medium initial pH is 3.5, and remains in the acidic range (pH of 5–6) after 5–8 days of cell culture.

The incubation conditions for all cell cultures were cycles of 12 h light (70 μmol quanta $m^{-2}\,s^{-1}$)/12 h dark at 20–25 °C. Cell growth and viability were determined by counting HCl-immobilized cells with a hemocytometer and incubating with 0.05% (w/v) trypan blue for 2 min at 25 °C, respectively.

2.2. Determination of dry weight

Dry weight was determined for $EgZn_{20}$, $EgZn_{200}$, $EgZn_{300}$, $EgZn_{400}$, $EgZn_{500}$ and $EgZn_{1000}$ cells grown for 5 and 8 days under control conditions and in media supplemented with 200 μM Cd^{2+}. Cells were harvested by centrifugation for 2 min at 1,464 *g* and 4 °C and washed with SHE buffer (225 mM Sucrose, 10 mM HEPES, 1 mM EGTA pH 7.3). Thereafter, cells were counted and aliquots of $0.5–2 \times 10^8$ cells were dried at 60 °C for 24 h, although no changes in dry weight (DW) were observed after 6 h heating. The equivalences obtained (see Table 3) were similar to values previously reported (Buetow and Levedahl, 1962).

2.3. Determination of the rates of photosynthesis and respiration

Cells were harvested by centrifuging for 1 min at 1,464 *g* and 4 °C and washed with KME buffer (120 mM KCl, 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 1 mM ethylene glycol-bis(aminoethyl ether)-*N*,*N*'-tetra acetic acid (EGTA), pH 7.2. The cells were re-suspended in KME buffer and stored in ice. A cell aliquot ($1–2 \times 10^7$ cells/mL) was added to the oxymeter chamber (which was placed inside a dark box) that contained KME buffer at 25 °C. A Clark-type electrode was used to determine the oxygen consumption/production rates. Initially, it was registered the cellular O_2 consumption rate in the dark (2–5 min), followed by irradiating a white light beam (10,000 μquanta $m^{-2}\,s^{-1}$) to the cellular suspension to determine the maximal cellular O_2 production rate (*i.e.*, photosynthesis). Thereafter, light was turned off to repeat once the same dark/light cycle to determine the rates of respiration and photosynthesis.

2.4. Determination of ascorbate peroxidase (APX) activity

Cells grown under control conditions, preconditioned to Zn^{2+} and $EgZn_{20}$ cells exposed to 200 μM Cd^{2+} were harvested after 5 days by centrifugation for 5 min at 1,464 *g* and 4 °C, and washed

twice with 50 mM KH_2PO_4 pH 6.3. The cellular pellet (100–200 × 10^7 cells for $EgZn_{20}$ cells and cells preconditioned to Zn^{2+}, and 60–120 × 10^7 cells for $EgZn_{20}$ cells exposed to 200 μM Cd^{2+}) was re-suspended in 50 mM KH_2PO_4 pH 6.3 and disrupted by 2 cycles of 1 min sonication (20% of maximal output in a Branson sonicator using a 3 mm tip diameter probe) with 1 min rest in ice. The cell extract was centrifuged for 10 min at 12,298*g* and 4 °C. The supernatant was then centrifuged for 45 min at 181, 213*g* and 4 °C; this second supernatant was considered the cytosolic-enriched fraction.

For determination of ascorbate peroxidase (APX) activity, 0.01–0.35 mg protein/mL of the cytosolic fraction was mixed with 0.2–0.6 mM L-ascorbate and 0.1 mM H_2O_2 in 50 mM KH_2PO_4 pH 6.3 at 32 °C (Ishikawa et al., 1996). Activity was determined from the consumption of L-ascorbate by APX following the absorbance change at 285 nm ($\varepsilon = 5.8\ mM^{-1}\ cm^{-1}$). Unspecific activity observed in mixtures without cytosol was subtracted from all determinations. APX activity was negligible in reaction mixtures without H_2O_2 or in presence of 0.1 mM cumene hydroperoxide.

2.5. Determination of GSH peroxidase (GPX) activity

The reaction mixture contained 0.16–0.9 mg protein of cytosolic-enriched fraction/mL, 0.8–1.3 mM GSH, 0.1 mM H_2O_2 and 1 mM EDTA in 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 8 at 32 °C. Activity was determined by consumption of GSH by GPX using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay (Overbaugh and Fall, 1985). At different times (1–5 min) an aliquot of reaction was used to oxidize the DTNB reagent following the total change in absorbance at 412 nm ($\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$). Activity non-attributed to GPX activity (reaction mixture without cytosol) was subtracted from all determinations. GPX activity was negligible in reaction mixtures without H_2O_2.

2.6. Determination of chlorophyll

Contents of chlorophylls a and b (Chl a+b) were determined as described elsewhere (Arnon, 1949). Cell samples of 0.1–1.3 × 10^6 cells were used to extract Chl a+b with 80% (v/v) acetone. Cellular debris was eliminated by centrifugation for 5 min at 1,464 *g* and 4 °C, and the supernatant was recovered and kept in ice until use.

2.7. Intracellular metal contents

Cells were harvested by centrifugation and washed twice with 0.1 M KH_2PO_4 pH 7.2 *plus* 5 mM EGTA. The cells were re-suspended in distilled water and digested with H_2SO_4/HNO_3 for 2 h at 100 °C. Intracellular cadmium, calcium, cobalt, cupper, iron, magnesium, manganese, and zinc were determined in the digested cell samples by atomic absorption spectrophotometry (Varian SpectrAA-640, Australia).

2.8. Determination of acid soluble thiol-metabolites

Cells were harvested, washed and stored (2–5 × 10^7 cells/mL) at −72 °C in the presence of 5 mM dithiothreitol (DTT) until use. Cells were thawed in the presence of 0.1% (v/v) Triton X-100 at room temperature for 20 min, and then mixed with 2.7% (v/v) perchloric acid (PCA), vigorously stirred for 1 min, and centrifuged for 2 min at 20,817*g* and 4 °C. The supernatants were recovered and filtered through a Millex-Millipore filter (0.45 μm pore diameter). A filtered supernatant aliquot was further mixed with PCA to a final concentration of 3% (v/v), and analyzed by HPLC/Vis (Waters 1525, Milford, MA, USA), using a reverse-phase C-18, 3.5 μm column of 4.6 mm x 75 mm (Symmetry, Waters, MA, USA). The column was equilibrated with a buffer composed of 99% trifluoroacetic acid (TFA; Sigma, St. Louis, MO, USA) in 0.1% (v/v) water, *plus* 1%(v/v) acetonitrile (JT Baker, Mexico) using a flow rate of 1 mL/min. Thiol-metabolites were eluted from the column by applying a discontinuous gradient as previously described (García-García et al., 2012). Thiol-metabolites were post-column derivatized with 0.1 mM DTNB dissolved in 0.1 M KH_2PO_4 pH 8 and detected at 412 nm (Waters 2489, USA). Mixtures of Cys, γ-EC, GSH, and PC_2, PC_3 and PC_4 (AnaSpec, Fremont, CA, USA) as internal standards were used to identify and quantify the thiol-metabolites in the cellular samples.

2.9. Determination of polyphosphates (polyPs)

PolyPs were determined using the following formula: polyPs = Total phosphate − Free phosphate.

Total phosphate was determined, as previously reported (Jasso-Chávez et al., 2015), in aliquots of 6–50 × 10^6cells, which were harvested by centrifugation and washed twice with 50 mM Tris pH 7.2. Cells were digested with 3% (v/v) PCA at 90 °C by 2 h. Thereafter, the lysates were immediately incubated in ice for 10 min, and further spun down to discard undigested material. Aliquots of supernatant were mixed with 1 mL of water and 1 mL of Solution A (0.25% (w/v) $CuSO_4{\cdot}5H_2O$, 4.6% (w/v) $CH_3COONa{\cdot}3H_2O$ and 2 N acetic acid, pH 4). The mixtures were strongly vortexed for 1 min, and then further additions of 2 mL water, 0.5 ml Solution B [5% (w/v) $(NH_4)_6Mo_7O_{24}.4H_2O$] and 0.5 ml Solution C [2% (w/v) 4-(methylamino)phenol hemisulfate and 5% (w/v) $Na_2S_2O_5$] were carried out. This last solution was vigorously agitated and incubated for 10 min at room temperature (~25 °C). Finally, the absorbance of this solution was measured at 870 nm. Phosphate was not detected in a mixture reaction with solutions A, B and C but without cells. Addition of external Cys (1 and 10 mM) to the complete reaction mix did not interfere with phosphate determination.

Free phosphate was determined following the previous protocol but digestion by perchloric acid was omitted and a centrifugation step at 3,880 *g* for 10 min at 4 °C was carried out before determining the supernatant absorbance. Analytical standard solutions of H_3PO_4 (Sigma-Aldrich, St. Louis, MO, USA) were used to calibrate the Total and Free phosphate determinations.

3. Results

3.1. Cell susceptibility to Zn^{2+}

Control cells ($EgZn_{20}$) cultured with 0.5 mM or lower Zn^{2+} concentrations showed unaltered growth rates and cell yields (Fig. 1A). At higher concentrations, the estimated half maximal inhibitory concentration of Zn^{2+} for cell growth (IC_{50Zn}) after 5 days was 1.7 mM Zn^{2+} (calculated from Fig. 1B).

Zn^{2+} concentrations around this $IC_{50\ Zn}$ value were chosen to carry out the Zn^{2+} preconditioning protocol. $EgZn_{20}$ cells were continuously cultured (at least for 2 subsequent subcultures) in the presence of different Zn^{2+}concentrations. The cell densities reached in the stationary growth phase (after 5 days culture) at each Zn^{2+} concentration (200–1,000 μM Zn^{2+}) remained constant after the first subculture (Fig. S1). The rates of cellular O_2 consumption, as a measurement of the mitochondrial functionality, were also unchanged at the different Zn^{2+} concentrations used (Fig. 1C). In contrast, the rates of cellular O_2 production, as a measurement of photosynthesis, decreased in cells cultured with 500 (moderately) and 1,000 μM Zn^{2+} (drastically) (Fig. 1C). Accordingly, the Chl a+b content was not significantly affected at 500 μM Zn^{2+} and decreased by 30% at 1,000 μM Zn^{2+} (Fig. 1D).

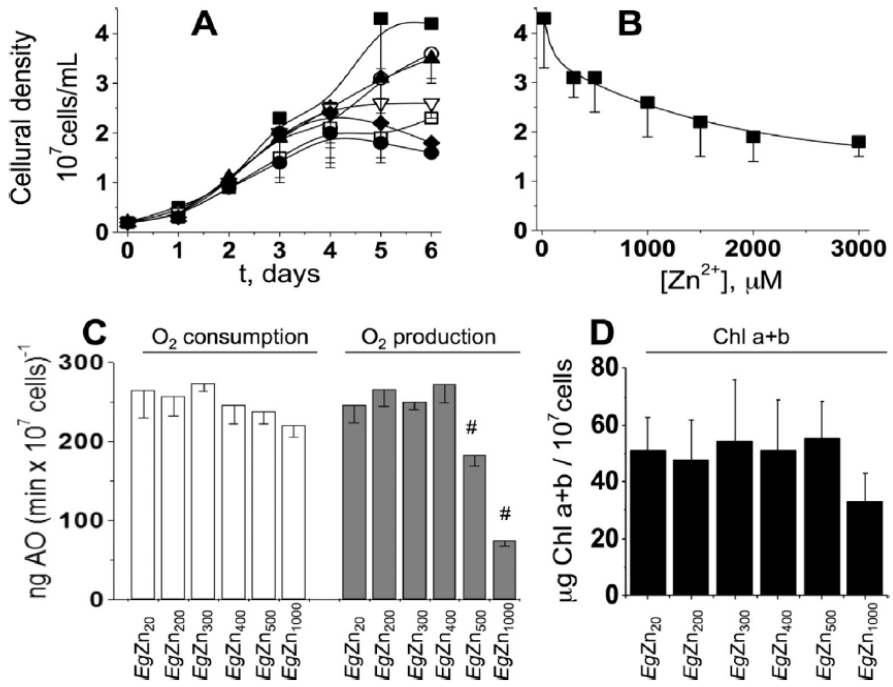

Fig. 1. Susceptibility of *E. gracilis* to Zn^{2+} and cellular functional status after Zn^{2+} preconditioning.
(A) Growth of control cells ($EgZn_{20}$) in the presence of 20 (■), 300 (○), 500 (▲), 1,000 (▽), 1,500 (◆), 2,000 (□) and 3,000 (●) μM Zn^{2+}. (B) Cell densities reached at different $ZnCl_2$ concentrations in the culture medium after 5 days (early stationary growth phase). Subsequently, $EgZn_{20}$ cells and cells preconditioned (*i.e.*, cells maintained at the indicated Zn^{2+} concentration for at least 2 subcultures) to 200 ($EgZn_{200}$), 300 ($EgZn_{300}$), 400 ($EgZn_{400}$), 500 ($EgZn_{500}$), and 1,000 ($EgZn_{1000}$) μM Zn^{2+} were used to determine (C) the rates of O_2 consumption (white bars), production (photosynthesis; gray bars) and (D) content of Chl a+b. Abbreviations: ng AO = nanogram atom of oxygen; Chl a +b = chlorophyll a and chlorophyll b. The data shown represent the mean ± SD of 3 (A), 4 (B), 4–5 (C), and 4 (D) independent preparations. Student *t*-test for non-paired samples; $^{\#}P<0.005$ *versus* $EgZn_{20}$ cells.

Oxidative stress was indirectly assessed by determining the activities of the antioxidant enzymes APX and GPX in control cells ($EgZn_{20}$) and cells preconditioned to 400 ($EgZn_{400}$) and 1,000 ($EgZn_{1000}$) μM Zn^{2+}. APX activity was similar in $EgZn_{20}$ and $EgZn_{400}$ cells, and increased 46% in $EgZn_{1000}$ (Fig. 2A); while GPX activity was unchanged in $EgZn_{20}$, $EgZn_{400}$ and $EgZn_{1000}$ (Fig. 2B). In contrast, APX and GPX activities increased 79 and 280% in $EgZn_{20}$ cells exposed to 200 μM Cd^{2+} for 5 days.

The intracellular content of total zinc substantially increased (25–54 times) with increasing external Zn^{2+} concentrations (Fig. 3); however, no changes in dry weight were observed (Table 3). The intracellular levels of other essential metals were in parallel determined to examine whether the high Zn^{2+} concentrations used perturb essential metal ion homeostasis. Indeed, with the increasing zinc intracellular levels, the calcium content increased by 2–27 times whereas the cobalt content decreased by 30–50% (Fig. 3). The contents of copper, magnesium and manganese remained constant at the different Zn^{2+} concentrations, except for a doubling in the copper content at 1,000 μM Zn^{2+}.

Kinetic characterization of recombinant *Eg*PCS showed that Zn-*bis*-glutathionate (Zn-GS_2) > Cd-GS_2 and GSH were its best co-substrates for PC_2 formation (García-García et al., 2014). Therefore, increasing intracellular zinc could stimulate PCS activity and induce changes in the homeostasis of thiol-metabolites. However, the contents of Cys, γ-EC, GSH and canonical PCs (PC_2-PC_4) remained unaltered (Table 1). Furthermore, a detailed analysis of the different polymers of GSH (Fig. S2) revealed that intracellular contents of PC_2, PC_3, PC_4 and non-canonical polymers of glutathione (nc-polyGSH; presumably homo-, hydroxymethyl- and *iso*-phytochelatins) did not change in cells grown chronically with 200–1,000 μM Zn^{2+} (Table 1). The contents of trypanothione ($T(SH)_2$) also remained constant (Table 1). Contents of PC_2-PC_4 represented 4, 5.4, 6.4, 7.8, 9.1, 11.7% of total polymers of GSH (PC_2-PC_4 *plus* nc-polyGSH) in $EgZn_{20}$, $EgZn_{200}$, $EgZn_{300}$, $EgZn_{400}$, $EgZn_{500}$ and $EgZn_{1000}$ cells, respectively (Table 1). PC_4 was the most abundant canonical phytochelatin.

The intracellular contents of polyPs were also determined to analyze their contribution as metal chelators because they have been involved in homeostasis of zinc, calcium, magnesium, and potassium in green algae and protozoan parasites (Docampo and Moreno, 2008; Lemercier et al., 2004; Ruiz et al., 2001). Surprisingly, the intracellular content of polyPs in *E. gracilis* was relatively high, in the millimolar range, and was not drastically perturbed by preconditioning with Zn^{2+} (Fig. 4).

3.2. Cell susceptibility to Cd^{2+}

$EgZn_{20}$ and Zn^{2+}-preconditioned cells were further exposed to 1–500 μM Cd^{2+} to determine whether Zn^{2+} affects Cd^{2+} toxicity and accumulation in *E. gracilis*. The IC_{50} values for Cd^{2+} were 60 μM for *E. gracilis* cells preconditioned with 400 μM (Fig. 5A and B) Zn^{2+} ($EgZn_{400}$), and 16 μM (Fig. 5B) for control ($EgZn_{20}$) cells, as previously reported (García-García et al., 2012). Unexpectedly, the IC_{50Cd} was 5 μM (Fig. 5B) for $EgZn_{400}$ cells further cultured with 20 μM Zn^{2+} (Figs. 5B and S2).

The rates of O_2 consumption increased by 80–95%, while the rates of O_2 production were not modified (Fig. 5C) and the content

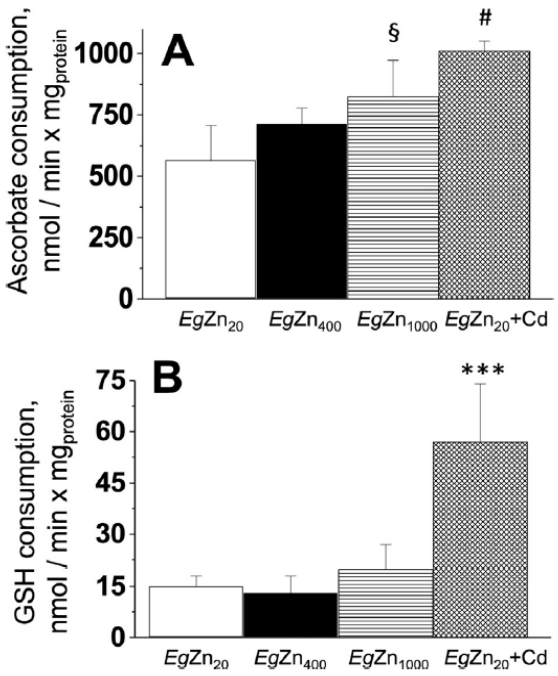

Fig. 2. Activities of APX and GPX in cells preconditioned to Zn^{2+} and stressed by Cd^{2+}.
(A) APX and (B) GPX activities were determined in $EgZn_{20}$, $EgZn_{400}$ and $EgZn_{1000}$ cells harvested after 5 days of growth as well as in $EgZn_{20}$ cells exposed to 200 μM Cd^{2+} for 5 days ($EgZn_{20}$ +Cd). The data shown represent the mean ± SD of at least four independent preparations. $^{\S}P < 0.05$; $^{\#}P < 0.005$ *versus* $EgZn_{20}$ cells; $^{***}P<0.001$ *versus* $EgZn_{20}$ cells.

of Chl a+b increased 2 times (Fig. 5D) in $EgZn_{20}$ and $EgZn_{400}$ cells exposed to 200 μM Cd^{2+} for 8 days.

3.3. Cadmium accumulation capacity

Intracellular accumulation of cadmium was significantly higher in $EgZn_{400}$ and $EgZn_{1000}$ cells *vs.* control $EgZn_{20}$ cells (Fig. 6A). When the high $ZnCl_2$ concentration used for pre-conditioning was removed and adjusted to only 20 μM for all cell cultures, the cadmium accumulation capacity was enhanced for all cells, with a maximum (doubling) reached by $EgZn_{400}$ cells (Fig. 6B) or $EgZn_{500}$ cells (Table 3). On the other hand, the zinc intracellular levels in Zn^{2+}-preconditioned cells under Cd^{2+} stress (i) increased with increasing $ZnCl_2$ concentrations reaching a maximum at 400 μM (Fig. 6A) or 1,000 μM (Table 3); and (ii) decreased by 40–80% at a homogeneously low $ZnCl_2$ concentration of 20 μM Zn^{2+} (Fig. 6B), as compared with the respective higher Zn^{2+} preconditioning concentrations (Fig. 3B). It should be noted that the DW values increased by 2–3 times in all cells exposed to Cd^{2+} independently of Zn^{2+} preconditioning regimen and further cultured with 20 μM Zn^{2+} (Table 3).

$EgZn_{20}$ and $EgZn_{400}$ cells were exposed to different Cd^{2+} concentrations to compare their intracellular cadmium accumulation capacities. Except for a significant increase of 99% at 200 μM Cd^{2+} in $EgZn_{400}$ cells, no other differences in cadmium accumulation were observed (Fig. 6C). The enhanced cadmium accumulation capacity of $EgZn_{400}$ cells was apparent after 2 days of exposure to 200 μM Cd^{2+} (Fig. 6D).

Homeostasis of divalent essential metal ions was perturbed by Cd^{2+} stress in both $EgZn_{20}$ and $EgZn_{400}$ cells. The calcium, cobalt and copper intracellular contents increased 2.7, 1.5 and 9.4 times, respectively, in $EgZn_{20}$ exposed to 200 μM Cd^{2+} for 8 days *vs.* $EgZn_{20}$ cells grown in the absence of Cd^{2+} (Fig. 7A; Table S1). Similarly, the copper and magnesium contents also increased, 11 and 2.8 times, while the calcium content remained high and unaltered, in $EgZn_{400}$ cells exposed to 200 μM Cd^{2+} for 8 days *vs.* $EgZn_{400}$ cells grown in the absence of Cd^{2+} (Fig. 7B; Table S1).

3.4. Intracellular levels of thiol molecules and polyPs

The biosyntheses of Cys, γ-EC, GSH, PCs, nc-polyGSH and $T(SH)_2$ were all induced by Cd^{2+} after 12 h of culture (Fig. 8) and correlated with increasing Cd^{2+} concentrations in $EgZn_{20}$ and $EgZn_{400}$ cells (Table 2). Cys and GSH achieved their maximal levels after 5 days of culture with Cd^{2+} (Figs. 8A and C), while the γ-EC maximal level was reached after 2 days (Fig. 8B). The contents of GSH, PCs, nc-polyGSH and $T(SH)_2$ were remarkably elevated in both $EgZn_{20}$ and $EgZn_{400}$ cells exposed to Cd^{2+} for 5 days *vs.* respective control cells (Fig. 8D; Table 2), correlating with their greater cadmium accumulation at the same time-point (Fig. 6D). In addition, at 200 μM Cd^{2+}, $EgZn_{400}$ cells showed higher PC_2-PC_4 and nc-polyGSH levels than $EgZn_{20}$ cells (Fig. 8D; Table 2). The PC_2-PC_4 contents were 6.4–22% of total PCs + nc-polyGSH molecules independently of Zn^{2+}-pretreatments and Cd^{2+} concentration.

The thiol molecules (Cys, γ-EC, GSH, PCs, nc-polyGSH, $T(SH)_2$)/accumulated cadmium ratios were high and sufficient (12-2.6) to warrant complete chelation and neutralization the toxic heavy metal ion at early culture times (3–24 h). In contrast, after 48 h and 120 h culture the thiol molecules/cadmium ratios of 1.8 and 1.5 (Figs. 6D and 8) indicated insufficient levels of thiol molecules for efficient neutralization of Cd^{2+}. Furthermore, after 192 h culture the ratio was ≈0.5, suggesting high Cd^{2+} toxicity or the synthesis of metal chelating molecules other than thiol-metabolites. Indeed, the intracellular content of polyPs was high and constant in the millimolar range in both $EgZn_{20}$ and $EgZn_{400}$ cells exposed to 200 μM Cd^{2+} for 8 days (Fig. 9). Further, $EgZn_{400}$ cells exposed to Cd^{2+} showed 55% (510 nmol phosphate equivalents/10^7cells) more free phosphate than non-stressed $EgZn_{400}$ cells.

4. Discussion

4.1. Accumulation of zinc does not induce severe damage to E. gracilis cells

Zinc and cadmium usually coexist in polluted water bodies and in their sediments (Augustynowicz et al., 2014; Luo et al., 2014; Magdaleno et al., 2014; Sharaf and Shehata, 2015; Zeng et al., 2015). In most cases, zinc concentrations are 18–131 times higher than those of cadmium (Augustynowicz et al., 2014; Luo et al., 2014; Sharaf and Shehata, 2015; Zeng et al., 2015). Although some other metals are present in polluted areas (chromium, cobalt, copper, lead, magnesium, and thallium) (Augustynowicz et al., 2014; Luo et al., 2014; Sharaf and Shehata, 2015; Zeng et al., 2015), the present study focused on the effects of chronic exposure to zinc on cadmium accumulation in *E. gracilis* because it has been documented that zinc can diminish this capacity in human cells, algae and plants (Aravind et al., 2009; Aravind and Prasad, 2004, 2005; Lavoie et al., 2012a,b; Li and Zhou, 2012; Tsuji et al., 2002). The zinc protection against cadmium toxicity in humans may have biomedical applications whereas, on the contrary, inhibition of cadmium accumulation by zinc would be an unfavorable side-effect for bioremediation purposes in plants and microorganisms.

The results of the present study showed that the protist *E. gracilis* was remarkably resistant to Zn^{2+}, with IC_{50} values in the millimolar range (using an acidic saline medium supplemented with glutamate and malate; see Materials and methods section for

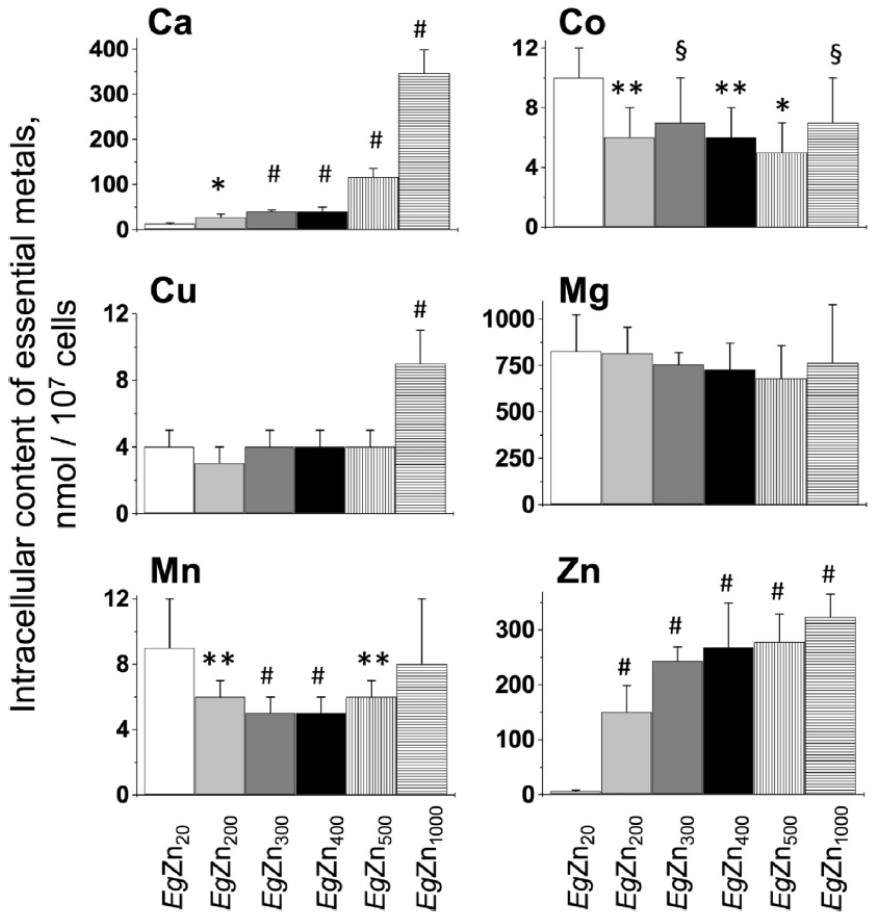

Fig. 3. Total content of divalent ion metals in *E. gracilis* preconditioned with Zn^{2+}.
Divalent metals (calcium, cobalt, copper, magnesium, manganese and zinc) were determined in cells harvested at the early stationary growth phase (5 days) by AAS. The data shown represent the mean ± SD of 3–6 independent preparations assayed. $^{\S}P < 0.05$; $^{**}P < 0.025$; $^{*}P < 0.01$; and $^{\#}P < 0.005$ *vs.* $EgZn_{20}$ cells.

further details). For comparison, several microalgae such as *Chlamydomonas reinhardtii*, *Chlorella ellipsoidea*, *Cyanidium caldarium*, *Ankistrodesmus fusiformis*, *Monoraphidium contortum*, and *Scenedesmus acuminatus*, mostly cultured in saline media at near-neutral pH with no carbon sources, are extremely sensitive to Zn^{2+} with IC_{50} values of 0.6–75 μM Zn^{2+} (Magdaleno et al., 2014; Mikulic and Beardall, 2014). As *C. ellipsoidea*, *A. fusiformis*, *M. contortum*, and *S. acuminatus* were isolated from polluted water bodies (Magdaleno et al., 2014), and hence these green algae have undergone population selection and resistance mechanisms have

Table 1
Contents of Cys, γ-EC, GSH, PC_{2-4} non-canonical polymers of GSH (nc-polyGSH), and tryopanothione ($T(SH)_2$) in *E. gracilis* cells preconditioned with increasing concentrations of Zn^{2+}.

	nmol Cys equivalents/10^7 cells							
	Cys	γEC	GSH	PC_2	PC_3	PC_4	nc-polyGSH	$T(SH)_2$
$EgZn_{20}$	27 ± 10 (5)	1.5 ± 0.3 (5)	16 ± 4 (5)	0.01 ± 0.005 (5)	0.03 ± 0.02 (5)	0.11 ± 0.06 (5)	3.6 ± 1.7 (5)	1.3 ± 0.7 (5)
$EgZn_{200}$	24 ± 6 (5)	2.1 ± 0.6 (5)	17 ± 5 (5)	0.01 ± 0.005 (5)	0.03 ± 0.03 (5)	0.11 ± 0.05 (5)	2.6 ± 1 (5)	1.8 ± 0.8 (5)
$EgZn_{300}$	20 ± 3 (4)	2.1 ± 0.6 (4)	16 ± 5 (5)	0.01 ± 0.007 (4)	0.03 ± 0.02 (4)	0.11 ± 0.08 (4)	2.2 ± 0.8 (4)	1.4 ± 1.1 (4)
$EgZn_{400}$	28 ± 8 (5)	2.8 ± 0.9 (5)	16 ± 8 (5)	0.02 ± 0.007 (5)	0.06 ± 0.03 (5)	0.15 ± 0.06 (5)	2.7 ± 0.6 (5)	2.7 ± 0.6 (5)
$EgZn_{500}$	22 ± 6 (3)	1.6 ± 0.4 (3)	15 ± 4 (3)	0.02 ± 0.03 (3)	0.03 ± 0.009 (3)	0.1 ± 0.04 (3)	1.5 ± 0.2 (3)	1.1 ± 0.9 (3)
$EgZn_{1000}$	30 ± 10 (3)	2.5 ± 0.8 (3)	20 ± 6 (3)	0.02 ± 0.01 (3)	0.03 ± 0.01 (3)	0.19 ± 0.11 (3)	1.8 ± 1.1 (3)	0.7 ± 0.3 (3)

Thiol-molecules were determined in cells grown for 5 days at the early stationary phase. PC_2, PC_3, PC_4, and $T(SH)_2$ were determined using internal standards as detailed in the *Materials and methods* section and Fig. S3. The data shown represent the mean ± SD of the number of independent preparations assayed between parentheses.

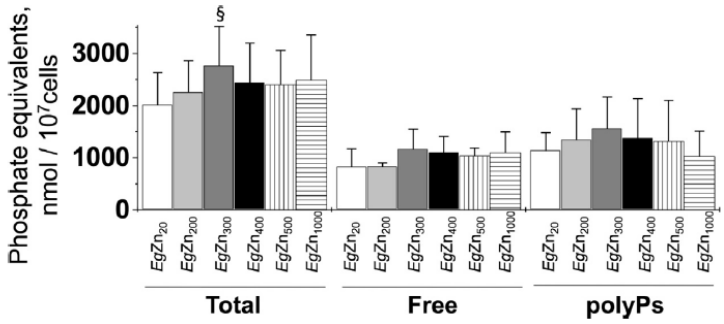

Fig. 4. Phosphated-molecules in *E. gracilis* preconditioned to Zn^{2+}.
Pi and polyPs were determined in cells grown for 5 days with the indicated Zn^{2+} concentration. The data shown represent the mean ± SD of at least four independent preparations assayed. $^{\S}P < 0.05$ *versus* $EgZn_{20}$ cells.

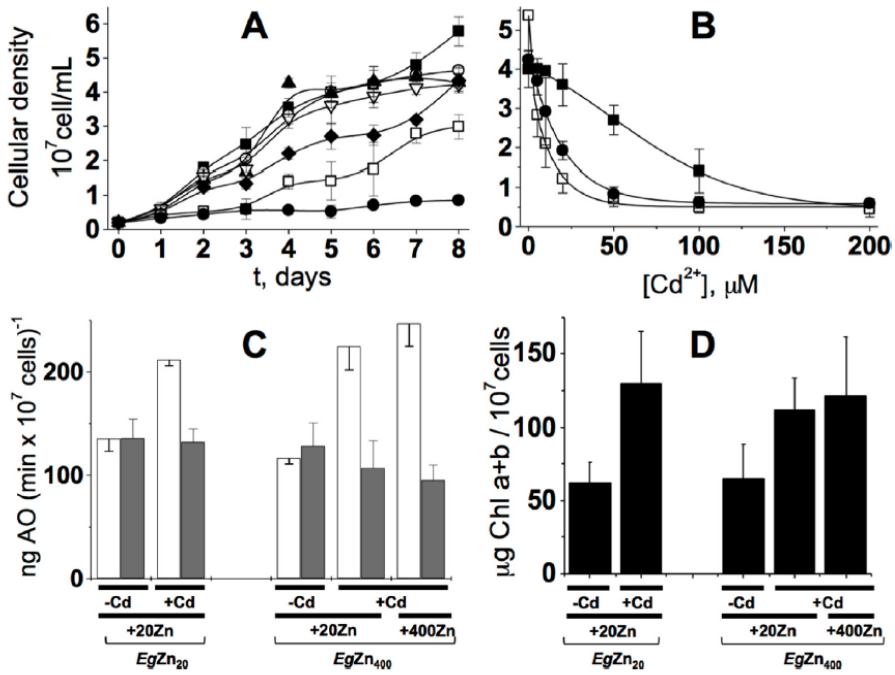

Fig. 5. Effect of chronic exposure to Zn^{2+} on Cd^{2+} susceptibility.
(A) Growth of $EgZn_{400}$ cells in the presence of 400 µM Zn^{2+} and with 0 (■), 5 (○), 10 (▲), 20 (▽), 50 (◆), 100 (□), and 200 µM (●) $CdCl_2$. (B) Cellular densities reached after 5 days (early stationary growth phase) in $EgZn_{20}$ (●) and $EgZn_{400}$ (■) cells exposed to Cd^{2+} (5–200 µM). $EgZn_{400}$ cells were also cultured in media with 20 µM Zn^{2+} (□). IC_{50} for Cd^{2+} was calculated by using a sigmoidal curve (■) or inverted hyperbole (□, ●). (C) Rates of O_2 consumption (white bars), production (photosynthesis; gray bars) and (D) content of Chl a+b were determined in $EgZn_{20}$ and $EgZn_{400}$ cells cultured without (−Cd) or with (+Cd) 200 µM Cd^{2+} for 8 days. $EgZn_{400}$ cells were cultured in media with 20 (+20Zn) or 400 (+400Zn) µM Zn^{2+}.

evolved, the differences in Zn^{2+} susceptibility *vs. Euglena* are quite remarkable. As the *E. gracilis* cells used in the present study proceeded from a laboratory strain cultured under optimal conditions for many years, it seems plausible that *E. gracilis* strains isolated from metal polluted sites might show even greater resistance to Zn^{2+} and other metal ions.

On the other hand, *E. gracilis* accumulated up to 3,017 mg zinc/Kg DW (Table 3), a value that exceeds the established worldwide standard reference concentration for accumulated zinc in plants (50 mg/Kg DW) (Ricachenevsky et al., 2015), as well as a previous classification that established 3,000 mg zinc/Kg DW as threshold for zinc-hyperaccumulators (Ali et al., 2013). Nevertheless, the zinc accumulation capacity of *E. gracilis* was lower than that reported for *Scenedesmus subspicatus* (6,539 mg zinc/Kg DW) exposed for 5 days to 10 µM zinc (Knauer et al., 1997). It should be noted that the zinc accumulation capacities determined in terrestrial plants have used soils or nutrient solutions that contain a broad range of zinc concentrations (0.065–600 ppm) (Jin et al., 2008; Lasat et al., 2000; Zhang et al., 2010); and the zinc concentrations used in the present study (1.3–65 ppm) were well within this range.

The cadmium accumulation capacity of *E. gracilis* (Table 3) also exceeded the respective standard reference for cadmium hyper-accumulators (100 mg cadmium/Kg DW; Ali et al., 2013), and was up to 33 times higher than the capacities determined for

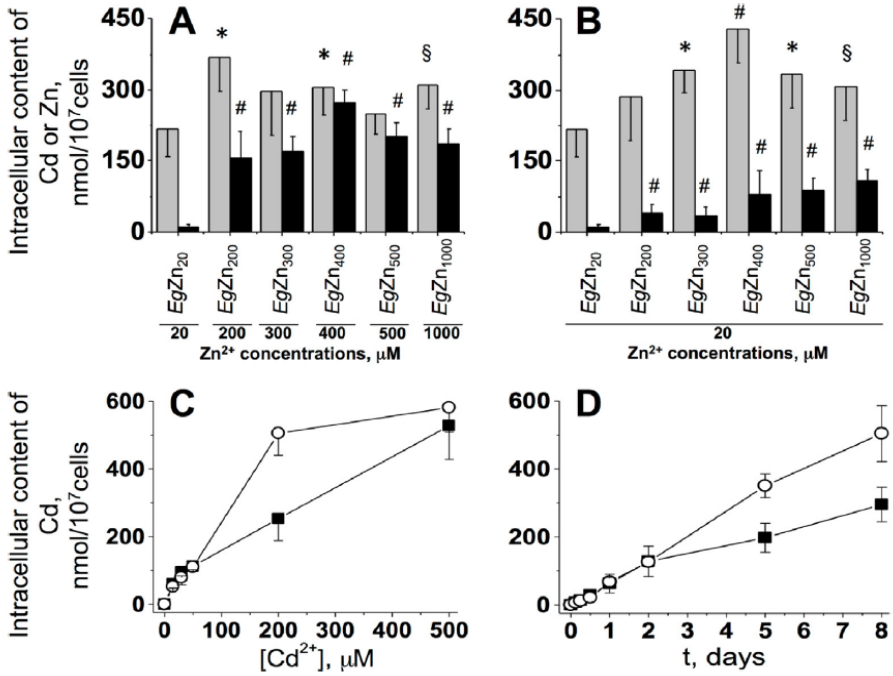

Fig. 6. Accumulation capacity of cadmium in Zn^{2+} preconditioned *E. gracilis* cells.
(A) Intracellular accumulation of cadmium (grey bars) and zinc (black bars) in cells exposed to 200 μM $CdCl_2$ for 8 days.
(B) Accumulations of cadmium and zinc were also determined in these Zn^{2+} pre-adapted cell cultures exposed to 200 μM $CdCl_2$ and a homogeneous $ZnCl_2$ concentration of 20 μM.
(C) Cadmium accumulation in $EgZn_{20}$ (■) and $EgZn_{400}$ (○) at increasing concentrations of Cd^{2+} for a fixed culture time of 8 days.
(D) Time-course of the cadmium accumulation in cells exposed to 200 μM Cd^{2+} in the presence of 20 μM $ZnCl_2$.
Vertical lines indicate the SD of at least 3 independent determinations. $^{§}P < 0.05$; $^{*}P < 0.01$; and $^{\#}P < 0.005$ *versus* $EgZn_{20}$ cells.

Chlamydomonas acidophila and *C. reinhardtii* (Nishikawa et al., 2006). Further, the cadmium accumulation capacities determined in plants have used high cadmium concentrations (1–200 ppm) (January et al., 2008; Shi and Cai, 2009; Zhang et al., 2010; Zhang et al., 2014); the cadmium concentrations used in the present study (5–22 ppm) were also well within this range.

Thus, the data of the present study suggested that *E. gracilis* may be considered as a zinc and cadmium hyperaccumulator microorganism, when exposed for a few days to high metal concentrations. *E. mutabilis* and *E. gracilis* seem dominant inhabitants of heavy metal polluted wastewaters derived from mining and other industrial activities (García-García et al., 2016; Olaveson and Nalewajko, 2000; Ruiz et al., 2004), and hence their zinc and cadmium accumulating capacities could be useful in bioremediation of wastewaters highly polluted by these metals.

Damage of photosynthesis by Zn^{2+} has been related to a decrease in the Chl a+b contents in algae and plants (Mikulic and Beardall, 2014; Subba et al., 2014), and to the replacement of Mg^{2+} by Zn^{2+} in the chlorophyll porphyrin ring where the Zn-chlorophyll derivatives are much less efficient for electron transfer (Gerola et al., 2011; Küpper et al., 2002). However, chlorosis was not apparent in $EgZn_{500}$ and the Chl a+b content decreased only by 30% in $EgZn_{1000}$ cells. Therefore, the replacement of Mg by Zn in the porphyrin ring or other unknown mechanisms were triggered by high Zn^{2+} to induce photosynthesis impairment.

Zn^{2+} (20–500 μM) seemed to be a weak oxidative stress inducer in *E. gracilis*, as judged by the negligible variation in the APX and GPX activities, which detoxify H_2O_2 in this catalase-lacking protist (Castro-Guerrero et al., 2008; Ishikawa et al., 1996; Shigeoka et al., 1980). GPX activity was 38 times lower than APX activity in *E. gracilis* control cells ($EgZn_{20}$), which was in agreement with previous reports (Ishikawa et al., 1996; Overbaugh and Fall, 1985; Shigeoka et al., 1980). In *Scenedesmus* sp., submerged plant *Hydrilla verticillata* and duckweeds (*Lemna gibba*, *Lemna minor* and *Spirodela polyrhiza*), increased APX activity correlates with increased malondialdehyde levels induced by Zn^{2+} (Tripathi et al., 2006; Uruç Parlak and Demirezen Yilmaz, 2012; Wang et al., 2009). On the other hand, increase of 46% in APX activity in *E. gracilis* preconditioned to 1,000 μM Zn^{2+} suggested induction of a moderate oxidative stress. In contrast, considerable induction of APX and GPX activities (79 and 280%) was observed in $EgZn_{20}$ cells exposed to 200 μM Cd^{2+}, which was in agreement with literature indicating that Cd^{2+} toxicity is associated with generation of severe oxidative stress in *E. gracilis*, algae and plants (Bajguz, 2010; Castro-Guerrero et al., 2008; Kumar et al., 2010; Singh et al., 2006).

As Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and the non-essential Cd^{2+} may share their plasma membrane transporters (Garnham et al., 1992; Reid et al., 1996), analysis of the contents of divalent essential metal ions was carried out to determine metal ion homeostasis perturbations by Zn^{2+}-preconditioning. The standard culture medium contained Co^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+} and Mg^{2+} (see Material and Methods section); thus, cellular uptakes of Co^{2+}, Cu^{2+} and Mn^{2+} could be compromised by Zn^{2+}-preconditioning. However, the intracellular levels of cobalt, copper, magnesium, and manganese were unchanged in $EgZn_{200}$, $EgZn_{300}$, $EgZn_{400}$, $EgZn_{500}$ and $EgZn_{1000}$ cells. Intracellular contents of calcium increased in all cellular cultures preconditioned with Zn^{2+}, suggesting that the homeostasis of Zn^{2+} and Ca^{2+} are linked in

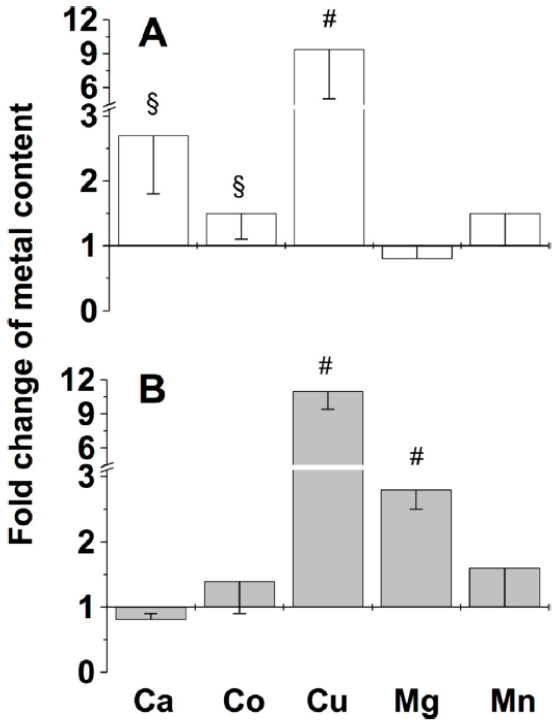

Fig. 7. Status of essential metals in $EgZn_{20}$ and $EgZn_{400}$ cells exposed to Cd^{2+}. The intracellular contents of calcium, cobalt, copper, magnesium and manganese were determined in (A) $EgZn_{20}$ and (B) $EgZn_{400}$ cells exposed to 200 μM Cd^{2+} for 8 days. The 1 value represents the normalized content of each metal (see Table S1 for absolute values). $^{§}P < 0.05$; $^{\#}P < 0.005$ *versus* $EgZn_{20}$ and $EgZn_{400}$ cells non-stressed by Cd^{2+}.

Euglena, and in eukaryotic cells (Colvin et al., 2010; Pitt and Stewart, 2015; Sharaf et al., 2015).

4.2. High intracellular contents of zinc (and calcium) provide protection to E. gracilis against Cd^{2+} stress

It has been described that zinc provides protection to algae, plants and mammalian cells against Cd^{2+} stress by inducing antioxidant enzymes (SOD, catalase, ascorbate peroxidase, GSH-S-transferase and GSH peroxidase) and inhibiting Cd^{2+} uptake (Aravind et al., 2009; Aravind and Prasad, 2004, 2005; Lavoie et al., 2012a,b; Li and Zhou, 2012; Tsuji et al., 2002).

Indeed, Zn^{2+} preconditioning conferred protection to *E. gracilis* growth against Cd^{2+} toxicity by enhancing the intracellular Zn^{2+} (Fig. 6A), thus shielding susceptible groups from interacting with Cd^{2+}. In contrast, removal of the high Zn^{2+}concentration used for preconditioning made the cell growth more sensitive to Cd^{2+}. As the cadmium accumulation was further increased in Zn^{2+} preconditioned cells by removal of the high Zn^{2+} in the culture medium (Fig. 6B and Table 3), it follows that the presence of a high external Zn^{2+} concentration, by inhibiting cellular Cd^{2+} uptake, also participates in the protection against Cd^{2+} stress (Lavoie et al., 2012a,b; Li and Zhou, 2012).

Therefore, to generate cells with enhanced capacity for cadmium accumulation, Zn^{2+} preconditioning followed by removal of high external Zn^{2+} should be applied. Or alternatively, to protect cells from Cd^{2+} stress, Zn^{2+} preconditioning and maintenance of the high external Zn^{2+} should be applied. Similarly, the high calcium contents in $EgZn_{400}$ cells could also help to attenuate the Cd^{2+} stress perhaps by a process mediated by a Ca^{2+}/Cd^{2+} exchange, as it has been reported in plants (Ahmad et al., 2015; Cho et al., 2012; Siddiqui et al., 2012).

In regard to the energy-related functions, the increased O_2 consumption rates and non-significant effects on photosynthesis induced by Cd^{2+} are in agreement with previous reports (Devars

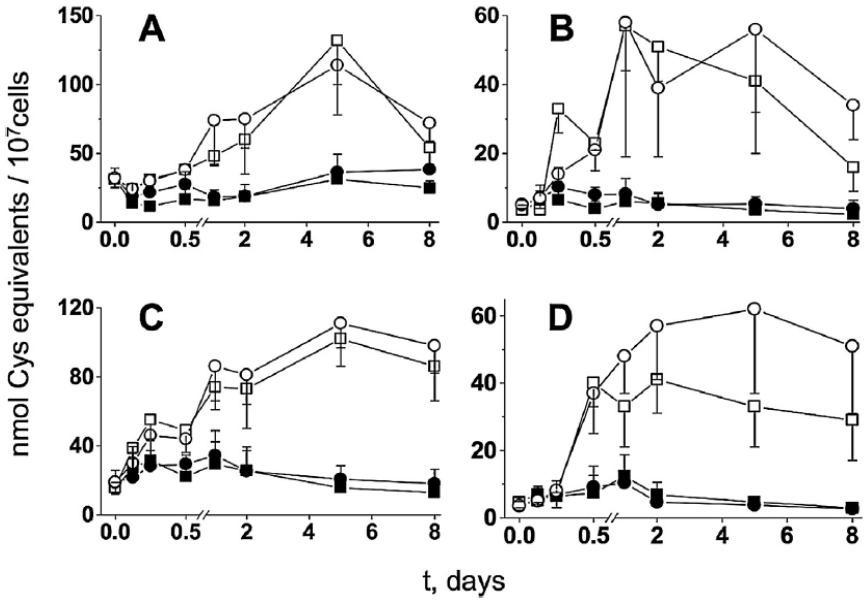

Fig. 8. Time-courses of the syntheses of Cys, γ-EC, GSH and GSH polymers in $EgZn_{20}$ and $EgZn_{400}$ cells exposed to Cd^{2+}. Contents of (A) Cys, (B) γ-EC, (C) GSH and (D) total polyGSH (PC_2-PC_4 *plus* nc-polyGSH) in $EgZn_{20}$ (squares) and $EgZn_{400}$ (circles) cells grown without (close symbols) and with 200 μM Cd^{2+} (open symbols). For these experiments $EgZn_{400}$ cells were transferred to culture media with 20 μM Zn^{2+}. The data shown represent the mean ± SD of at least three independent preparations assayed.

Table 2
Contents of Cys, γ-EC, GSH, PCs_{2-4}, nc-polyGSH and $T(SH)_2$ in $EgZn_{20}$ and $EgZn_{400}$ cells exposed to different Cd^{2+} concentrations for 8 days.

	nmol Cys equivalents/10^7 cells							
	Cys	γEC	GSH	PC_2	PC_3	PC_4	nc-polyGSH	$T(SH)_2$
$EgZn_{20}$	27 ± 3 (5)	2.3 ± 0.6 (5)	14 ± 5 (5)	0.03 ± 0.02 (5)	0.04 ± 0.02 (5)	0.11 ± 0.05 (5)	2.5 ± 0.8 (5)	0.4 ± 0.1 (5)
$EgZn_{400}$	36 ± 9§ (5)	2.1 ± 0.4 (5)	17 ± 7 (5)	0.05 ± 0.05 (5)	0.1 ± 0.09 (5)	0.15 ± 0.09 (5)	2.2 ± 1 (5)	0.4 ± 0.3 (5)
$EgZn_{20}$ + 50Cd	39 ± 10§ (3)	8.6 ± 5.2§ (3)	44 ± 17* (3)	0.2 ± 0.1* (3)	0.4 ± 0.2* (3)	1.3 ± 0.9** (3)	6.8 ± 1.7 (3)	0.9 ± 0.8 (3)
$EgZn_{400}$ + 50Cd	30 ± 6 (4)	6.1 ± 4.5 (4)	40 ± 17** (4)	0.04 ± 0.02 (4)	0.1 ± 0.1 (4)	0.3 ± 0.1 * (4)	6.4 ± 2.6 (4)	2.5 ± 1.7 (4)
$EgZn_{20}$ + 200Cd	50 ± 13§ (5)	12 ± 8§ (5)	73 ± 20# (5)	0.2 ± 0.1* (5)	1.1 ± 0.7* (5)	1.5 ± 0.4# (5)	13 ± 6 (5)	12 ± 7 (5)
$EgZn_{400}$ + 200Cd	71 ± 17§ (3)	17 ± 7§ (3)	68 ± 32* (3)	0.5 ± 0.4§ (3)	2.2 ± 0.9# (3)	3 ± 1.8** (3)	23 ±10 (3)	16 ± 2 (3)

Thiol-molecules contents were determined in acidic extracts from $EgZn_{20}$ and $EgZn_{400}$ cells grown for 8 days under 50 (50Cd) and 200 (200Cd) μM Cd^{2+}. The data shown represent the mean ± SD of the number of independent preparations assayed between parentheses. Student *t*-test for non-paired samples; #$P<0.005$; *$P<0.01$; **$P<0.025$; and §$P<0.05$ *versus* $EgZn_{20}$ cells.

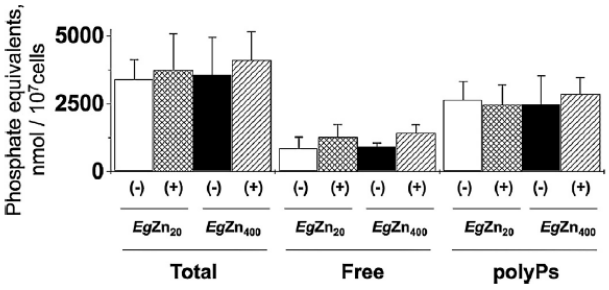

Fig. 9. Phosphated-molecules in *E. gracilis* preconditioned to Zn^{2+} and exposed to Cd^{2+}.
Pi and polyPs were determined in $EgZn_{20}$ and $EgZn_{400}$ cells non-stressed (–) and stressed with 200 μM Cd^{2+} (+) for 8 days. For these experiments $EgZn_{400}$ cells were transferred to culture media with 20 μM Zn^{2+}. The data shown represent the mean ± SD of at least five independent preparations assayed.

et al., 1998; Mendoza-Cózatl et al., 2002); and Zn^{2+} preconditioning did not prevent the Cd^{2+} effect on respiration. Stimulation of O_2 consumption could be linked to uncoupling of the respiratory chain activity from the ATP synthesis. Most (62%) of the cadmium accumulated in *E. gracilis* is neutralized and stored in chloroplasts after 8 days of exposure to 25–200 μM Cd^{2+} (conditions similar to those used in the present work), as Cd-High Molecular Weight Complex (Cd-HMWC) (Mendoza-Cózatl et al., 2002; Mendoza-Cózatl et al., 2006c), which correlated with the lack of effect on photosynthesis by Cd^{2+}.

4.3. Intracellular binding of cadmium by thiol-molecules and polyPs

It is well documented that cadmium (25–300 μM) induces the biosynthesis of PCs in plants such as *A. thaliana, Brassica juncea, Glycine max*, *Lotus japonicus*, and tobacco plants (Brunetti et al., 2011; Heiss et al., 2002; Li et al., 2004, 2006; Oven et al., 2002; Ramos et al., 2007). Similarly, zinc (15–1,000 μM) also stimulates the synthesis of PCs in plants and algae (Harmens et al., 1993; Hirata et al., 2001; Pawlik-Skowrońska, 2003a; Tennstedt et al., 2009; Tsuji et al., 2003). However, the contents of Cys, γ-EC, GSH and polyGSH (including PC_2, PC_3, PC_4, and nc-polyGSH) and $T(SH)_2$ were not altered by Zn^{2+}-preconditioning in *E. gracilis*, although intracellular zinc increased.

Zn^{2+} was not an efficient inductor of GSH and PCs biosyntheses, perhaps because Zn^{2+} did not induce severe oxidative stress and hence it was not highly toxic for this protist (Fig. 2). Enhanced synthesis of PCs and cadmium accumulation capacity induced by Zn^{2+} preconditioning have been reported for the algae *Stigeoclonium tenue* and *D. tertiolecta*, isolated from water bodies polluted with Zn^{2+} and other metals (Tsuji et al., 2002; Pawlik-Skowrońska, 2001). Exposure of the marine alga *D. tertiolecta* to 200 μM Zn^{2+} for 24 h triggers an increased ROS production and

Table 3
Dry weight/cell number equivalences and maximal zinc and cadmium accumulation capacities in *E. gracilis* preconditioned to Zn^{2+} and exposed to Cd^{2+}.

	Zinc preconditioning		Cadmium exposure		
	Equivalences, mg DW/10^7 cells	Zinc accumulation, mg zinc/Kg DW	Equivalences, mg DW/10^7 cells	Cadmium accumulation mg cadmium/Kg DW	Zinc accumulation mg zinc/Kg DW
Eg_{Zn20}	6 ± 0.5 (3)	65	17 ± 4 (3)	1,435	42
Eg_{Zn200}	7 ± 0.6 (3)	1401	14 ± 1 (3)	2,304	187
Eg_{Zn300}	7 ± 0.2 (3)	2269	14 ± 2 (3)	2,746	163
Eg_{Zn400}	7 ± 1 (3)	2503	18 ± 1 (3)	2,691	287
Eg_{Zn500}	7 ± 0.3 (3)	2597	12 ± 2 (3)	3,119	485
Eg_{Zn1000}	7 ± 0.7 (3)	3017	14 ± 2 (3)	2,481	504

DW values were determined using cells grown with the indicated Zn^{2+} concentrations for five days (*Zinc preconditioning*; see Materials and methods for details) or exposed to 200 μM Cd^{2+} for 8 days in presence of 20 μM Zn^{2+} (*Cadmium exposure*). Maximal accumulation capacities were calculated using the respective mean values of DW equivalence and mean cell number values shown in Fig. 3 (for zinc accumulation during *Zinc preconditioning*) and Fig. 6B (for cadmium and zinc accumulation during *Cadmium exposure*).

biosynthesis of PC_2-PC_5, which were higher than those induced by 400 μM Cd^{2+} (Hirata et al., 2001; Tsuji et al., 2003). Synthesis of PC_2-PC_4, and chlorosis, were also induced in the green alga *S. tenue* exposed to 15 μM Zn^{2+} by 48 h (Pawlik-Skowrońska, 2003b). A relationship between ROS production, antioxidant defense imbalance and PCs synthesis, has been well established for plants and algae under Cd^{2+} stress (Cho and Seo, 2005; Kumar et al., 2012; Pinto et al., 2003).

It is possible that oxidative stress was not induced by Zn^{2+} in *E. gracilis* because this protist has a robust anti-oxidant machinery. This issue has not been yet examined in this heavy-metal hyperaccumulator microorganism. PCs biosynthesis has not been characterized in plants and algae exposed only to H_2O_2 either.

On the other hand, it is well documented the enhanced biosyntheses of Cys, γ-EC, GSH and PCs induced by Cd^{2+} stress in *E. gracilis* (García-García et al., 2012; Mendoza-Cózatl et al., 2002; Mendoza-Cózatl et al., 2006b). However, the data of the present study indicated that the presence of high Zn^{2+} did not synergize the Cd^{2+} activating effect on Cys, GSH and PCs biosyntheses. These results suggested that the sulfur assimilation pathway and glutathione synthesis are activated by Cd^{2+} but not by Zn^{2+} in *E. gracilis*.

Furthermore, in Zn^{2+} preconditioned *E. gracilis*, the intracellular concentration of GSH remained in the 2–3 mM range (values calculated from Table 1 and using the relationship 7.2 μL = 10^7 cells estimated from Avilés et al., 2003). In marked contrast, GSH levels increased by 4.8-12 times when cells were exposed to Cd^{2+} (see Table 2 and Mendoza-Cózatl et al., 2002). Thus, it seemed that in $EgZn_{400}$ cells *Eg*PCS activity was low, whereas in $EgZn_{400}$ cells exposed to Cd^{2+} the *Eg*PCS activity was high, because the *Eg*PCS K_m for free GSH is 14–22 mM (García-García et al., 2014). Therefore, other factors such asincreased levels of ROS may be required to stimulate, physiologically, the *Eg*PCS activity. The stronger interactions of Cd^{2+} with GSH, other thiol-molecules and cysteine residues in proteins, than those of Zn^{2+} (Sillen and Martell, 1964; Vatamaniuk et al., 2000), might be also involved in the differential effects of Zn^{2+} and Cd^{2+} on GSH and PCs syntheses.

The contents of total phosphate, free phosphate (Pi) and polyPs were determined in $EgZn_{20}$ and Zn^{2+}-preconditioned cells in order to examine their possible roles as Cd^{2+} binding elements. It is known that homeostasis of polyPs is perturbed by metals, extracellular Pi and cellular growth phase in microorganisms and plants (Docampo and Moreno, 2008; Keasling, 1997; Seufferheld and Curzi, 2010). The levels of free Pi and polyPs (micromollar ranges) were 2.3-53 times greater than previous reports in photosynthetic *E. gracilis* cells grown under different heterotrophic conditions (Santiago-Martínez et al., 2015; Smillie and Krotkov, 1960). No differences in the polyPs contents were observed in Zn^{2+} preconditioned cells, and in $EgZn_{20}$ and $EgZn_{400}$ cells exposed to Cd^{2+}, whereas a 55% difference in free phosphate content was found between non-stressed and Cd^{2+}-stressed $EgZn_{400}$ cells. These observations suggested that (i) Pi and polyPs can be non-specific defense mechanisms against Cd^{2+}; and (ii) the polyPs metabolism is not altered by Zn^{2+} in *E. gracilis*, but it is responsive to Cd^{2+}.

5. Concluding remarks

1) *E. gracilis* may now be classified as a zinc hyperaccumulator microorganism under laboratory culture conditions.
2) Preconditioning to > 500 μM Zn^{2+} impairs photosynthesis in *E. gracilis*, but it does not affect cell growth and respiration.
3) The intracellular zinc content reaches a maximum of approximately 300 nmol/10^7 cells in *E. gracilis* cultured with 400 μM $ZnCl_2$; higher $ZnCl_2$ concentrations did not lead to greater intracellular zinc levels. This observation suggests the presence of a tight homeostasis regulation system for Zn^{2+} in this protist.
4) Increased intracellular zinc did not activate the PCs biosynthesis, but it provided protection against Cd^{2+} toxicity and enhanced cadmium accumulation.
5) Polyphosphates are intracellular metal ion chelators which are in high concentrations in *E. gracilis* and can contribute to the greater resistance and accumulation of cadmium.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2016.06.009.

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Supplementary material

Accumulation of zinc protects against cadmium stress in photosynthetic *Euglena gracilis*.

Rosina Sánchez-Thomas, Rafael Moreno-Sánchez, Jorge D. García-García.
Environmental and Experimental Botany. 2016. 131:19-31.
doi: 10.1016/j.envexpbot.2016.06.009.

Table S1. Contents of Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, and Mn^{2+} in *Eg*Zn_{20} and *Eg*Zn_{400} cells grown for 8 days in the presence of 20 or 400 μM Zn^{2+}.

	nmol/10^7cells				
	Ca^{2+}	Co^{2+}	Cu^{2+}	Mg^{2+}	Mn^{2+}
Eg$_{Zn20}$	10 ± 1 (3)	6 ± 1 (3)	2 ± 0.4 (3)	822 ± 147 (3)	8 ± 2 (3)
Eg$_{Zn400}$	28 ± 4 (3)	4 ± 1 (3)	1 ± 0.1 (3)	575 ± 95 (3)	5 ± 1 (3)

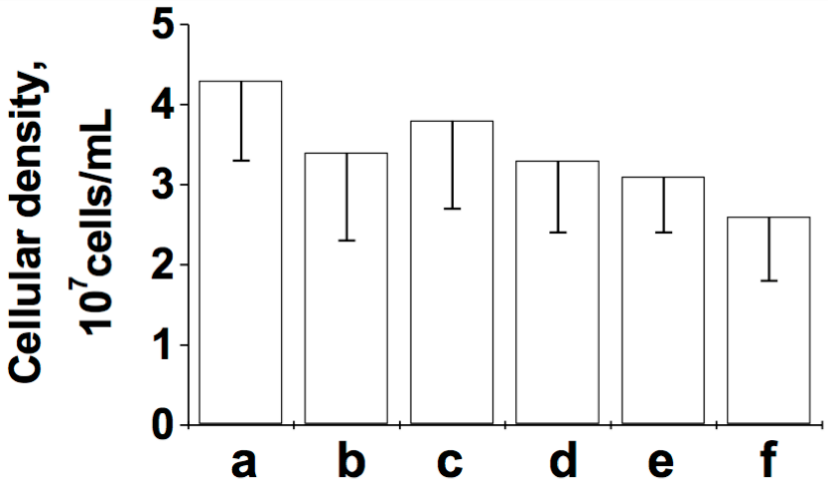

Figure S1. Cellular densities of *E. gracilis* cells grown chronically in the presence of increasing concentrations of Zn^{2+}.

The cell inoculum, transferred from *Eg*Zn_{20} cell cultures, was grown in culture media supplemented with (a) 20, (b) 200, (c) 300, (d) 400, (e) 500 or (f) 1,000 μM $ZnCl_2$. Cellular densities were determined at the early stationary growth phase (5 days) after, at least, 2 subcultures.

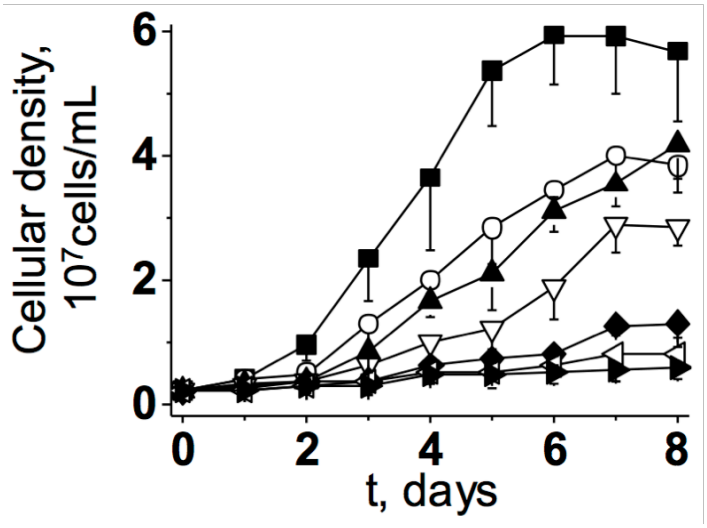

Figure S2. Growth of *Eg*Zn$_{400}$ cells exposed to Cd^{2+} in low Zn^{2+} culture media.
Growth of *Eg*Zn$_{400}$ cells in the presence of 20 μM Zn^{2+} and with 0 (■), 5 (○), 10 (▲), 20 (▽), 50 (◆), 100 (◁), and 200 μM (▶) $CdCl_2$.

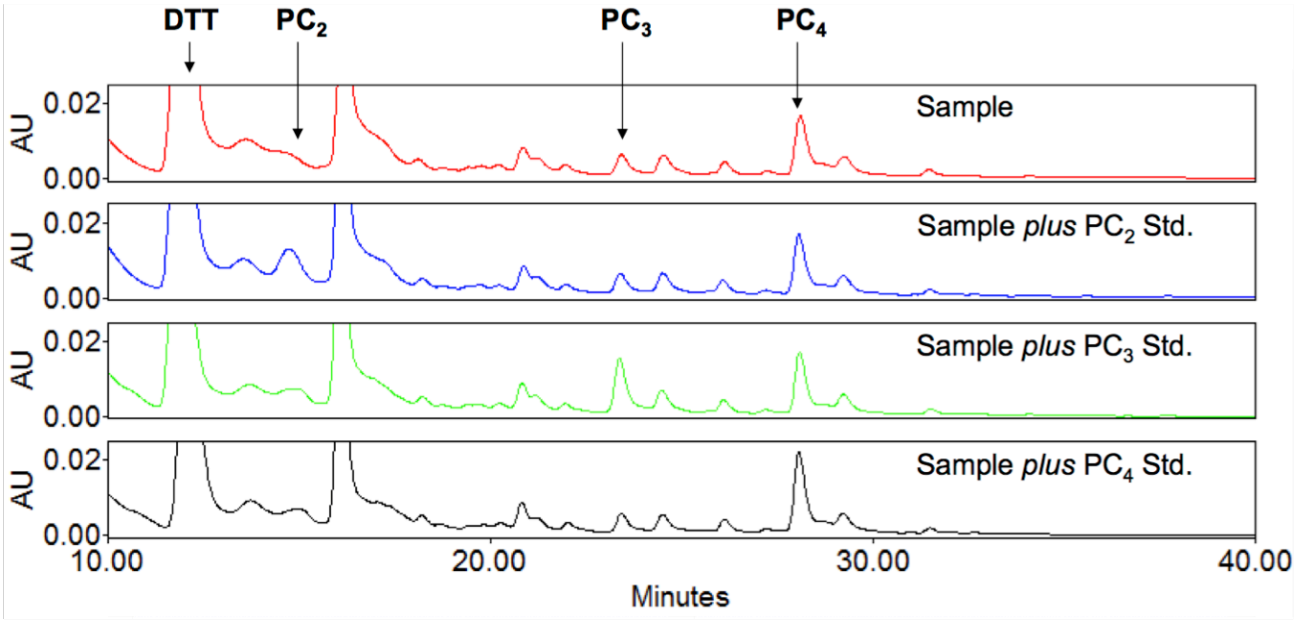

Figure S3. HPLC/Vis chromatograms to identify PC_2, PC_3 and PC_4 in *Eg*Zn$_{20}$ exposed to 200 μM Cd^{2+} for 8 days.
Typical chromatogram (red) of an acid extract of *Eg*Zn$_{20}$ cells grown in the presence of 200 μM Cd^{2+} for 8 days. PC_2 (blue), PC_3 (green) and PC_4 (black) standards were mixed with the sample to identify PCs as was detailed in *Materials and methods* section.

Response to Reviewers

Reviewer 1:

"1) The claim that E. gracilis is a Zn hyperaccumulator is false, or premature, at least. One might argue that the Zn content exceeds the nominal threshold of 3000 mg/Kg DW for Zn hyperaccumulation in plants, but this is only the case under exposure to an extremely high Zn concentration. Terrestrial Zn hyperaccumulators typically also hyperaccumulate Zn from non-metalliferous substrates, and also from nutrient solutions with normal nutritional Zn concentrations (1 - 5 µM). In contrast, E. gracilis, even when grown at 20 µM Zn, which is, in my experience, in fact toxic for most "normal plants", exhibits a perfectly normal intracellular Zn content. It seems to me that the results argue in favor of "artificially induced" hyperaccumulation, rather than "natural hyperaccumulation". Has Zn hyperaccumulation in nature ever been observed in E. gracilis? If not, then there is no reason, whatsoever, left to claim that E. gracilis would be a Zn hyperaccumulator, according to the generally accepted criteria for terrestrial plants (van der Ent et al., 2012)."

R: We agree with the reviewer's arguments that is premature to state that *Euglena gracilis* is a zinc hyperaccumulator microorganism. Indeed, *E. gracilis* was cultured with high $ZnCl_2$ concentrations to achieve elevated intracellular zinc levels (1.3-65 ppm). Certainly, we have not done yet experiments of chronic exposure to low concentrations of heavy metals trying to resemble the experimental conditions used with plants. Therefore, the claims in the paper that *E. gracilis* is a zinc hyperaccumulator have been mostly erased throughout the manuscript and a note of caution has been added on the interpretation of the elevated intracellular levels of zinc and cadmium found for cells cultured with high $ZnCl_2$ (p. 18, l. 415-417 in the marked manuscript). However, studies with plants have also used high zinc concentrations up to 600 ppm (Jin *et al*., 2008; Lasat *et al*., 2000; Zhang *et al*., 2010). In addition, there are water bodies and soils polluted by extremely high zinc concentrations (see modified text in p. 3, 1st paragraph; p. 17, 2nd paragraph for references).

On the other hand, growth has been described for *Euglena* species in heavy metal polluted sites, although specific data for zinc are scarce. A concise statement about this environmental feature of *Euglena* species was inserted in p. 18, l. 417-421.

"2) The claim of Cd hyperaccumulation is even more absurd. A decisive criterion for natural hyperaccumulation is that plants can do it under non-toxic exposure levels. In your study, even the lowest Cd concentration applied is apparently toxic, so there is no case at all for Cd hyperaccumulation capacity in E. gracilis."
"4) The Cd tolerance level, on the other hand, is comparable with that in normal (non-metallicolous) plants, which is another argument against Cd hyperaccumulation capacity in E. gracilis."

R: We have now also erased the claims that *E. gracilis* is a cadmium hyperaccumulator throughout the paper, including the title, except for three paragraphs in Discussion (p. 17-18 in the marked manuscript).

"3) It occurs to me that the strain used in this study is extremely Zn-tolerant, comparable with terrestrial Zn hyperaccumulators, and even more Zn-tolerant than non-Zn-hyperaccumulating "metallophytes" from Zn mine tailings. I would like to know more about the origin of this unusual Zn tolerance level. Has it been evolved in nature, or has it been selected in the lab? What is a "Zn-like strain"? Is it a more Zn-tolerant strain, or has it an enhanced requirement for Zn, or both? I need a better description of your material here."

R: The history of our *E. gracilis* strain regarding zinc content in the culture media was described in the Methods section (p. 5, l. 121-, p. 6, 125 in the marked manuscript). The *E. gracilis* strains B and Z (no Zn) are the main laboratory strains used for research.

"5) The Cd concentrations chosen to compare the biochemical parameters between high-Zn-acclimated and non-acclimated cells, 50 and 200 µM, are unrealistically high; such concentrations do not exist in nature, which casts doubts on the ecological and practical relevance of these data. Moreover, at 200 µM Cd, the high-Zn-acclimation is apparently no longer beneficial in terms of growth (there is no growth at all, irrespective of the Zn pretreatment), meaning that any biochemical effects of the pretreatment can no longer be related to some fitness parameter (I mean, this concentration is not informative in relation to the research question)."

R: Polluting Cd^{2+} concentrations of up to 1,896 ppm have been reported in some mining areas (Yangun *et al*., 2005. *Environ. Int*. 31, 755-762). Studies on plants classified as

cadmium hyperaccumulators have also used high cadmium concentrations of 1-200 ppm. Therefore, we think the present data have indeed great biotechnological and ecological relevance. These arguments were further emphasized in p. 3, l. 63-66; and p. 17, l. 403-p. 18, l. -414 in the marked manuscript.

"6) Canonical PCs apparently make out no more than an inconsiderable fraction of the total of Cd-induced "polyGSH" plus trypanothione. I suggest not to include trypanothione in this category, because (1) it is neither a PC, nor a polyGSH, and (2) it makes it impossible to check whether it contributes to the Cd-induced increase of "polyGSH", or in other words, it makes it impossible to check the contribution of "non-canonical PCs", which are apparently the most prominent Cd chelators."

R: In agreement with this reviewer's observation, Tables 1 and 2 were modified in order to show the contents of PC_2, PC_3, PC_4, non-canonical polyGSH and trypanothione separately. A few additional thiol-metabolite determinations were also incorporated (p. 12, l. 286-p. 13--293 in the marked manuscript p. 15, l. 349). Figure 8D was not modified because already showed only PC_2-PC_4 *plus* nc-polyGSH; however, this information was now clearly detailed in the legend to Fig. 8.

"7) There is a wealth of plant literature on metal-induced PC synthesis, metal tolerance, and metal hyperaccumulation. Virtually none of it has been cited. It seems to me that you may have been unaware of some of the most relevant papers. This is a pity, because a knowledge of this literature would probably have improved your experimental designs (choice of concentrations) and results interpretations."

R: We are fully aware of the plant literature on Cd^{2+} tolerance/accumulation and PCs. In fact, we have published several reviews on the subject citing "plant literature" for relevant comparisons (Cervantes *et al*., 2001. *FEMS Microbiol. Rev*. 25, 335-347; Mendoza-Cózatl *et al*., 2005; Mendoza-Cózatl *et al*., 2006; García-García *et al*., 2016. *Biotechnol. Adv.* In Press.). We initially judged that this information was peripheral to the main goals of our present study. Nevertheless, references of plant literature have been now added in the revised manuscript, as suggested (p.4, l. 87-90 in the marked manuscript). Regarding the

criticism on the experimental design, it should be noted that biosynthesis of phytochelatins has been studied in *A. thaliana, Brassica juncea, Glycine max*, *Lotus japonicus*, and tobacco plants using 25-300 μM cadmium (Brunetti *et al*., 2011; Heiss *et al*., 2003; Li *et al*., 2004; Li *et al*., 2006; Oven *et al*., 2002; Ramos *et al*., 2007). The 2nd paragraph in p. 21 was modified to include information with plants.

Reviewer 2:

"I appreciate the sincere efforts of the authors to revise their manuscript. They added valuable new information and improved some parts of the manuscript. However, there are still several parts of the manuscript that are hard to follow and coherence between sentences could be improved (especially in the abstract and the introduction). Moreover, the authors apparently did not understand my concerns regarding the notation of the free or the total metal ion since throughout the manuscript, the authors frequently used Zn2+ even though they mean the total Zn concentration added in solution. As a whole, I think that this manuscript provides several new information on the binary effect of Zn and Cd on Euglena gracilis, but the text and data interpretation need to be improved before publication. I would strongly advise the authors to model cadmium and zinc speciation in the medium since the toxicity effects of metals are strongly related to metal speciation (Campbell et al 2002; Lavoie et al 2014).For instance, the high Zn and Cd concentrations they used in the submitted manuscript might be above (in some cases) the solubility limit of Cd and Zn and therefore the bioavailable free Zn and Cd concentration could be very sensitive to changes in total Zn or Cd concentrations. Also, if the authors want to compare Zn and Cd bioavailability in E. gracilis to Zn and Cd bioavailability measured in other species and other reports available in the literature, they would need to know Zn and Cd speciation in their culture medium as metal bioavailability and toxicity is strongly related to the free metal ion concentration (Campbell et al 2002; Lavoie et al 2014).Moreover, I would advise to compare the amount of cellular Zn internalized in E gracilis to the cellular Zn concentrations measured in other freshwater algae species in order to better evaluate whether or not E. gracilis is an hyperaccumulator.

In conclusion, there is still a lot of work to do on this manuscript before it can be publishable in an international scientific journal. Perhaps it would be better for the authors to contact a specialist in environmental Chemistry/ecotoxicology and seek help in environmental chemistry and metal ecotoxicology. I congratulate the authors for their work! I sincerely believe that, with more work, this manuscript could be a good significant advance in metal ecotoxicology."

R. It should be noted that no Zn^{2+} and Cd^{2+} speciation or Zn^{2+} solubility problems (see figure below) are expected in the culture medium because its initial pH is 3.5 and after 5 days the medium pH is still in the acidic range of 5-6. We agree that these pH details must be further emphasized in the revised manuscript (p. 6, l. 137-140 in the marked manuscript).

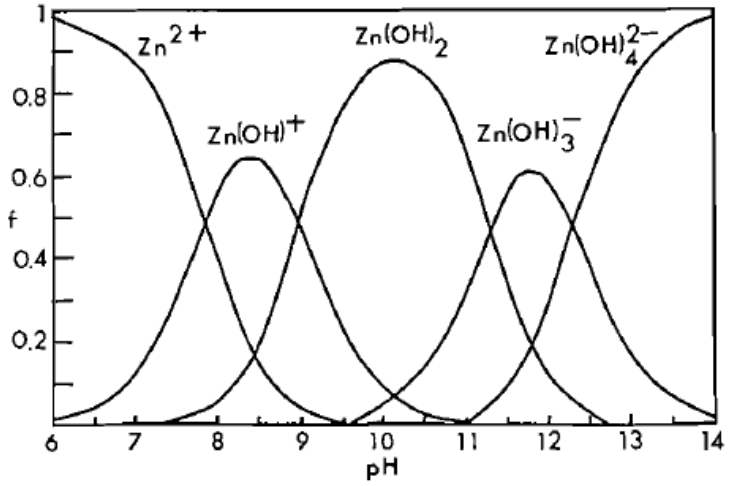

FIG. 1. Graph of fraction *f* of Zn(II) existing as $Zn^{2+}(aq)$, $Zn(OH)^{-}(aq)$, $Zn(OH)_2(aq)$, $Zn(OH)_3^{-}$, and $Zn(OH)_4^{2-}(aq)$ over a range of p*H* at 25 °C.

Reichle, R.A., McCurdy, K.G., Hepler, L.G. 1975. Zinc hydroxide: solubility product and hydroxyl-complex stability constants from 12.5-75 C. Can. J. Chem. 53, 3841-3845,

On the other hand, we have also made theoretical determinations of metal speciation in culture media and reaction mixtures. According to the stability constants values (Sillen and Martell, 1964) of the multi-equilibrium reactions of heavy metals with ligands such as orthophosphate, glutamate and malate (the main components of the *Euglena* culture medium), calculated by the Chelator software (Schoenmakers *et al*., Biotechniques, 1992), which we have used in previous published papers (Lira-Silva *et al*., J Hazard Mater 2011; García-García *et al*., Metallomics, 2014; Santiago-Martínez *et al*., J Hazard Mater, 2015), only at **pH values above 7**, significant binding of metal ions by the indicated ligands occurs.

Metal speciation may certainly develop in the intracellular milieu which has a pH around 7. At pH values near or above 7, thiol-compounds strongly bind heavy metal ions leaving free ion concentrations below the undetectable picomolar range, when micromolar concentrations of both metal and thiol-compound are mixed. Thus, in these last conditions

it may be assumed that all intracellular heavy metal is not free and is bound to biomolecules mainly thiol-compounds.

Comparisons of intracellular zinc and cadmium contents between *E. gracilis* and algae were now made; this information is shown in p. 17, l. 401-403 and p. 17, l. 407-p. 18, l. 410 in the marked manuscript. Previous statements on calling *E. gracilis* a zinc or cadmium hyperaccumulator microorganism were mostly deleted, except for three paragraphs (p. 17-18) in which this possibility is discussed.

2.3 La osmorregulación durante la exposición a metales pesados en *E. gracilis*.

Con el objetivo de describir otros mecanismos implicados en la acumulación del Cd^{2+} que aún no se han identificado plenamente, se realizó una revisión de la literatura sobre organismos hiperacumuladores de metales pesados. Los antecedentes sugerían que un organismo resistente e hiperacumulador de Cd^{2+} sería capaz de contender de manera eficiente contra los principales efectos tóxicos que genera este metal. En general, el Cd^{2+} en las plantas genera inhibición del crecimiento, daño fotosintético (clorosis), estrés oxidante, desequilibrio de la homeostasis de iones esenciales y un desbalance en la cantidad de agua (Clemens, 2006). Con respecto a esto último, no sólo el aumento en la síntesis de moléculas quelantes, sino también el aumento en la síntesis de metabolitos compatibles (osmolitos) se ha descrito como un efecto de la toxicidad del Cd^{2+}. En este sentido, al observar un aumento en la cantidad de prolina durante la exposición a Cd^{2+} en concentraciones que no generan estrés osmótico (μM), se propuso que el aumento de osmo-metabolitos como la prolina podría ser un mecanismo para contender con las alteraciones en el balance hídrico en plantas y microalgas (Schat *et al*., 1997; Siripornadulsil *et al*., 2002). Dicho de otra manera, el estrés por Cd^{2+} activa la participación de mecanismos osmorreguladores. Relacionado con lo anterior, se ha propuesto que plantas que habitan en ambientes con un estrés salino y/o hídrico (halófitas) son modelos para la fitorremediación de los metales pesados, debido a su innata capacidad de osmorregulación.

La osmorregulación es una estrategia biotecnológica que ha sido poco muy estudiada en el área de la biorremediación, pero existe información que sugiere su participación en la acumulación de metales pesados en los organismos. Las plantas halófilas poseen las facultades genéticas y metabólicas para contender contra un estrés por iones, por lo que consiguen sobrevivir en ambientes que presentan elevados indices de estrés osmótico, salino y/o hídrico (Manousaki y Kalogerakis, 2011; Lutts y Lefèvre, 2015; Van Oosten y Maggio, 2015; Sruthi *et al*., 2017). En la Fig. 5 se representan los mecanismos que les permiten a las plantas halófilas sobrevivir en ambientes extremos y que se han asociado con su resistencia y una hiperacumulación de metales pesados: (i) la formación de estructuras especializadas de excreción llamadas glándulas salíferas o

tricomas localizadas en las hojas y que normalmente excretan iones (Na^+, Cl^-, Ca^{2+}, Zn^{2+}, Pb^{2+} Cu^{2+}); (ii) la síntesis de metabolitos quelantes; (iii) la sobreproducción de osmo-metabolitos como sacáridos, betainas, aminoácidos (Pro, His, Ala, Gln), poliaminas; (iv) cambios en la permeabilidad de la membrana plasmática (canales Na^+/K^+ y transportadores de iones) para disminuir la captación de iones que se encuentran en altas concentraciones; y (v) mecanismos para estabilizar el contenido de agua intracelular o regular la transpiración para controlar la posible pérdida o un exceso de agua (Van Oosten y Maggio, 2015; Lutts y Lefèvre, 2015; Rucińska-Sobkowiak *et al*., 2016; Sruthi *et al*., 2017; Nikalje y Suprassana, 2018). Estos mecanismos y propiedades podrían servir también para contender contra un estrés por metales pesados. Esta hipótesis se sustenta en el hecho de que la exposición de estas plantas a metales pesados modifica su potencial osmótico y el contenido de agua o turgencia de la planta (Rucińska-Sobkowiak *et al*., 2016), indicando una relación estrecha entre ambos tipos de estrés.

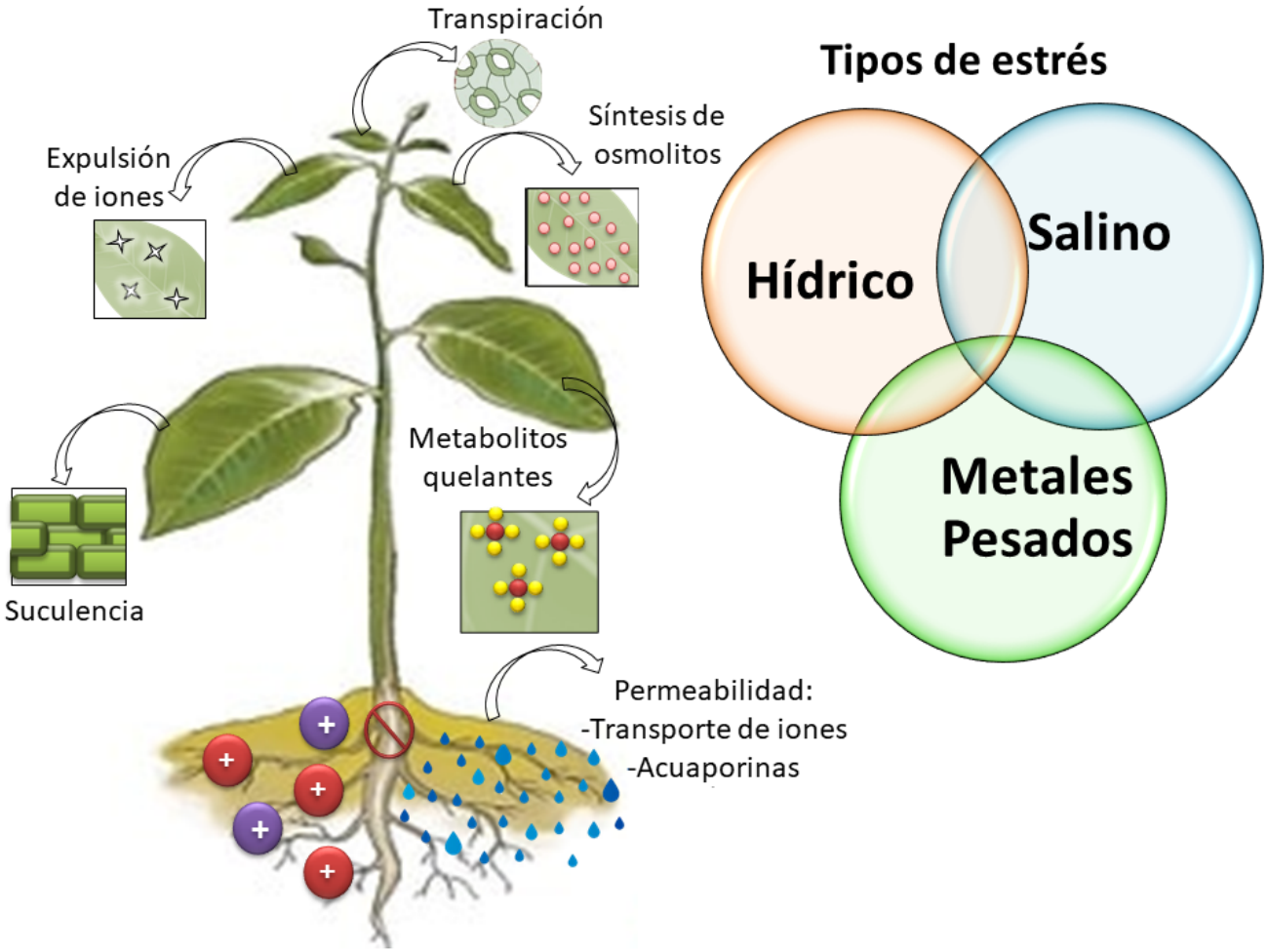

Figura 5. Mecanismos asociados a un estrés hídrico y/o salino que se han propuesto que contribuyen en la resistencia y la acumulación de metales pesados en plantas halófilas.

Con respecto a los efectos de tipo osmótico en microorganismos, se ha establecido que las células pueden modificar el volumen celular como una respuesta a un estrés osmótico, el cual provoca daño celular por hinchamiento o compresión (Chengappa *et al*., 2018). Las células de *E. gracilis* heterotrófica expuestas a 100 µM de Cd^{2+} durante 5 días muestran un aumento en el volumen intracelular de agua de 2.8 veces (6.4 µL $H_2O/10^7$células) con respecto a las células control (2.3 µL $H_2O/10^7$células) (Avilés *et al*., 2003). Estos antecedentes indican que el Cd^{2+} sí provoca cambios de tipo osmótico en *Euglena*. Aquí cabe señalar que la participación de los mecanismos osmoreguladores, cómo son los ajustes en el volumen intracelular del agua, con la acumulación de metales pesados no se ha caracterizado en plantas o levaduras, tal vez porque estos organismos poseen una pared celular rígida que impide cambios en el volumen celular. En cambio, los organismos del género *Euglena* carecen de pared celular, por lo cual pueden cambiar de forma y volumen celular rápidamente. De igual forma, *E. gracilis* expuesta a un estrés osmótico con 200 mM de NaCl también aumenta su volumen intracelular de agua 1.6 veces (4.7 µL/10^7células) con respecto a las células no tratadas con sal (2.9 µL/10^7células) (González-Moreno *et al*., 1997). También se ha descrito que durante el estrés osmótico inducido con NaCl en *E. gracilis*, la reserva de paramilo (polisacárido de almacén) disminuye y la cantidad del osmolito trehalosa aumenta 12 veces (792 nmol/10^7células). Los niveles de otros posibles osmolitos, tales como aminoácidos, ácidos orgánicos, y/o disacáridos no se modifican, ni los contenidos de proteína y lípidos (Takenaka *et al*., 1997).

Estos antecedentes indican que las respuestas inducidas con un estrés osmótico y causado por metales pesados puede estar mediado por mecanismos similares. Con el propósito de entender la relación mecanística entre el manejo del estrés osmótico y la hiperacumulación de metales pesados, en el presente proyecto se evaluó en *E. gracilis* fotosintética expuesta a 50 y 200 µM de Cd^{2+} (i) los efectos de tipo osmótico durante la acumulación del Cd^{2+}, (ii) los mecanismos asociados al estrés osmótico que facilitan la acumulación del Cd^{2+} y del Zn^{2+}; y (iii) si la resistencia al estrés osmótico mejora la acumulación de los metales pesados.

3. HIPÓTESIS

Los mecanismos de osmorregulación, principalmente el volumen intracelular de agua y los niveles de osmolitos, contribuyen a la acumulación del cadmio y del zinc en Euglena gracilis.

4. OBJETIVOS

4.1 General

Determinar cómo el volumen de agua intracelular y los niveles de osmolitos contribuyen a la capacidad de acumulación del cadmio y del zinc en *E. gracilis*.

4.2 Particulares

1. Determinar los cambios de tipo osmótico durante el proceso de acumulación del cadmio en *E. gracilis.*

2. Determinar si los mecanismos osmoreguladores también participan en la acumulación del zinc.

3. Identificar los mecanismos bioquímicos osmoreguladores que modulan la acumulación del cadmio, tales como el cambio en el volumen intracelular.

5. METODOLOGÍA

En la Fig. 6 se muestra la estrategia experimental general que se planteó para realizar esta segunda parte del proyecto de doctorado. Se utilizaron células de *Euglena gracilis* fotoheterotrófica cultivadas con y sin Cd^{2+} para determinar los cambios de tipo osmótico generados durante la acumulación de este metal y así establecer una relación entre ambos tipos de estrés, los cuáles podrían derivar de una respuesta generalizada debido al estrés oxidante inducido por el Cd^{2+}.

Posteriormente, el proyecto se enfocó en evaluar cómo los cambios en el volumen intracelular podrían estar involucrados en la acumulación del Cd^{2+}, para lo cual se modificó la osmolaridad del medio de cultivo, y se utilizaron inhibidores de acuaporinas como el Hg^{2+} y la pentamidina para modular el volumen intracelular y observar su repercusión en la acumulación del Cd^{2+}.

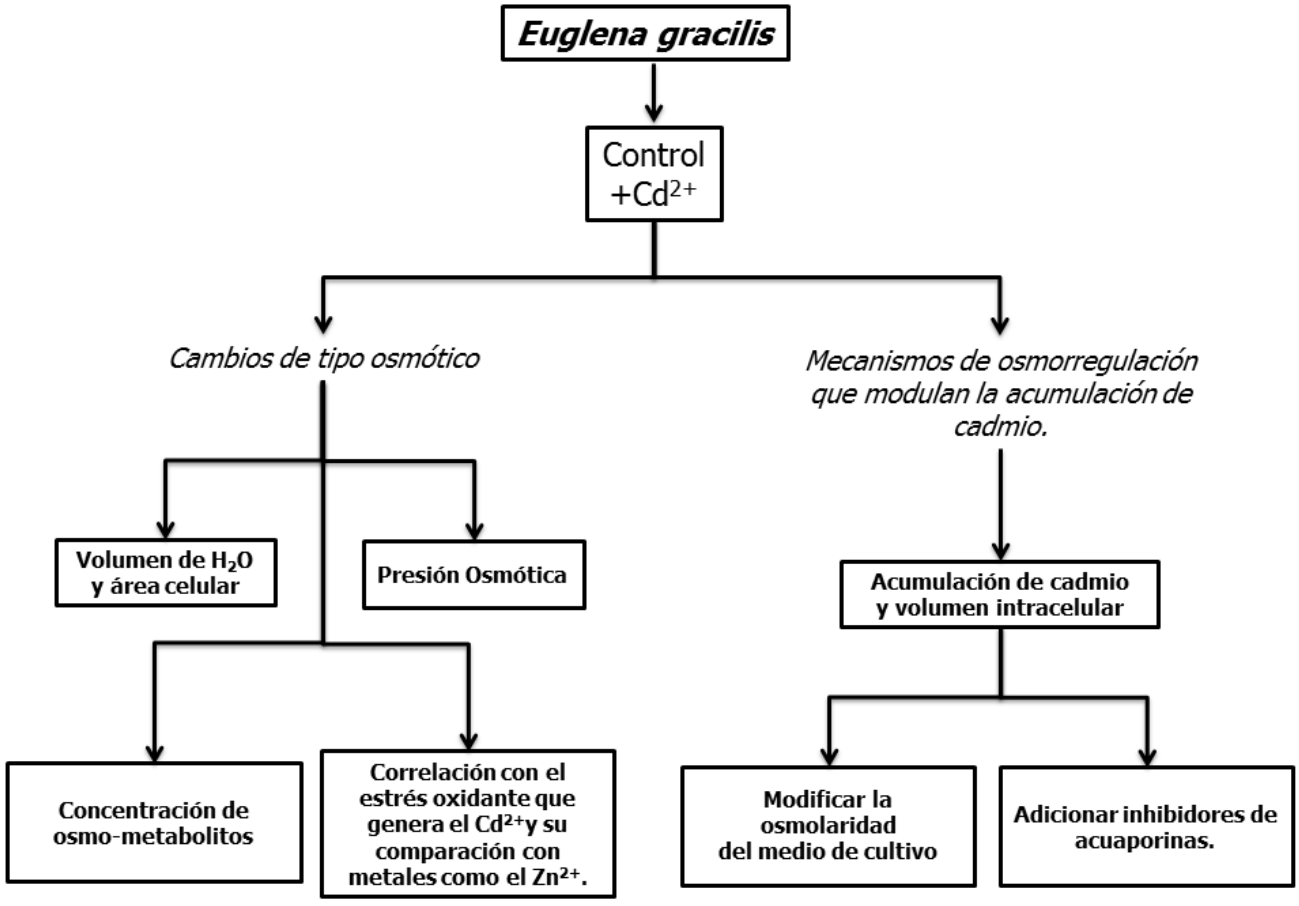

Figura 6. Estrategia experimental para caracterizar los mecanismos de osmorregulación asociados a la acumulación del Cd^{2+} en *E. gracilis*.

5.1 Condiciones de crecimiento de *E. gracilis*

Se utilizó la cepa fotosintética de *E. gracilis* Klebs var. Z, la cual fue originalmente obtenida hace aproximadamente 35 años del Departamento de Parasitología de la Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional de México. La cepa utilizada se considera variedad "Z" debido a su tiempo generacional, su tendencia a"blanquearse" (perder los cloroplastos y la clorofila) cuando se cultiva a 35ºC y su incapacidad para utilizar galactosa como fuente de carbono (Santiago-Martínez *et al*., 2015). Las células se cultivaron en condiciones fotoheterotróficas, con ciclos de luz/obscuridad de 12 h mediante lámparas que emiten luz blanca a una intensidad de 42-90 µmol quanta $m^{-2}s^{-1}$ y en un intervalo de temperatura de 24 y 26ºC, con variaciones intrínsecas debido a la cámara de luz/obscuridad que se utiliza para el crecimiento de las células.

La composición del medio de cultivo que se utilizó está basada en el medio Hutner modificado en 1971 por Schiff (Schiff *et al*., 1971). El medio consta de los siguientes componentes: 34 mM ácido glutámico, 15 mM ácido málico, 2 mM $CaCO_3$, 1.5 mM $(NH_4)_2HPO_4$, 2 mM $MgSO_4 \cdot 7H_2O$, 3 mM KH_2PO_4, minerales traza A (0.02 mM $ZnSO_4 \cdot 7H_2O$, 0.01 mM $MnSO_4 \cdot H_2O$, 0.1 mM $Na_2MoO_4 \cdot 2H_2O$, y $7x10^{-3}$ mM $CoCl_2 \cdot 6H_2O$) y minerales traza B ($3x10^{-3}$ mM $CuSO_4 \cdot H_2O$, 0.01 mM H_3BO_3 , $2x10^{-4}$ $Na_2Vo_4 \cdot 16H_2O$ y 0.03 mM $Fe(NH_4)_2(SO_4)$) ajustado a pH 3.5 utilizando NaOH o HCl. El medio de cultivo se esteriliza en el autoclave, y antes de la adición de las células se agrega 1 mL de vitaminas (3×10^{-3} mM tiamina, 0.2 µg/mL cianocobalamina y 0.02 mM $FeCl_2$) por cada 100 mL de medio de cultivo.

Cuando aparece contaminación en los cultivos líquidos de *E. gracilis*, se hacen placas de agar al 1.5% sin los componentes del medio de cultivo, donde se estrían células de los cultivos dañados y se mantienen dentro de la cámara fotosintética. Después de una semana aproximadamente se observa el crecimiento de las células en el agar. Entonces se utiliza un palillo para colectar células de la zona más alejada del inicio del estriado, las cuales se inoculan en un matraz con medio de cultivo fresco para recuperar el cultivo axénico.

5.2 Volumen de H_2O y área celular

Para determinar el volumen intracelular de agua es necesario generar un sistema que funcione como filtro del medio de cultivo mediante el uso de soluciones con diferentes densidades. En tubos Eppendorf de 1.5 mL se debe colocar de abajo hacia arriba (**A**) 300 μL de ácido tricloroacético (TCA) 30%, (**B**) 300 μL de 1-bromododecano 97% y (**C**) 300 μL de medio de cultivo Hutner pH 3.5. Al colocar la última capa se pueden formar burbujas, por lo que se recomienda dispensar el volumen cuidadosamente y al final centrifugar a 14,000 rpm durante 1 minuto para que se separen claramente las 3 capas y se eliminen microburbujas (Figura 7).

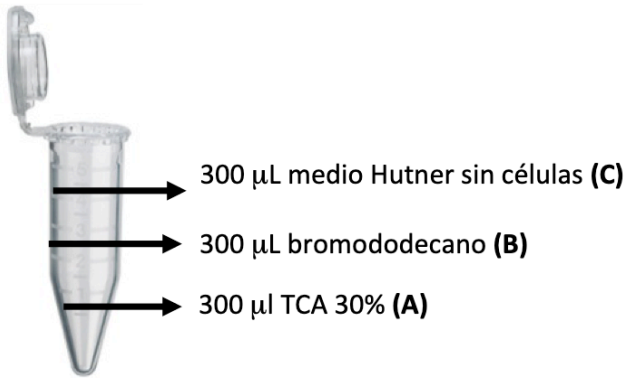

Figura 7. Tubo tipo Eppendorf que representa las capas necesarias para llevar a cabo la determinación del volumen intracelular.

Las células que se desea analizar se cosechan de los medios de cultivo por centrifugación a 3500 rpm durante 1 minuto a 25ºC; el sedimento celular se re-suspende a una alta concentración (~ 250 x 10^6 células/mL) en su propio medio de cultivo ya usado, que es el sobrenadante obtenido después de la centrifugación. Este paso es esencial, ya que las células son susceptibles a cambios en su volumen interno cuando se varía la osmolaridad del medio exterior. A continuación, las células se diluyen con medio Hutner fresco (2–5 × 10^6células/mL) y se incuban por 1 min con ^3H_2O (542–2532 Bq/μL) o [^{3}H]-inulina (650–2911 Bq/μL). La suspensión celular con el isotopo radiactivo se vierte cuidadosamente en la capa superior del tubo Eppendorf (Fig. 7), y se centrifuga inmediatamente a temperatura ambiente a 14,000 rpm por 1 min en una microcentrífuga (Benchmark, Edison, NJ, USA). Después, se retiran alícuotas de 0.2 mL de la capa de arriba del bromododecano (**C** en Fig. 7) y de la capa que contiene TCA debajo del

bromododecano (**A** en Fig. 7), y se mezclan con una solución de centelleo que contiene tolueno 60%, arkopal N-100 o Triton X-100 33% y M-flúor 7% [2,5-difeniloxazol 0.45 M y p-bis-(o-metilestiríl) benceno 6.4 mM disueltos en tolueno]. Para tomar la segunda alícuota, se remueve cuidadosamente la capa (**C**) y después, una punta amarilla nueva (para micropipeta de 0-200 μL) se introduce a través de la capa (**B**) hasta llegar a la capa (**A**) para poder retirar la alícuota correspondiente.

La radioactividad de las muestras se determina en un contador de centelleo Beckman Coulter (Brea, CA, USA) en cuentas por minuto (CPMs). El volumen intracelular se calcula al introducir las CPMs obtenidas en la fórmula de Rottenberg (1979) que se muestra a continuación:

$$\text{Volumen intracelular} = \left(\frac{{}^3H_2O_P}{{}^3H_2O_{Sn}} - \frac{{}^3Inul_P}{{}^3Inul_{Sn}} \right) * Vol_{Sn}$$

$^3H_2O_P$ = cantidad de ^3H_2O en la capa **A** de TCA (pellet)

$^3H_2O_{Sn}$ = cantidad de ^3H_2O en la capa **C** de medio sin células (sobrenadante)

$^3Inul_P$ = cantidad de ^{3}inulinaen la capa **A** de TCA (pellet)

$^3Inul_{Sn}$ = cantidad de ^{3}inulinaen la capa **C** de medio sin células (sobrenadante)

Vol_{Sn} = Volumen total de la capa **C** (medio sin células + suspensión celular)

5.3 Contenido de aminoácidos

En esta segunda parte del presente proyecto se implementó una metodología para cuantificar estos metabolitos. La determinación de aminoácidos se llevó a cabo mediante HPLC acoplado a un detector de fluorescencia (Waters 1525/2475, Milford, MA, USA), mediante el uso de una columna C-18 en fase reversa tipo Columbus de 5 μm de tamaño de partícula, 110 Å y con dimensiones de 150 x 4.6 mm (Phenomenex, CA, USA). Las células (5-34 x10^6 cells/mL) se cosecharon, lavaron con 0.1 M KH_2PO_4 pH 7.4 suplementado con 5 mM EGTA y guardaron a -20ºC hasta su uso. Posteriormente, las células se descongelaron en presencia de 0.1% (v/v) Tritón X-100 a temperatura ambiente durante 20 min y se rompieron al adicionar 2.7% (v/v) de ácido perclórico y agitar vigorosamente por 1 min. Después de centrifugar por 5 min a 13,000 rpm, los sobrenadantes ácidos se neutralizaron con 3 M KOH/0.1 M Tris, se centrifugaron y se congelaron para asegurar la remoción total del $KClO_4$.

A continuación, las muestras se descongelaron, se volvieron a centrifugar y se derivatizaron por incubación con 1 volumen de *o*-phtalaldehído (OPA; 37 mM *o*-phtalaldehído, 5% metanol, 5% β-mercaptoetanol disuelto en ácido bórico 0.4 M pH 9.9), por 1 min a temperatura ambiente. La reacción de derivatización se detuvo por dilución con 0.5 mL de 40 mM NaH_2PO_4 pH 7.8, y la solución se filtró a través de un filtro Millex-Millipore (0.45 μ diámetro del poro) para su análisis por HPLC. La columna se mantuvo a una temperatura de 40ºC, y fue equilibrada con 40 mM NaH_2PO_4 pH 7.8 (solución A) y 45% acetonitrilo (ACN), 45% metanol (MeOH) y 10% H_2O (solución B) con un flujo de 1 mL/min. Los metabolitos se separaron y eluyeron utilizando el protocolo de elución que se muestra en la Tabla 1. Los aminoácidos se detectaron por fluorescencia a una $\lambda_{excitation}$ de 340 nm y $\lambda_{emisión}$ de 460 nm. Se utilizaron estándares de alanina (Ala), β-alanina (β–Ala), glicina (Gli) e histidina (His) (Sigma Aldrich, St. Louis, MO, USA) para identificar los metabolitos en las muestras celulares. Las concentraciones utilizadas para las curva patrón fueron 10-200 nmoles para Pro y 0.25-2.5 nmoles para Ala, β-Ala, Gli e His, que representan la zona lineal de detección para cada aminoácido.

Tabla 1. Protocolo de elución para la determinación de aminoácidos por HPLC.

Tiempo (minutos)	**Solución A NaH_2PO_4 40 mM pH 7.8 %**	**Solución B ACN:MeOH:H_2O %**
0-40.5	100	0
40.5-41	59.5	40.5
41-43	39	61
43-44	39	61
44-44.5	18	82
44.5-46.5	0	100
46.5-47	0	100
47-49	100	0
fin	100	0

Para la determinación de prolina se utilizó un protocolo con ninhidrina (Ábrahám *et al*., 2010), ya que este aminoácido es incapaz de interactuar con el OPA. Para esto, se

utilizaron 300 μL de extracto neutralizado, 200 μL de ácido acético 100% y 200 μL de una solución de ninhidrina (140 mM ninhidrina, 60% ácido acético, 2.5 M ácido ortofosfórico; cubierta de la luz). La mezcla se calentó durante 1 hora a ≈95ºC en tubos de crioconservación, y se enfrió en hielo por 10 min. Posteriormente, se añadieron 2 mL de tolueno, se mezcló vigorosamente y se dejó reposar por 5 min a temperatura ambiente dentro de una campana de extracción. Se extrajo la fracción colorida y se midió en celdas de vidrio a 520 nm. Para calcular las concentraciones de prolina se utilizó como referencia una curva patrón de prolina (10-100 μg/mL). Por otro lado, la ninhidrina es un compuesto que puede interaccionar con otros aminoácidos, no sólo con la prolina, por lo que se evaluó la interferencia de los aminoácidos que se encuentran en mayor proporción en *E. gracilis* como la Ala, la Cys y la Gly; sin embargo, no se encontró interferencia para la determinación de prolina.

5.4 Contenido de osmo-metabolitos

Las células (13-37 x 10^6 células/mL) fueron cosechadas, lavadas y guardadas a -20ºC hasta su uso. Posteriormente, se obtuvieron los extractos neutralizados tal y cómo se describe en el apartado 5.3. A continuación, se describe la metodología establecida en el laboratorio durante la segunda parte del presente proyecto de doctorado para la determinación de diferentes metabolitos:

Trehalosa

La trehalosa se hidrolizó a glucosa mediante la adición de 1 U de trehalasa (Sigma Aldrich, St. Louis, MO, USA) a 5-10 μL del extracto neutralizado en buffer de KH_2PO_4 160 mM pH 6 a 37ºC, incubando toda la noche. La cantidad de glucosa obtenida se determinó mediante un ensayo acoplado con1 U de hexocinasa y 1 U de glucosa 6-fosfato deshidrogenasa (Roche) en presencia de 5 mM $MgCl_2$, 1 mM ATP y 0.5 mM $NADP^+$. La determinación se hizo obteniendo los cambios de absorbancia a 340 nm por reducción del $NADP^+$. Para calcular la cantidad de trehalosa se consideró que 2 nmol de glucosa (2 nmol NADPH) corresponden a 1 nmol de trehalosa, después de corregir la cantidad de glucosa

libre que se obtiene en el extracto sin incubación con trehalasa. La curva patrón de trehalosa que se utilizó fue con concentraciones de 50-1500 nmoles.

Betaínas

El contenido total de betaínas en *E. gracilis* se determinó como ya se había descrito previamente en plantas (Grieve y Grattan, 1983). Extractos ácidos provenientes de 10-72 x 10^6 células/mL se diluyeron 1:1 con 2 N H_2SO_4. Esta mezcla se mantuvo en hielo durante 1 h y entonces se adicionó una solución fría de yodo (1 M yodo y 1 M yoduro de potasio) en proporciones 1:10 y se mezcló vigorosamente. Las muestras se incubaron en agua con hielo a 0-1ºC toda la noche. Posteriormente, las muestras se centrifugaron a 17,000 x *g* por 30 min a 2ºC. El sobrenandante se removió cuidadosamente dentro de un cuarto frío para evitar que las sales de periodato se solubilicen. Por último, los cristales se disolvieron en 1,2-dichloroetano. Se determinó la absorbancia a 365 nm y se utilizó una curva patrón de glicina-betaína con 5-100 nmol/mL como referencia.

Poliaminas

Estos metabolitos se determinaron como ya se había descrito anteriormente (Olin-Sandoval *et al*., 2012). Se utilizaron extractos ácidos que se neutralizaron utilizando bicarbonato de sodio en polvo. Después, 50 μL de dicho extracto se calentó durante 2 horas a baño maría hasta desecación. Para este paso se utilizó un termo-block con agua y tubos Eppendorf abiertos. A continuación, la muestra seca se disolvió en 80 μL de 0.05 N HCl y se mezcló en un tubo de vidrio de 13 x 100 mm con 400 μL de 0.1 M bicarbonato de sodio pH 9.1 y 800 μL de cloruro de dansilo 3 mM, agregando una perla de ebullición. Los tubos se cubrieron con aluminio y parafilm, se hicieron orificios a la tapa y se calentaron durante 15 min a 70ºC. Posteriormente, 650 μL de muestra se diluyeron con 1 mL de metanol 100% grado HPLC, se filtraron y se inyectaron 50 μL al HPLC acoplado a un detector de fluorescencia (Waters 1525/2475, Milford, MA, USA). Se utilizó una columna C-18 en fase reversa tipo Spherisorb de 5 μm de tamaño de partícula, 110 Å y con dimensiones de 250 x 4.6 mm (Waters, Mildford, USA). La columna se mantuvo a una temperatura de 40ºC, y fue equilibrada con metanol 100% (solución A) y H_2O milli-Q

(solución B) con un flujo de 1 mL/min; los metabolitos se separaron y eluyeron utilizando el protocolo de elución que se muestra en la Tabla 2. Las poliaminas fueron detectadas por fluorescencia a una $\lambda_{excitation}$ de 365 nm y $\lambda_{emisión}$ de 510 nm. Se utilizaron estándares de espermidina (Spd), putrescina (Put) y cadaverina (Cad) (Sigma Aldrich, St. Louis, MO, USA) para su identificación en las muestras celulares.

Tabla 2. Programa de elución para la determinación de poliaminas por HPLC.

Tiempo (minutos)	**Solución A Metanol %**	**Solución B H_2O %**
0-1	40	60
1-24	40	60
24-28	5	95
28-30	5	95
30-40	0	100
4042	0	100
42-55	40	60
Fin	40	60

Por último, es importante mencionar que en cada determinación es necesario procesar muestras con estándares de poliaminas con el objetivo de corregir por la pérdida de estos metabolitos durante el procesamiento de las muestras. Las concentraciones de poliaminas utilizadas para la curva patrón fueron de 0.1-1 nmoles de espermidina, putrescina y cadaverina.

5.5 Determinación de metales por espectrofotometría de absorción atómica

Las células se obtuvieron como se detalló en el inciso 5.3. Para romper a las células y eliminar residuos orgánicos, se hizo una digestión agregando 3 mL de una mezcla ácida (2.5 mL HNO_3/0.5 mL H_2SO_4) por cada mililitro de muestra, y utilizando una plancha de

calentamiento durante 2 horas, alternando cada 15 minutos con alta intensidad (100-110°C) y 10 minutos con baja intensidad (95-100°C). Al final, las muestras se aforaron a 5 mL con H_2O mQ. La cantidad intracelular total de Cd^{2+} se determinó por espectrofotometría de absorción atómica (EAA). Se utilizó un equipo Varian Spectra AA 640 con una mezcla de aire-acetileno a un flujo de 13.5:2 mL/min. Se utilizó una lámpara marca Varian que emite a una longitud de onda de 228.8 nm para medir Cd. La concentración de las muestras se obtuvo mediante la interpolación de una curva patrón con un ajuste lineal utilizando distintas concentraciones de Cd (0.5-2 ppm).

5.6 Métodos de análisis estadístico

Cada uno de los experimentos se llevaron a cabo al menos 3 veces con muestras celulares independientes. Los datos obtenidos fueron presentados como media ± desviación estándar. Para determinar diferencias estadísticamente significativas entre 2 grupos independientes se utilizó un análisis *t* de Student. Por otro lado, para establecer diferencias estadísticamente significativas en experimentos con comparaciones entre más de dos grupos se utilizó el estadístico one-way ANOVA/post hoc Scheffé. Dichos análisis fueron realizados con el software IBM SPSS Statistics versión 25 (IBM Corp.; NY, USA). Para ambos tipos de análisis se utilizó un valor de $P \leq 0.05$ como criterio de significancia.

6. RESULTADOS

6.2 Principales resultados de la publicación 2.

La publicación fue de gran importancia para los objetivos del proyecto, ya que aportó información relevante y novedosa con respecto a los mecanismos de acumulación del Cd^{2+} en microorganismos. Al inicio de esta parte del proyecto se determinó que una de las principales respuestas osmóticas durante la acumulación del Cd^{2+} en *E. gracilis* era el aumento en el volumen intracelular del agua (Fig. 8A), que se alcanzaba desde las primeras 24 h de exposición al Cd^{2+}. A pesar de esto, los cambios en el volumen intracelular no correlacionaron con la cinética de la acumulación del Cd^{2+} (Fig. 8A inserto). Sin embargo, el aumento en el volumen intracelular fue evidente al observar a las células al microscopio (Fig. 8B).

Con respecto a la caracterización de los efectos osmóticos que se inducen por la exposición al Cd^{2+}, se determinaron los contenidos intracelulares de diferentes osmolitos para estimar los cambios en la osmolaridad interna, los cuáles se muestran en la Fig. 8C. El contenido de trehalosa aumentó significativamente en las células expuestas a 200 μM de Cd^{2+} (Control = 15 mM; 200Cd = 67 mM). También los contenidos de paramilo, aminoácidos (Pro, Gly, Ala, β-Ala e His) y poliaminas (Spd, Put y Cad) se mantuvieron en concentraciones altas en las células expuestas al Cd^{2+} con respecto a las células control. Además, se observó que la betaína fue el osmolito menos abundante en *E. gracilis*. Por último, se determinó que el contenido de Na^+ y K^+ se mantuvo constante, tanto en las células control como en las expuestas al Cd^{2+}. La estimación teórica de los osmolitos se corroboró experimentalmente con el uso de un osmómetro. La osmolaridad interna de las células control fue de $\approx$182 mOsM, mientras que para las células expuestas a 50 y 200 μM de Cd^{2+} fue 1.5 y 2.2 veces mayor (50Cd = 292 ± 66 mM; 200Cd = 425 ± 127 mM), lo cual permitió establecer una correlación con la acumulación proporcional del Cd^{2+}. Los resultados de la osmoralidad se utilizaron para calcular la presión osmótica, lo cual indicó claramente que la acumulación del Cd^{2+} incrementa la presión osmótica en *E. gracilis* (Fig. 8C).

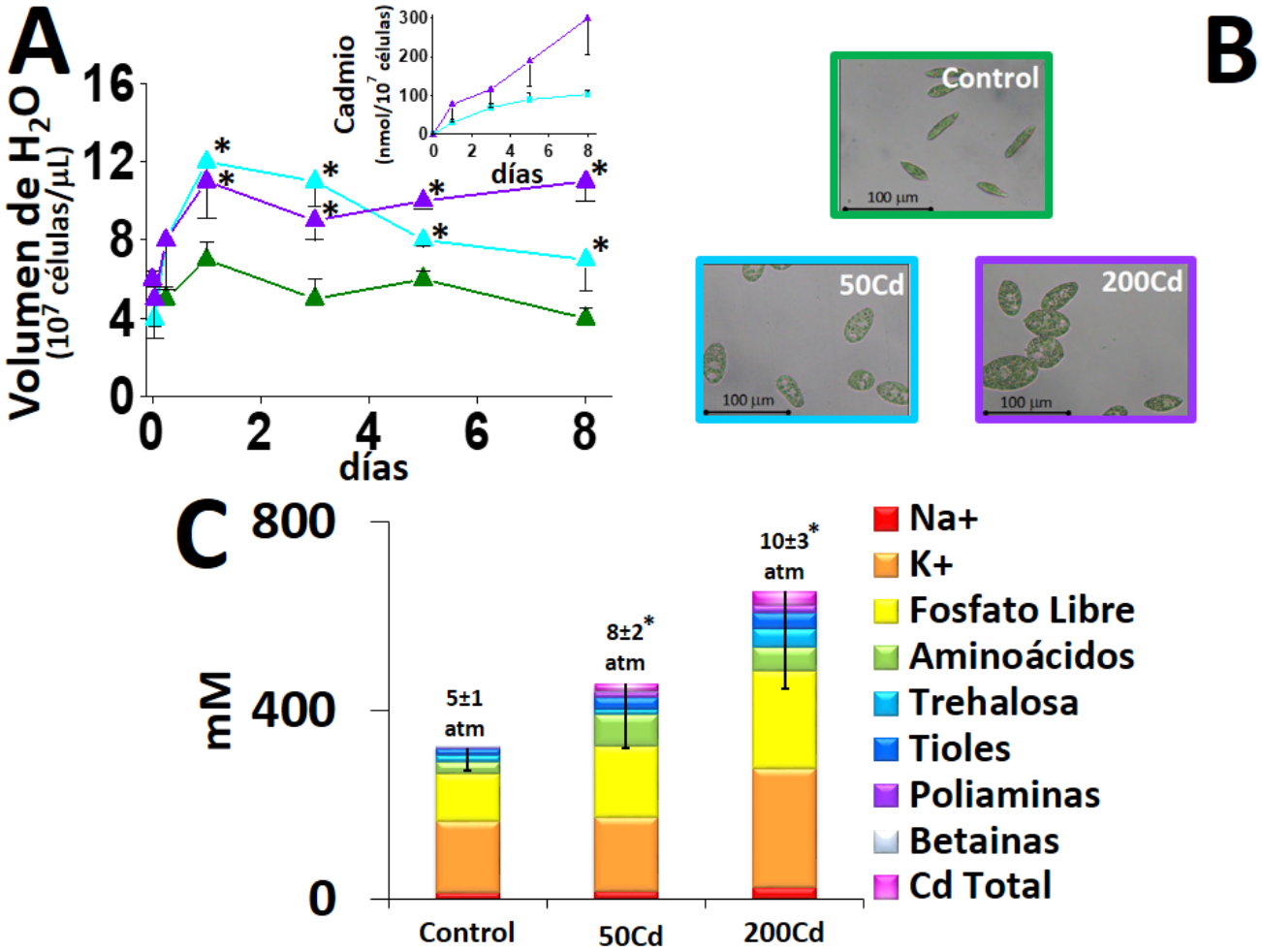

Figura 8. Efecto de la acumulación del Cd^{2+} en el volumen intracelular de agua, en el tamaño celular y en el contenido de osmo-metabolitos en *E. gracilis*.

(A) Volumen intracelular de agua en células control (verde) y expuestas a 50 (azul) o 200 (morado) µM de Cd^{2+} durante 8 días. (B) Tamaño celular y (C) contenido de osmo-metabolitos en *E. gracilis* control y expuesta a 50 o 200 µM de Cd^{2+} durante 5 días. El valor encima de los bloques representa la presión osmótica calculada a partir de las determinaciones de la osmolaridad interna en cada una de las condiciones. Las barras verticales corresponden a la desviación estándar obtenida de al menos 3 determinaciones independientes. *P< 0.05 *versus* control (ANOVA/*post hoc*Scheffé).

Los efectos de tipo osmoregulador derivados de la acumulacion del Cd^{2+} se compararon con aquellos observados en células de *E. gracilis* expuestas a 200 µM de Zn^{2+}, con el objetivo de analizar si estos cambios también se presentan durante la acumulación de otros metales pesados y es por lo tanto un mecanismo universal (Fig. 9). Sin embargo, la acumulación del Zn^{2+} no modificó el volumen intracelular del agua o el contenido de trehalosa, a pesar de que las concentraciones intracelulares de ambos metales alcanzaron valores similares (50Cd= 13 ± 1; 200Cd= 21 ± 2; 200Zn= 36 ± 5 mM). A partir de estos

resultados, se propuso que los cambios de tipo osmótico podrían estar relacionados con el estrés oxidante que genera la acumulación del Cd^{2+} con respecto al Zn^{2+}. De acuerdo con esta idea, los marcadores de estrés oxidante como la relación GSH/GSSG y la actividad de enzimas antioxidantes como APx, GR y GPx, se incrementaron significativamente en la condición con Cd^{2+}, mientras que en presencia de Zn^{2+} no hubo cambios con respecto a células control.

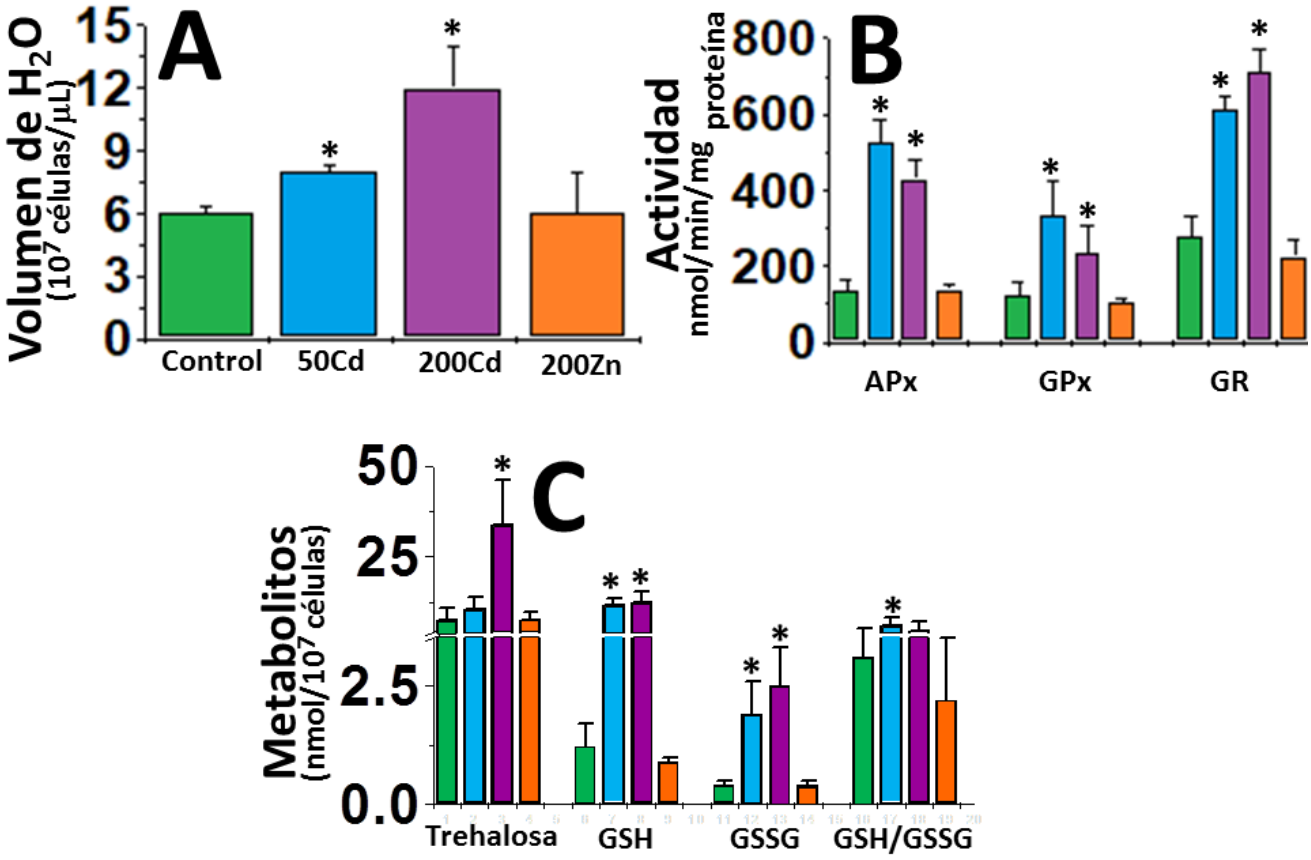

Figura 9. Efecto de la acumulación del Cd^{2+} y del Zn^{2+} en el volumen intracelular de agua, en la actividad de las enzimas antioxidantes y en el contenido de metabolitos en *E. gracilis*.

(A) Volumen intracelular de agua, (B) actividad enzimática de APx, GPx y GR, y (C) contenido de trehalosa y GSH/GSSG en células control (verde) y expuestas al Cd^{2+} (50-200 µM) o Zn^{2+} (200 µM) durante 5 días. Las barras verticales corresponden a la desviación estándar obtenida de al menos 3 determinaciones independientes. **P*< 0.05 *versus* control (ANOVA/*post hoc* Scheffé).

Los efectos de la modulación del volumen intracelular en la acumulación del Cd^{2+} en *E. gracilis* se evaluaron al modificar la osmolaridad del medio de cultivo. La acumulación

del Cd^{2+} y el volumen intracelular de agua no se modificó en células cultivadas en un medio hipo-osmótico (35 mOsM) con respecto al medio de cultivo control (75 mOsM). Sin embargo, las células cultivadas ya sea en un medio iso- o hiper-osmótico (300 y 400 mOsM) acumularon 20-47% menos Cd^{2+} y mostraron un volumen intracelular 50-72% menor, indicando que existe una relación entre los cambios en el volumen intracelular y la acumulación del Cd^{2+}. Debido a esto, se trató de determinar algunas de las características del transportador de agua involucrado en los cambios en el volumen intracelular. Por lo que se determinó la acumulación del Cd^{2+} en presencia de una muy baja concentración de Hg^{2+} (0.5 μM) o de pentamidina (100-500 μM) como inhibidores de las acuaporinas. Los resultados mostraron que el Hg^{2+} bloquea moderadamente los cambios en el volumen intracelular inducidos por el Cd^{2+}, lo cual correlacionó con una disminución en la acumulación del Cd^{2+}, mientras que la pentamidina disminuyó la acumulación del Cd^{2+} (22-51%) y bloqueó por completo el aumento en el volumen intracelular (Fig. 10A y B).

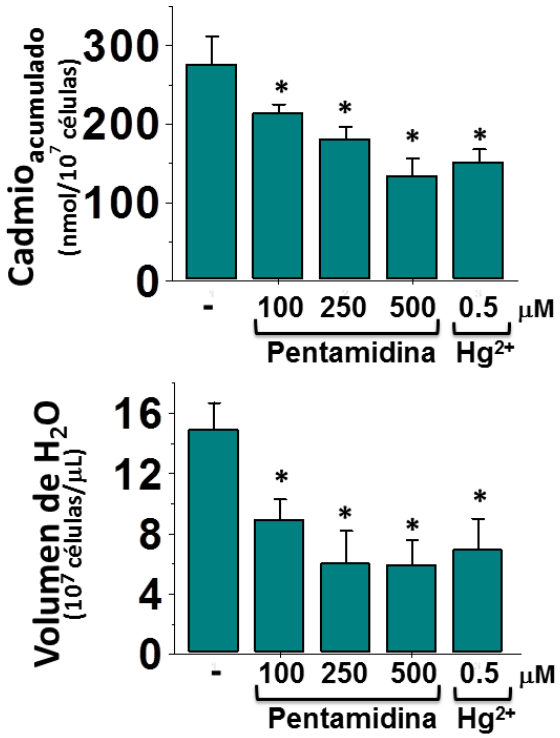

Figura 10. El efecto de la pentamidina y el Hg^{2+} en la acumulación del Cd^{2+} en *E. gracilis*.

(A) Acumulación del Cd^{2+} y (B) volumen intracelular de agua en células expuestas a 200 μM de Cd^{2+} en ausencia o presencia de pentamidina y Hg^{2+} durante 5 días. Las barras verticales corresponden

a la desviación estándar obtenida de al menos 3 determinaciones independientes. *P< 0.05 *versus* control (ANOVA/*post hoc* Scheffé).

Además, se estableció una correlación entre un estrés osmótico y un estrés por Cd^{2+}. Las células expuestas a 50 μM Cd^{2+} durante 5 días al ser re-cultivadas en presencia de 200 mM NaCl, fueron capaces de crecer significativamente mejor que las células control (Fig. 11).

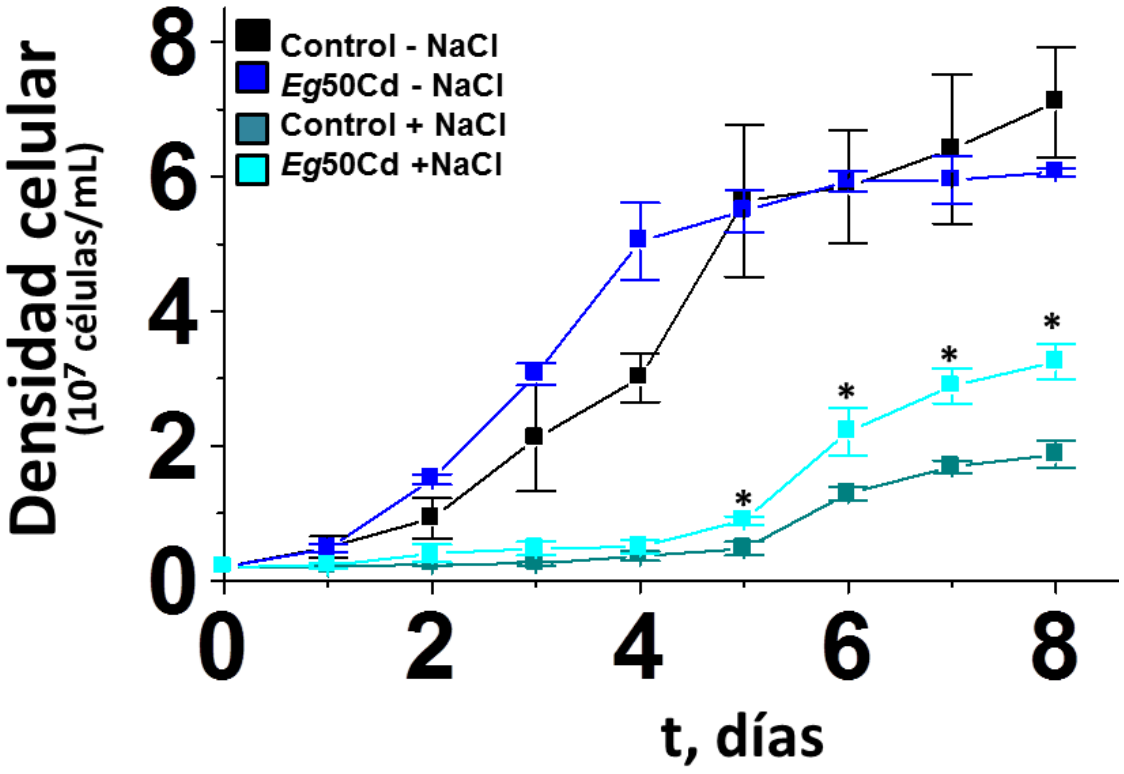

Figura 11. Cinética de crecimiento de células de *E. gracilis* control y pre-expuestas a 50 μM de Cd^{2+}.

Crecimiento de células control y pre-expuestas durante 5 días a 50 μM Cd^{2+} (*Eg*50Cd), en presencia de 0 y 200 mM de NaCl. Las barras verticales corresponden a la desviación estándar obtenida de al menos 3 determinaciones independientes. *P< 0.05 *versus* control (ANOVA/*post hoc* Scheffé).

Por último, como resultados posteriores a esta publicación se evaluó la posibilidad de que otros metales pesados pudieran tener efectos tipo osmótico similares a los que indujo el Cd^{2+}. En la Tabla 2 se muestra el volumen intracelular obtenido durante la exposición distintos metales, donde se observó que el volumen intracelular no cambia en exposición a Pb^{2+} pero incrementa en presencia de Cu^{2+}, Ni^{2+} y Co^{2+}. De igual forma, se determinó que la pentamidina aumenta la acumulación de Pb^{2+} y disminuye la acumulación

de Ni^{2+} y Co^{2+} (Fig. 12); en dichos experimentos se utilizó a células sin metal añadido, y células expuesta a Cd^{2+} ó Zn^{2+} como control.

Tabla 3. Volumen intracelular de agua y acumulación en células de *E. gracilis* expuestas de distintos metales pesados.

Metal	µM	Volumen intracelular (µL/10^7 células)	Acumulación (nmol/10^7células)
-	–	6 (2)	–
Cd^{2+}	200	14.1 (2)	308 ± 39
Zn^{2+}	200	4.3 (2)	125 ± 44
Pb^{2+}	200	6.1 (2)	84 ± 22
Cu^{2+}	200	11.2 (2)	173 ± 86
Ni^{2+}	50	10.4 (2)	12 ± 6
Co^{2+}	200	13.7 (2)	251 ± 46

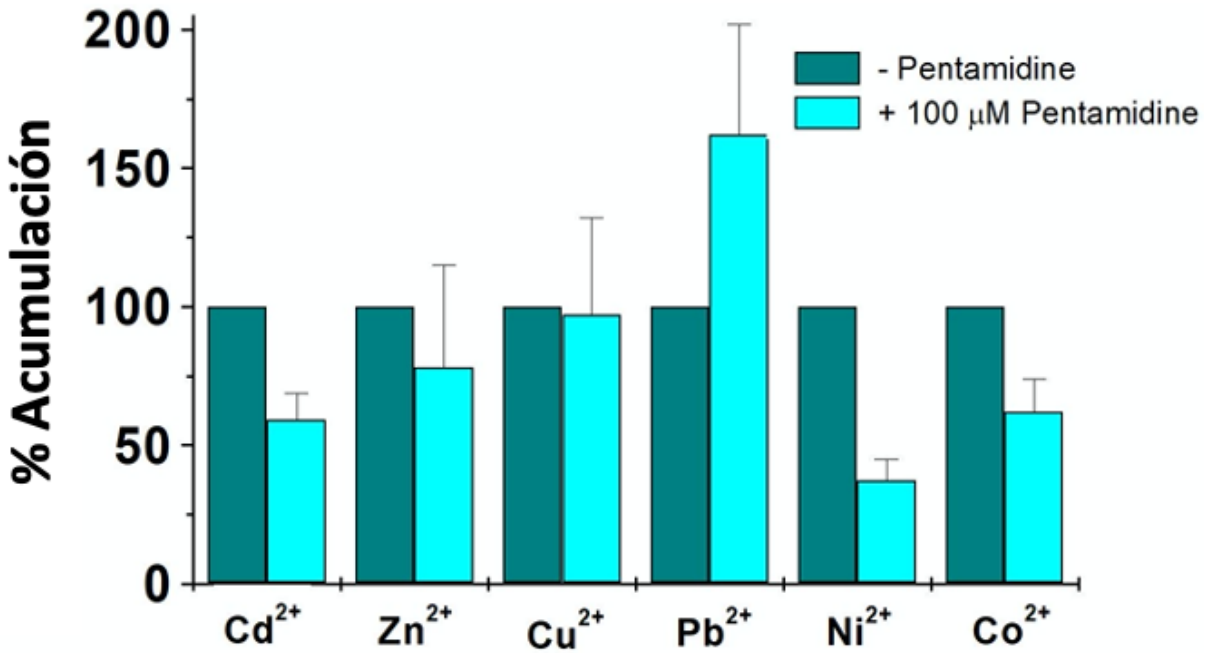

Figura 12. Efecto de la pentamidina en la acumulación de diferentes metales pesados.

Publicación 2:

Los resultados de este trabajo se publicaron en enero del 2020 en el artículo "**The intracellular volume modulates the accumulation of cadmium in *Euglena gracilis*"**

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The intracellular water volume modulates the accumulation of cadmium in *Euglena gracilis*

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ABSTRACT

In order to describe a relatively novel mechanism associated to heavy metal resistance in *Euglena gracilis*, the osmotic responses during cadmium accumulation were analyzed. Several physiological/biochemical parameters were assessed in *E. gracilis* exposed to 50 and 200 μM Cd^{2+}, concentrations well within the range found in polluted soils and aquatic environments. The Cd^{2+} accumulation attained after only 24 h exposure induced marked increases in both the intracellular water volume and cellular size, which were maintained for several days; these changes correlated with (i) an increased intracellular osmolarity (*i.e.* osmotic pressure) driven by a generalized increase in the osmo-metabolites (trehalose, phosphate-molecules, amino acids, thiol-molecules, betaines and polyamines) content; and (ii) the triggering of antioxidant defenses (increased GSH/GSSG ratios and GPx, GR and APx activities). In contrast, no changes in intracellular volume, cellular size and antioxidant status were observed under hyperaccumulation of Zn^{2+}. Furthermore, *E. gracilis* cultured in a hypoosmotic medium promoted greater Cd^{2+} accumulation and water volume, compared to a hyperosmotic medium. These changes were significantly decreased by the aquaporin inhibitors Hg^{2+} and pentamidine. The results suggested that the cell responses to Cd^{2+} and osmotic stress share biochemical mechanisms in this algae-like protist, in which Cd^{2+} accumulation closely correlates with the intracellular water volume changes in an oxidative stress-mediated process.

1. Introduction

Cadmium pollution is an eminent problem throughout the world, as a result of the increase in mining and industrial activities. For instance, areas surrounding mine industries may reach Cd^{2+} concentrations of 11–80 ppm (96–711 μM Cd^{2+}) [1–3]. The physicochemical methods used so far have been unable to amend this environmental problem, which has prompted the search for alternative "green" solutions to decrease cadmium pollution [4–6]. Attempts to improve the Cd^{2+} bioremediation strategies in plants by genetic manipulation of presumed rate-limiting steps of processes that provide Cd^{2+} resistance have been largely unsuccessful [7–11]. Therefore, non-canonical cellular responses under Cd^{2+} stress need to be further characterized to better understand and then improve the Cd^{2+} accumulation/removal abilities of organisms with biotechnological potential for use in Cd^{2+} bioremediation [12].

Plants, algae, protists and yeast have developed genetic and biochemical mechanisms for survival under different abiotic stress challenges [13–16]. Osmotic stress prompts changes in the intracellular water volume associated to mechanisms developed to prevent cellular

Abbreviations: AAS, atomic absorption spectrophotometry; β-Ala, β-alanine; Ala, alanine; APx, ascorbate peroxidase; AQP, aquaporin; Cad, cadaverine; Cys, cysteine; γ-EC, gamma-glutamylcysteine; Gly, glycine; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; His, histidine; MTG, MitoTracker Green; PCA, perchloric acid; PolyGSH, total polymers of glutathione; PolyPs, polyphosphates; Put, putrescine; ROS, reactive oxygen species; Spd, spermidine; TCA, trichloroacetic acid; *t*-BHP, *tert*-butyl hydroperoxide; 50Cd, 50 μM Cd^{2+}; 200Cd, 200 μM Cd^{2+}

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damage by burst or shrinkage. The proposed mechanisms that block the movement of water across biological membranes are (i) inhibition at the transport level (*e.g.* H^+ and/or Na^+/K^+ pumps and aquaporins), (ii) the use of storage organelles (vacuole and contractile vacuole), and (iii) the synthesis of osmolytes [17].

Different halophytes (*i.e.*, *Atriplex halimus*, *Mesembryanthemum crystallinum* and *Iris ensata*) have been proposed as models for Cd^{2+}, Cu^{2+} and Pb^{2+} phytoremediation, arguing that these plants have fully developed the required genetic and biochemical mechanisms, due to their innate osmoregulatory capacity to survive in saline environments [18–24]. In addition, saline stress may confer increased Cd^{2+} tolerance in *Sesuvium portulacastrum* [25]. However, studies focused on understanding the molecular or biochemical mechanisms that halophytes have to accumulate heavy metals are scarce. A taxonomic and phylogenetic analysis carried out to determine whether tolerance to salinity and accumulation of heavy metals is a wide-spread phenotype in plants indicated that such phenotype has no evolutionary link but rather an environmental one [26].

The role of osmoregulatory mechanisms, including modulation of water volume, on the intracellular accumulation of Cd^{2+} has not been established. Halophytes have developed mechanisms favoring heavy metal accumulation, such as the synthesis of chelating metabolites, alteration of the plasma membrane permeability, maintenance of water status and the overproduction of compatible solutes [22,24,27]. Exposure of plants to Cd^{2+} and other heavy metals modifies their osmotic potential and water content [27]. For instance, the osmotic potential and the leaf fresh weight or water content significantly diminish in *Phaseolus vulgaris* and *Kosteletzkya virginica* after Cd^{2+} or Cu^{2+} exposure [28,29]. Thus, although it is still not clear how mechanisms of osmotic protection may contribute to the accumulation of Cd^{2+}, there are similar cellular responses that are activated in both stresses.

E. gracilis is a mixotrophic protist that can hyperaccumulate Cd^{2+}, Zn^{2+} and Ni^{2+} [30–32]; this free-living unicellular, alga-like microorganism lacks cell wall. The mechanisms involved in the intracellular Cd^{2+} accumulation are the syntheses of chelating thiol-molecules (Cys, GSH and poly-GSH), histidine and polyphosphates, and the subsequent formation of Cd-thiol molecule complexes and their compartmentalization into chloroplasts and mitochondria [31,33–36]. In addition, Cd^{2+} accumulation in heterotrophic *E. gracilis* induces significant changes in intracellular water volume [37]. The intracellular water volume also increases [38], as well as the trehalose content [39,40], when *E. gracilis* is subjected to saline stress.

Therefore, it seems that the cellular responses induced by both osmotic and Cd^{2+} stress share some common mechanisms. To elucidate whether there is a mechanistic relationship between the management of osmotic stress with the cadmium hyperaccumulation, in the present work it was assessed in mixotrophic *E. gracilis* which osmotic stress-associated mechanisms facilitate the accumulation of Cd^{2+}.

2. Materials and methods

2.1. Growth conditions

E. gracilis Klebs (a *Z*-like strain) was originally obtained over 35 years ago from the collection of the Parasitology Department, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional de México; due to their generation time, tendency to bleach at 35 °C and lack of growth with galactose as carbon source, these cells are considered a *Z*-like strain. *E. gracilis* cells were grown under axenic photoheterotrophic conditions, in a modified Hutner medium (initial pH 3.5) as previously reported [31].

The culture medium osmolarity was theoretically calculated (75 mOsM) and verified with a cryoscopic osmometer (see Section 2.8). For hyposmotic condition experiments (35 mOsM), the concentrations of glutamate and malate were adjusted to 7 and 3 mM respectively, while 230–330 mM sorbitol or mannitol was added to the culture medium for the isosmotic (300 mOsM) and hyperosmotic (400 mOsM) conditions. The osmolarity of the modified culture media was also experimentally verified.

2.2. Intracellular contents of cadmium, zinc, sodium and potassium

Cells were harvested by centrifugation at 1900 ×*g* and 4 °C and washed twice with 0.1 M KH_2PO_4 pH 7.4 supplemented with 5 mM EGTA for removal of external Cd^{2+} and Zn^{2+}. For Na^+ and K^+, the washing buffer was 225 mM sucrose, 10 mM Hepes and 1 mM EGTA pH 7.2 (SHE buffer). Thereafter, cells were re-suspended in Milli-Q water and digested with 0.5 mL of 99% (v/v) H_2SO_4/2.5 mL of 70% (v/v) HNO_3 for 2 h at 100 °C. The ion contents were determined in the acid-digested cellular extracts by AAS (Varian SpectraAA-640, Australia).

2.3. Determination of intracellular volume, cell surface area and protein

Cells were harvested at room temperature and re-suspended in their own cell-free medium. Cells (2–5 × 10^6 cells/mL) were incubated with ^3H_2O (542–2532 Bq/μL) or [^{3}H]-inulin (650–2911 Bq/μL). After 1 min incubation, the cell suspension was carefully layered in a microfuge tube containing from bottom to top 0.3 mL 30% (v/v) TCA, 0.3 mL 1-bromododecane and 0.3 mL of cell-free medium harvested from the respective cell cultures. Cells were centrifuged at room temperature for 1 min at 14,000 ×*g* through the 1-bromododecane layer into the TCA layer using a microcentrifuge (Benchmark, Edison, NJ, USA). Then, a 0.2 mL aliquot from the top and bottom layers were carefully taken and mixed with scintillation cocktail. Afterwards, radioactivity was determined in a Beckman Coulter Scintillation Counter (Brea, CA, USA). The intracellular water volume was calculated according to Rottenberg [41] (Eq. 1).

$$\underset{Volume}{Intracellular} = \left(\frac{^3H_2O_P}{^3H_2O_{Sn}} - \frac{^3Inul_P}{^3Inul_{Sn}}\right) * Vol_{Sn} \quad (1)$$

where $^3H_2O_P$ = water concentration in the TCA layer (pellet)

$^3H_2O_{Sn}$ = water concentration in the top of the 1-bromododecane layer (supernatant)

$^3Inul_P$ = inuline concentration in the TCA layer (pellet)

$^3Inul_{Sn}$ = inuline concentration in the top of the 1-bromododecane layer (supernatant)

Vol_{Sn} = Total volume (cell free medium *plus* cell suspension) in the top of the 1-bromododecane layer (supernatant).

The surface area of the cell was determined assuming an oval-shaped form. Taking into account the formula to obtain the area of an oval (Eq. (2)), the two diameters of the cell were determined using an eyepiece ruler of an inverted microscope (Carl Zeiss; Oberkochen, Germany).

$$Area = \left(\frac{d^1}{2}\right)\cdot\left(\frac{d^2}{2}\right)\cdot\pi \quad (2)$$

where d^1 = horizontal diameter

d^2 = vertical diameter

$\pi = 3.1416$

The method described by Murphy & Kies [42] was used to determine protein content.

2.4. Mitochondria and chloroplasts detection

Euglena cells (0.5 × 10^6 cells) exposed to 0 (control), 50 and 200 μM Cd^{2+} were loaded with 10 μM MitoTracker Green for 30 min at room temperature and washed three times with Hutner medium pH 3.5. Afterwards, cells were re-suspended in fresh medium and placed in glass coverslips in the presence of HCl (0.1% v/v; pH ~ 1.8) to restrain cell movement. Confocal microscopy images were collected with a Zeiss

LSM 510 meta inverted laser scanning confocal microscope (Carl Zeiss; Oberkochen, Germany) using 63× oil 1.4 N.A. plan apochromat objective lens. MTG $\lambda_{excitation}$ of 488 nm was provided by an argon laser and $\lambda_{emission}$ of 500–550 nm was used. For chlorophyll autofluorescence detection, a $\lambda_{excitation}$ of 488 nm and $\lambda_{emission}$ of 695 nm was used. Laser excitation energy was attenuated 1000-fold to minimize photobleaching and photodamage.

2.5. Content of compatible solutes

Cells (13–37 × 10^6 cells/mL) were harvested, washed and stored at −20 °C until use. Afterwards, cells were thawed in the presence of 0.1% (v/v) Triton X-100 at room temperature for 20 min, and then mixed with 2.7% (v/v) PCA, vigorously stirred for 1 min, and centrifuged for 2 min at 17,000 ×*g* and 4 °C. The acidic supernatants were recovered and neutralized with 3 M KOH/0.1 M Tris. After centrifugation to remove $KClO_4$, the neutralized samples were stored at −70 °C until use.

Trehalose [43,44], paramylon [45] and proline [46] contents were determined as previously reported. Determination of Gly, Ala, β-Ala and His as well as thiol-molecules (Cys, γ-EC, GSH and GSH polymers) was carried out by HPLC as described elsewhere [31,32]. Total amount of betaines [47], polyamines [48] and phosphate-molecules [31] was determined as previously described.

2.6. GSH/GSSG ratios

Cells were harvested after 5 days by centrifugation for 1 min at 1900 ×*g* and 4 °C and washed twice with 100 mM KH_2PO_4 and 5 mM EGTA pH 7.4. The cellular pellet (30–60 × 10^6 cells/mL) was resuspended in water and mixed with 2.7% (v/v) PCA, vigorously stirred for 1 min and centrifuged for 3 min at 17,000 ×*g* and 4 °C. The acidic extracts were neutralized with 3 M KOH/0.1 M Tris and immediately frozen in N_2 and then thawed at room temperature. The samples were further centrifuged at 17,000 ×*g* and the supernatants used for determination of the GSH and GSSG contents at 30 °C in KME buffer (120 mM KCl, 20 mM MOPS, 1 mM EGTA pH 7.2) *plus* 1 U commercial GR/mL, 1 U commercial GPx/mL, 30 mM GSH, 0.15 mM NADPH and 100 μM *t*-BHP. To correct for metabolite loss (and GSH oxidation), known amounts of GSH and GSSG were also subjected in parallel to the same procedure described above.

2.7. Enzyme activities

Cells (≈100 × 10^6 cells) were harvested after 5 days by centrifugation for 1 min at 1900 ×*g* and 4 °C and washed twice with 100 mM KH_2PO_4 and 5 mM EGTA, pH 7.4. The cellular pellet was resuspended in 25 mM Tris-HCl *plus* 1 mM PMSF, 1 mM EDTA and 5 mM DTT pH 7.4 and disrupted by 3 cycles of 1 min sonication (20% of maximal output in a Branson sonicator using a 3 mm tip diameter probe) with 1 min rest in ice. The cellular extracts were centrifuged at 4 °C for 10 min at 12,298 ×*g*, and the supernatants centrifuged for 45 min at 181, 213 ×*g*. The last supernatant was considered as the cytosolic-enriched fraction and was stored in 10% glycerol at −70 °C until use.

The enzyme activities of GPx, GR and APx were determined spectrophotometrically at 340 or 285 nm in KME buffer, pH 7.20 at 30 °C as previously described [31,49]. The GPx activity assay was corrected for the non-enzymatic reaction of peroxide with GSH, adding 1 mM *t*-BHP to the assay mixture prior to protein (0.02–0.3 mg protein/mL). The GR activity assay was carried out with 0.03–0.15 mg protein/mL. The specific reaction was started by adding 1 mM GSSG. APx activity was determined with 0.03–0.4 mg protein/mL.

2.8. Osmotic pressure and medium osmolarity

Cells were harvested by centrifugation and washed with deionized water. The concentrated cell pellet (≈2 × 10^8 cells/mL) was frozen and ground with glass beads (diameter ~1.0 mm) by centrifugation at 15,542 ×*g* for 15 min at 4 °C. The osmolarity of the cell sap and cell-free medium was determined in a cryoscopic osmometer (Osmomat 030; Genotec, Berlin, Germany).

The experimental osmolar concentration of the cellular sap was used to calculate the osmotic pressure by the van't Hoff equation for osmotic pressure (Eq. (3)).

$$\pi = MiRT \quad (3)$$

where M is the molar concentration, R is the universal gas constant (0.082 atm·L·K^{-1}·mol^{-1}), T is the absolute temperature (298.15 K) and i, the van't Hoff factor with value = 1. The van't Hoff factor represents the number of ions a solute forms when dissolved in water. As the cryoscopic osmometer yields the total osmolarity given by the osmotically active species in the cellular sap, i was set to a value of 1.

2.9. Statistical data analysis

Experiments were performed at least with three independent cell samples. Data shown represent mean ± standard deviation. To establish significant differences between two groups, Student's *t*-test analysis was used. For the detection of significant differences between more than two groups, one-way ANOVA/*post hoc* Scheffé analysis, using IBM SPSS Statistics software version 25 (IBM Corp.; NY, USA), was performed. For both statistical analyses, P values < 0.05 or lower were used to determine statistical significance.

3. Results

3.1. Osmotic effects during cadmium accumulation

3.1.1. Intracellular water content

First, it was assessed which osmotic-type responses were revealed during Cd^{2+} stress. Changes in the intracellular water volume were used as the main parameter to establish cellular osmotic-type responses during the cadmium accumulation process. Control cells showed a constant internal volume of 4–6 μL $H_2O/10^7$ cells throughout the growth curve, whereas it was significantly greater (Fig. 1A) in cells incubated with 50 (50Cd) and 200 (200Cd) μM Cd^{2+}. Similarly, the cell surface area was also significantly wider in cells exposed to Cd^{2+} (Fig. 1 inset B); the protein content *per* cell did not change in the presence of Cd^{2+} (Fig. S1). The volume changes peaking after 1 day did not correlate with the accumulation of cadmium, which reached a maximum by the fifth and eighth day of culture for 50Cd and 200Cd cells respectively (Fig. 1B), as previously reported [31].

Changes in cell size were evident after 5 days of Cd^{2+} exposure (Figs. 1C and D). The chlorophyll autofluorescence (indicated by orange-red color) showed that Cd^{2+} stress altered the chloroplasts' shape and apparently increased their number (Fig. 1D). Because chlorophyll also shows autofluorescence at wavelengths used to detect MTG-loaded mitochondria, it was difficult to analyze the effect of Cd^{2+} stress on *in situ* mitochondria. Nevertheless, several green-fluorescence zones (presumably MTG-loaded mitochondria) that did not co-localize with chlorophyll fluorescence signal (Fig. 1D) were observed in both control and Cd^{2+} treated cells. Interestingly, the presumed mitochondrial green-fluorescence was much lower in cadmium-loaded cells *vs.* control cells.

3.1.2. Compatible solutes synthesis

The biosynthesis of “osmolytes” is the most common response of cells subjected to osmotic/hydric stress. In this regard, the trehalose content indeed raised significantly in 200Cd cells, reaching 67 ± 9 mM after 8 days of exposure, whereas control and 50Cd cells maintained their trehalose contents at around 25–33 mM (Fig. 2A). The paramylon content was high in the three cell cultures (≈2500 mM), although in

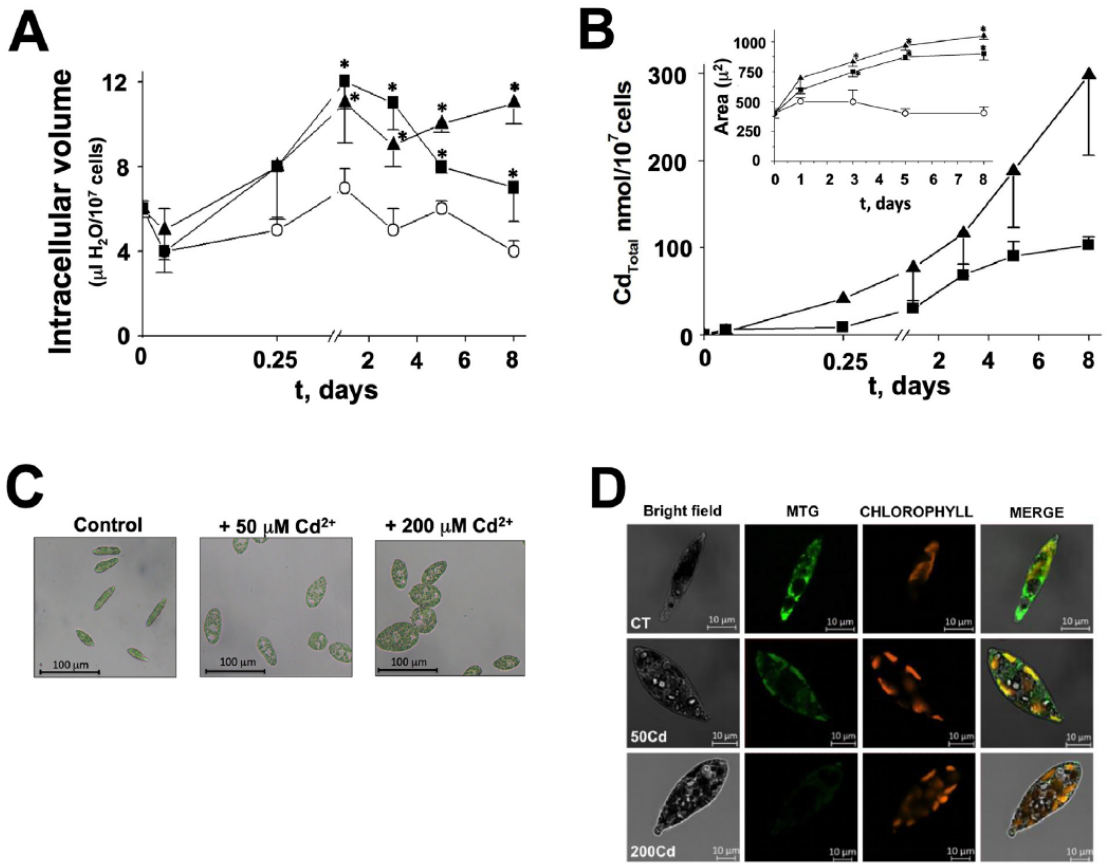

Fig. 1. Effect of Cd^{2+} accumulation on cell volume and size in *E. gracilis*.
(A) Intracellular volume and (B) Cd^{2+} accumulation in control cells (○) and in cells exposed to 50 (■) or 200 (▲) µM Cd^{2+}. Inset in panel B shows cell surface area. (C) Changes in cell size induced by Cd^{2+} exposure for 8 days *versus* control conditions. (D) Chloroplast and mitochondria changes by confocal microscopy. Vertical lines represent SD of at least 3 independent determinations; *$P < 0.05$ *versus* control cells (ANOVA/*post hoc* Scheffé).

control cells it was rapidly consumed and markedly decreased by the third day of culture (< 800 mM), while in the 50Cd and 200Cd cells paramylon was maintained at high levels by at least 8 days of culture (Fig. S2).

The Pro + Gly + Ala + β-Ala + His content (Fig. 2B) was also higher in 50Cd and 200Cd cells after the 3rd day of culture; Gly and Ala were the most abundant with 57–88% of the total amino acid content (Fig. S3). The polyamines (Spd + Put + Cad) increased 6 times in Cd^{2+}-exposed cells during the proliferative phase, while in the stationary phase their concentration decreased by almost half, but remained higher with respect to control cells (Fig. 2C). Spd was the most abundant polyamine with 43–84% of the total, followed by Put with 10–50% and Cad with 1.2–6.9% (Fig. S4). The betaines content was the least abundant osmolyte in *E. gracilis* (Fig. 2D), being almost ten times lower than the contents of the other osmolyte groups analyzed.

The Na^+ (Fig. 3A) and K^+ (Fig. 3B) concentrations remained unchanged in control and Cd^{2+}-exposed cells. Also, as previously reported [31,36], the total thiol content, mainly the GSH pool, was greatly enhanced in Cd^{2+}-exposed cells (Table 1).

3.1.3. Osmotic pressure

To determine whether the Cd^{2+} accumulation induced changes in the intracellular osmolarity, the cellular osmotic pressure was determined. First, a theoretical internal osmolarity was calculated using the concentrations of the metabolites experimentally determined; second, the intracellular osmolarity was determined by using a cryoscopic osmometer (Table 1). The theoretical intracellular osmolarity of control cells was ≈321 mOsM. For 50Cd and 200Cd cells, the internal osmolarity was 1.5 and 2.2 times higher, despite growing in a hypo-osmotic medium of 50–80 mOsM (Fig. S5). Experimental data further established the increased intracellular osmolarities in Cd^{2+} stressed cells. These results were used to calculate the cellular osmotic pressure with the van't Hoff equation, which indicated that the accumulation of cadmium induced an increase in the intracellular osmotic pressure (Table 1).

3.2. Relationship between heavy metal accumulation and the intracellular water content

3.2.1. Osmotic effects induced by zinc accumulation

To determine whether the changes in the intracellular volume and osmotic pressure were specific for Cd^{2+}, cells were also exposed to 200 µM Zn^{2+} for 5 days. However, the accumulation of Zn^{2+} did not modify the water volume and the trehalose and GSH contents (Table 2), despite reaching greater intracellular zinc concentrations than those of cadmium. Exposure to higher Zn^{2+} concentrations (0.5–1 mM) did not induce changes in the intracellular volume either (data not shown).

To identify the mechanistic reason why Cd^{2+} accumulation was able to induce osmotic-related changes, whereas Zn^{2+} did not, oxidative stress markers were assessed. Indeed, the oxidized glutathione (GSSG) levels as well as the activities of the anti-oxidant enzymes APx, GR and GPx were significantly increased by Cd^{2+} but not by Zn^{2+},

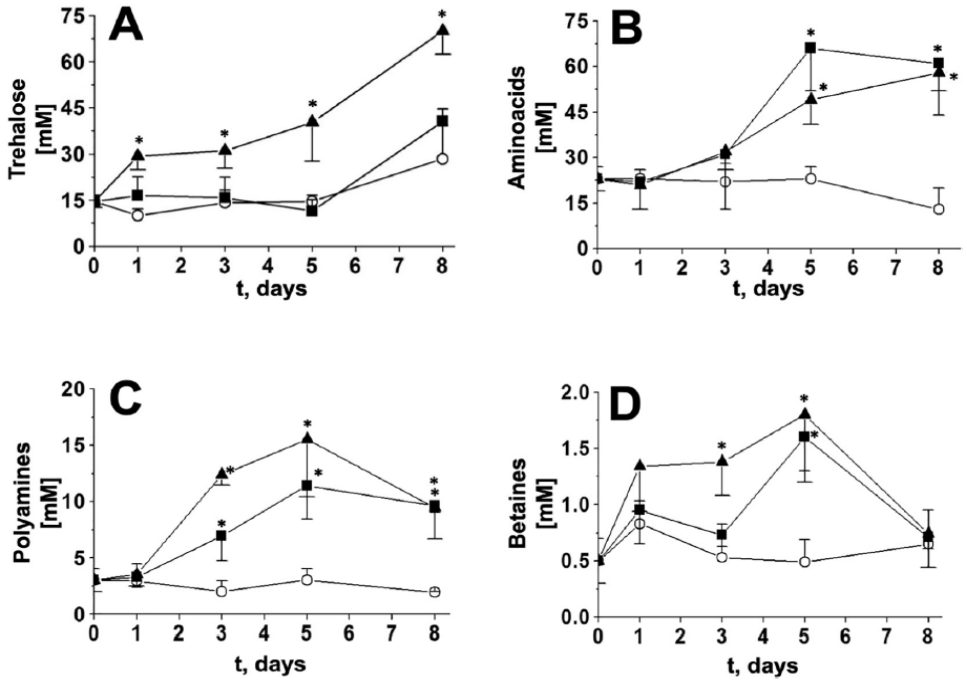

Fig. 2. Osmolytes content in *E. gracilis* exposed to Cd^{2+}.
Concentrations of (A) trehalose, (B) sum of proline, glycine, alanine, histidine and β-alanine in *E. gracilis*, (C) polyamines (spermidine, putrescine and cadaverine) and (D) betaines of control cells (○), and of cells exposed to 50 (■) and 200 (▲) μM Cd^{2+} for 8 days. The intracellular content of each metabolite was related to its respective intracellular volume to calculate the millimolar concentration. Vertical lines represent SD of at least 3 independent determinations; *$P < 0.05$ *versus* control cells (ANOVA/*post hoc* Scheffé).

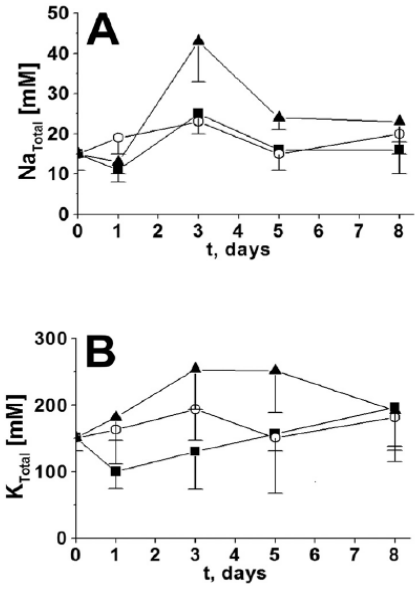

Fig. 3. Sodium and potassium content in *E. gracilis* exposed to Cd^{2+}.
Intracellular contents of Na^+ (A) and K^+ (B) in control cells (○) and in cells exposed to 50 (■) or 200 (▲) μM Cd^{2+} for 8 days.

Table 1
Theoretical and experimental calculation of osmotic pressure.

Metabolite or ion [mM]	Condition		
	Control (5 days culture)	+50 μM Cd^{2+}	+200 μM Cd^{2+}
K_{Total}	151 ± 20 (4)	157 ± 90 (4)	252 ± 63* (4)
Na_{Total}	15 ± 4 (3)	16 ± 5 (3)	24 ± 3* (3)
Free phosphate	100 ± 14 (3)	152 ± 18* (3)	208 ± 98 (3)
Trehalose	14 ± 2 (4)	11 ± 3 (4)	40 ± 11* (4)
Amino acids (Pro, Gly, Ala, βAla, His)	24 ± 4 (4)	66 ± 14* (4)	49 ± 8* (4)
Thiol molecules (Cys, γ-EC, GSH, PCs)	13 ± 2 (6)	25 ± 3* (4)	33 ± 11* (3)
Polyamines (Spd, Put, Cad)	3 ± 1 (4)	12 ± 3* (4)	16 ± 5* (4)
Betaines	0.5 ± 0.2 (3)	1.6 ± 0.3* (3)	1.8 ± 0.6* (3)
Cd_{Total}	–	15 ± 1 (4)	28 ± 6 (4)
Theoretical total osmolarity	321 ± 47	456 ± 137	652 ± 206
Experimental total osmolarity	182 ± 46 (4)	292 ± 66 (4)	425 ± 127 (4)
Osmotic pressure	5 ± 1 (4)	8 ± 2* (4)	10 ± 3* (4)

The data shown represent the mean ± SD of the number of preparations assayed between parentheses.
* $P < 0.05$ *versus* control cells (ANOVA/*post hoc* Scheffé).

Table 2
Effect of Zn^{2+} and Cd^{2+} accumulation on water volume, trehalose and glutathione contents, antioxidant enzyme activities in *E. gracilis*.

	Water volume	Metal accumulation	Metabolites				Enzymes		
			Trehalose	GSH	GSSG	GSH/GSSG	APx	GPx	GR
	$\mu L_c/10^7$ cells	[mM]	[mM]				nmol/min * mg $_{prot}$		
Control	6 ± 0.4 (4)	–	8 ± 3 (3)	1.2 ± 0.5 (3)	0.4 ± 0.1 (3)	3.1 ± 2.2 (3)	134 ± 28 (5)	123 ± 38 (5)	278 ± 52 (5)
50 µM Cd^{2+}	8 ± 0.3* (4)	13 ± 1* (3)	11 ± 3^{NS} (3)	12.1 ± 1.5* (3)	1.9 ± 0.7* (3)	6.4 ± 1.8* (3)	526 ± 60* (3)	334 ± 89* (3)	612 ± 37* (3)
200 µM Cd^{2+}	12 ± 2* (4)	21 ± 2* (3)	34 ± 12* (3)	12.7 ± 2.7* (3)	2.5 ± 0.8* (3)	5 ± 2.3^{NS} (3)	429 ± 53* (5)	234 ± 75* (5)	710 ± 61* (5)
200 µM Zn^{2+}	6 ± 2 (4)	36 ± 5 (3)	8 ± 2 (3)	0.9 ± 0.1 (3)	0.4 ± 0.1 (3)	2.2 ± 1.3 (3)	139 ± 11 (3)	103 ± 14 (3)	226 ± 46 (3)

Cells were cultured for 5 days and the data shown represent the mean ± SD of the number of preparations assayed between parentheses.
* $P < 0.05$ *versus* determined in cells exposed to Zn^{2+} (ANOVA/*post hoc* Scheffé). NS = not significantly different from cells exposed to Zn^{2+}.

indicating oxidative stress induced by Cd^{2+}. A counter-intuitive observation was the increased GSH/GSSG ratios induced by Cd^{2+} since oxidative stress usually results in decreased ratios.

3.2.2. Cadmium accumulation and water volume under different medium osmolarities

The interplay between Cd^{2+} levels and water volume was further assessed by modifying the osmolarity of the incubation medium. The intracellular volume did not change significantly in control cells cultured in different medium osmolarity, although there was a tendency to decrease as external osmolarity increased (Fig. 4). Cell growth under 35 mOsM and 25 mOsM (culture medium with no carbon sources, *i.e.* strict phototrophic conditions) was lower than that attained under 75 mOsM (standard culture medium), probably due to the carbon source deficit, although this effect was not observed in the presence of Cd^{2+} (data not shown). The Cd^{2+} accumulation and intracellular volume in cells grown in the 35 mOsM medium were similar to those attained in the usual 75 mOsM culture (Fig. 4); cells grown under strict phototrophic conditions accumulated 221 ± 32 nmol $Cd^{2+}/10^7$ cells ($n = 4$) and displayed an intracellular volume of 6.8 µL/10^7cells (n = 2). In contrast, cells accumulated significantly 20–47% less Cd^{2+}, and showed a 50–72% smaller intracellular volume in the iso- and hyperosmotic media (300 and 400 mOsM) (Fig. 4). In general, cell viability was > 95%, except in the 400 mOsM condition in which viability was about 75%. Replacement of sorbitol by mannitol to modify the osmolarity of the culture medium rendered the same results (data not shown). These data indicated a correlation between higher cell volume and greater cadmium accumulation, suggesting a mechanistic relationship.

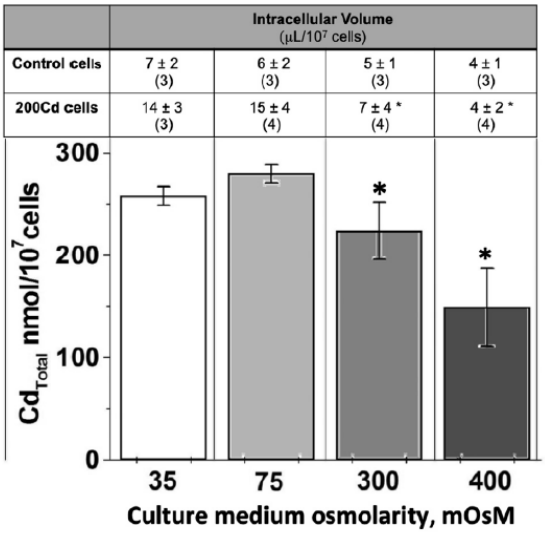

Fig. 4. Accumulation of Cd^{2+} under different culture medium osmolarities. This experiment aimed to modulate the intracellular volume of *E. gracilis* by modifying the osmolarity of the culture medium and determining how this affects the accumulation of cadmium. The table above shows the values of intracellular volume in cells cultured for 5 days at the indicated medium osmolarities with or without 200 µM Cd^{2+}; the bars represent the accumulation of cadmium in cells cultured for 5 days at the indicated osmolarities with 200 µM Cd^{2+}. * $P < 0.05$ *versus E. gracilis* cells with 200 µM Cd^{2+} in 75 mOsM (standard culture medium) by an ANOVA/*post hoc* Scheffé analysis.

3.2.3. Aquaporin role on cadmium accumulation and intracellular water volume

To assess whether the water transport across the plasma membrane is involved in the cadmium accumulation, Hg^{2+} (0.5 µM) and pentamidine (100–500 µM) were used as aquaporin inhibitors [50–52]. Hg^{2+} indeed blocked the increase in water volume induced by Cd^{2+}, correlating with a slight but significant inhibition of Cd^{2+} accumulation (Figs. 5A and B). The increment in water volume induced by salt stress [38] was also partially inhibited by Hg^{2+} (Table S2). Pentamidine also diminished Cd^{2+} accumulation (22–51%), and fully blocked the increase in intracellular water volume induced by Cd^{2+} (Fig. 6A and B). On the other hand, the low Hg^{2+} concentration used did not affect the cellular growth in the presence or absence of Cd^{2+} (data not show), whereas pentamidine promoted an increased cell growth in the presence of Cd^{2+} (Fig. 6C). In addition, no significant differences were found between control *vs.* Hg^{2+}, and Cd^{2+} *vs.* Hg^{2+} *plus* Cd^{2+} exposures on oxidative stress markers (Table S1).

3.3. Relationship between the biochemical mechanisms activated by osmotic and heavy metal stress

Finally, to establish a mechanistic relationship between Cd^{2+} accumulation and osmotic stress responses, cells were exposed to 50 µM Cd^{2+} for 5 days, and then further re-cultivated in the presence of 200 mM NaCl. The growth of *Eg50Cd* cells under salt stress was significantly higher *versus* control (no Cd^{2+}) cells under salt stress (Fig. 7). However, salt-stressed cells did not show greater growth under Cd^{2+} stress (data not shown).

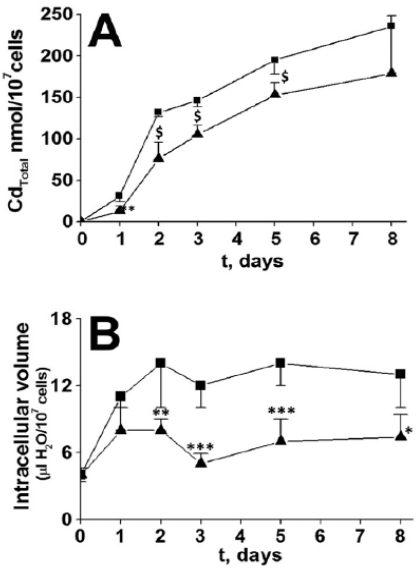

Fig. 5. Inhibition of Cd^{2+} accumulation and intracellular water volume increase by Hg^{2+} in *E. gracilis*.
(A) Cadmium accumulation and (B) intracellular volume in cells exposed to 200 µM Cd^{2+} in presence of 0 (■) and 0.5 µM Hg^{2+} (▲) for 8 days. *$P < 0.05$; **$P < 0.025$; ***$P < 0.01$; $$P < 0.005$ *versus* 200Cd cells (no Hg^{2+} added) by Student's *t*-test.

4. Discussion

4.1. Cadmium accumulation induces osmotic stress-related cell responses

It has been documented that the accumulation of heavy metals induces effects similar to those triggered by osmotic stress, even when the concentrations of these metals are not osmotically stressful. In plants, the heavy metal exposure induces a decrease in the water content of different tissues, whereas the concentration of solutes and osmotic pressure increase [27–29,53,54]. The results of the present study showed that the intracellular osmolarity also increased in *E. gracilis* exposed to Cd^{2+}. The difference between the theoretical and experimental intracellular osmolarity values was very likely due to the strong interaction of some molecules and ions, which was not accounted for in the theoretical estimation. For instance, accumulated Cd^{2+} is not expected to be osmotically active because it is tightly bound by thiol-molecules (the stability constant of the Cd-$(GS)_2$ complex is $2.24 \times 10^{15}\,M^{-2}$ [55]).

Avena sativa, *Ulva lactuca*, *Chlorella sp* and *Trichosporon cutaneum* increase the synthesis of several osmolytes such as amino acids, betaines, saccharides and polyamines when exposed to 0.04–2.5 mM Cd^{2+}, Cu^{2+} or Cr^{6+} [56–58]. Under osmotic stress due to salinity, osmo-metabolites like trehalose behave as cellular stabilizers, optimizing the internal water content by equalizing the intracellular osmolarity with that of the surrounding medium. Thus, osmo-metabolites provide protection to proteins, nucleic acids, and organelles by maintaining the arrangement and level of water molecules, and may also act as ROS scavengers [59–61]. Furthermore, it has been suggested that the synthesis of osmo-metabolites is a general cell response that organisms have developed to cope with other stresses, besides osmotic stress [59].

E. gracilis exposed to Cd^{2+} increased trehalose to levels similar to those attained under osmotic stress due to salinity [39,40]. A similar pattern was also observed for thiol-molecules, polyamines, amino acids and betaines. Also, metabolites like glucose were transformed to their less active osmotic form (paramylon, a β1–3 glucose polymer), perhaps as part of the osmotic regulation of the cell under stress.

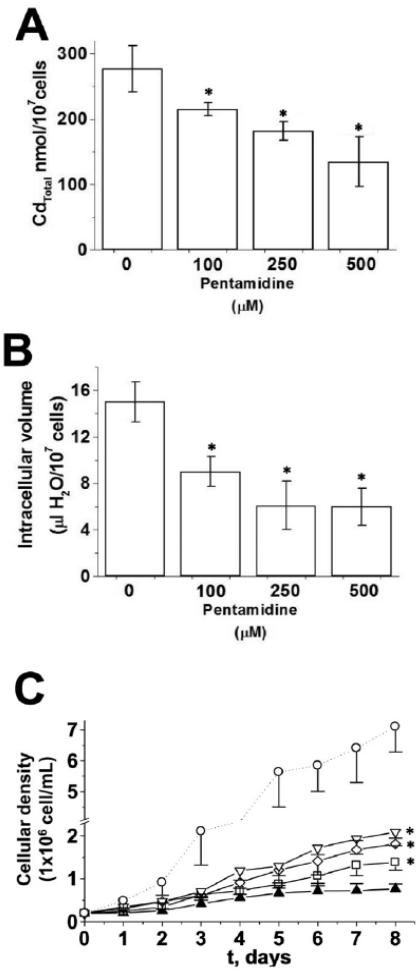

Fig. 6. The effect of pentamidine on Cd^{2+} accumulation, water volume and cellular growth of *E. gracilis*.
(A) Cadmium accumulation and (B) intracellular volume in the presence of 0, 100, 250 and 500 µM of pentamidine after 5 days of exposure to 200 µM Cd^{2+}. (C) Growth of *E. gracilis* with 200 µM of Cd^{2+} and 0 (▲), 100 (□), 250 (◇) and 500 (▽) µM of pentamidine. Control cells (without Cd^{2+}) growth is shown with dash-dot line. *$P < 0.05$ *versus* growth of *Eg200Cd* cells (ANOVA/*post hoc* Scheffé).

The present study clearly showed that *Euglena* cells exposed to Cd^{2+} increased cell size and volume. The significant increased intracellular volume attained by *E. gracilis*, in comparison to some green algae, plants and yeasts, may be related to the lack of a rigid cell wall in this protist [62]. Furthermore, confocal microscopy analysis revealed a decrease in the mitochondrial energization of Cd^{2+}-exposed cells, as a diminished MTG signal was observed; MTG accumulates in the mitochondrial matrix when a H^+ electrical gradient ($\Delta\psi_m$) across the inner mitochondrial membrane is formed. Confocal microscopy also showed an increased chloroplasts number in 50Cd and 200Cd cells. These observations correlated with the enhanced total oxygen consumption (*i.e.*, a uncoupled cellular respiration and low $\Delta\psi_m$) and increased chlorophyll content in *E. gracilis* cells exposed to 200 µM Cd^{2+} for 8 days [31].

E. gracilis accumulated two-fold more zinc than cadmium. However, Zn^{2+} was not toxic for *Euglena* at the concentrations used [31], and

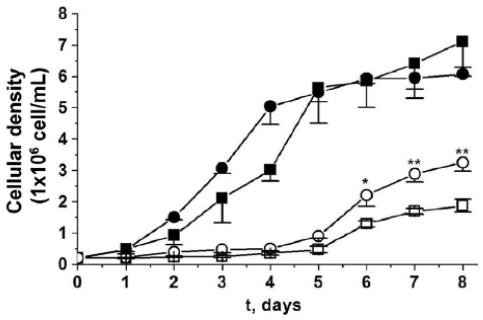

Fig. 7. *Eg50Cd* (and control) cell growth under salt stress.
Growth of *E. gracilis* with 0 (■, ●) and 200 mM NaCl (□, ○). Cells were previously cultures with no Cd^{2+} (■, □) or with 50 μM Cd^{2+} (●, ○) for 5 days (*Eg50Cd*). $^{*}P < 0.01$; $^{**}P < 0.005$ *versus* growth of control cells *plus* 200 mM NaCl (Student's *t*-test).

hence Zn^{2+} did not induce osmotic changes. Therefore, the increased cell volume induced by Cd^{2+}, and not by Zn^{2+}, could be associated to severe oxidative stress by Cd^{2+} (as revealed by the marked increased antioxidant enzyme activities and the increased GSSG levels) and strong interaction, and inactivation, of reactive cysteine residues in proteins. Indeed, the cysteine affinity constant for Cd^{2+} ($7.76 \times 10^{9} M^{-1}$) is greater than for Zn^{2+} ($3.16 \times 10^{8} M^{-1}$) [61]. Similarly, histidine and GSH also have 2–3 orders of magnitude greater affinity for Cd^{2+} than for Zn^{2+} [63]. The GSH/GSSG ratio, a typical and sensitive oxidative stress marker, did not decrease under Cd^{2+} stress but it was rather increased. However, as GSH forms high-affinity complexes with Cd^{2+}, which drastically decreases both the ***free*** Cd^{2+} and GSH concentrations, the GSH/GSSG ratio then seems a less reliable oxidative stress marker under Cd^{2+} stress.

4.2. Water transport contributes to the accumulation of cadmium

In plants, the exposure and accumulation of heavy metals generate an imbalance in the cell water homeostasis [27,28]. The pea mutant *SGECd*, generated by random chemical mutation with ethylmethane sulfonate [64], shows a greater tolerance to and accumulation of Cd^{2+} in comparison to wild-type plants; this mutant also exhibits a more efficient water transport in the vascular system (xylem), increased stomata conductance, and enhanced osmotic and hydric potential, turgency and transpiration [54]. Likewise, *Phytolacca americana* shows ability to hyperaccumulate Cd^{2+} because it modifies tonicity, transpiration and the stomata functioning [65]. Inhibition of Cd^{2+} accumulation by Hg^{2+} suggested a role for aquaporins in the development of the *Phytolacca* phenotype. It has also been proposed that Cd^{2+} and other heavy metal ions can affect water transport by changing aquaporin conductivity, leading to decreased intracellular water content and increased solutes content in plants [27].

In contrast, the increased intracellular water volume of *E. gracilis* induced by Cd^{2+} may be involved in regulating (and decreasing) the Cd^{2+} concentration to thus attenuating its toxicity. This is illustrated by the observation that the cadmium concentration was kept constant, or within a narrow range, in cells grown under different medium osmolarities, but the total amount of nmoles of accumulated cadmium was clearly greater when the cells increased their water volume.

It has been proposed that the water transport by aquaporins is tightly linked to the ability of plants to resist different types of stress, such as drought and heavy metal stress [66,67]. Indeed, expression of aquaporins is regulated by different abiotic stresses, including drought, salinity, low temperatures, oxidative and osmotic stress [68–70]. It is known that the main mechanisms of post-translational activation in aquaporins to modulate their gating involve phosphorylation, pH, divalent cations (Ca^{2+}) and oxidation of its amino acid residues by ROS [69,71,72]. Increased mRNA levels of aquaporin isoforms have been observed in *Phytolacca americana* and *Nicotiana tabacum* in response to Cd^{2+} exposure [73,74]. As ROS have been described as second messengers for aquaporins transcription [69,71,72], then oxidative stress triggered by Cd^{2+} could also regulate expression of aquaporins. The transport of H_2O_2 by aquaporins provides another regulatory link with ROS signaling [69].

Moreover, it has been shown that the aquaporins from *Pteris vittata, Hydrangea macrophylla and Hordeum vulgare* can transport arsenite, aluminium and boron [75–77]. Moreover, overexpression of *Triticum aestivum* aquaporins in *A. thaliana* and tobacco increases Ca^{2+} accumulation [78,79], a divalent cation with a similar ionic radius to that of Cd^{2+}. A genome-wide analysis in maize unveiled a correlation between the expression patterns of the aquaporin gene *Zm*NIP2;3 with those of a gene involved in Cd^{2+} transmembrane transport [80], implying a relationship between aquaporins and Cd^{2+} transport. Indeed, the blockade of aquaporins by Hg^{2+} and pentamidine in *E. gracilis* cells exposed to Cd^{2+} was associated with a significant decrease in the accumulation of Cd^{2+}, suggesting that a fraction of Cd^{2+} uptake might be mediated by aquaporins.

5. Conclusions

The present work shows for the first time that water volume changes play an important regulatory role on Cd^{2+} accumulation in *E. gracilis*. Indeed, Cd^{2+} accumulation, but not Zn^{2+} accumulation, was able to induce osmotic changes such as increased intracellular water volume, cellular size, osmotic pressure and osmo-metabolites concentration in a process apparently mediated by oxidative stress and dependent on aquaporins. These osmotic changes were more evident in cells incubated in a hypoosmotic medium.

CRediT authorship contribution statement

Rosina Sánchez-Thomas: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization.**Jorge D. García-García:** Conceptualization, Writing - review & editing, Visualization.**Álvaro Marín-Hernández:** Methodology, Resources, Writing - review & editing.**Juan P. Pardo:** Conceptualization.**Sara Rodríguez-Enríquez:** Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Funding acquisition.**Rosario Vera-Estrella:** Methodology, Resources.**Ambar López-Macay:** Methodology, Formal analysis, Resources.**Rafael Moreno-Sánchez:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

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Supplementary material

The intracellular volume modulates the accumulation of cadmium in *Euglena gracilis*.

Rosina Sánchez-Thomas, Jorge D. García-García, Álvaro Marín-Hernández, Juan P. Pardo, Sara Rodríguez-Enríquez, Rosario Vera-Estrella, Ámbar López-Macay, Rafael Moreno-Sánchez.

Algal Research. 2020. 46: 101774. doi: 10.1016/j.algal.2019.101774.

Table S1. Effect of Hg^{2+} on APx, GPx, GR activities and thiol contents in *E. gracilis* exposed to 200 μM Cd^{2+} for 5 days.

	Activity ($nmol/min/mg_{protein}$)			
Enzyme or metabolite	Control	0.5 μM Hg^{2+}	+200 μM Cd^{2+}	+200 μM Cd^{2+} + 0.5 μM Hg^{2+}
APx	134 ± 28 (5)	87 ± 12 (3)	429 ± 53 (5)	424 ± 21 (3)
GPx	123 ± 38 (5)	143 ± 16 (3)	234 ± 75 (5)	353 ± 50 (3)
GR	278 ± 52 (5)	232 ± 44 (3)	733 ± 57 (5)	710 ± 61 (3)
Total content of thiols ($nmo/10^7$cells)	58 ± 6 (3)	62 ± 14 (3)	188 ± 26 (3)	211 ± 32 (3)

Table S2. Hg^{2+} inhibits the increment in the intracellular volume induced by NaCl.

	IntracellularVolume $\mu L/10^7$cells	
	48h	120h
Control	6 ± 2 (3)	4 ± 1 (3)
200 μM NaCl	14 ± 4 (3)	9 ± 3 (3)
200 μM NaCl + 0.5 μM Hg^{2+}	6 ± 3 (3)	6 ± 2 (3)

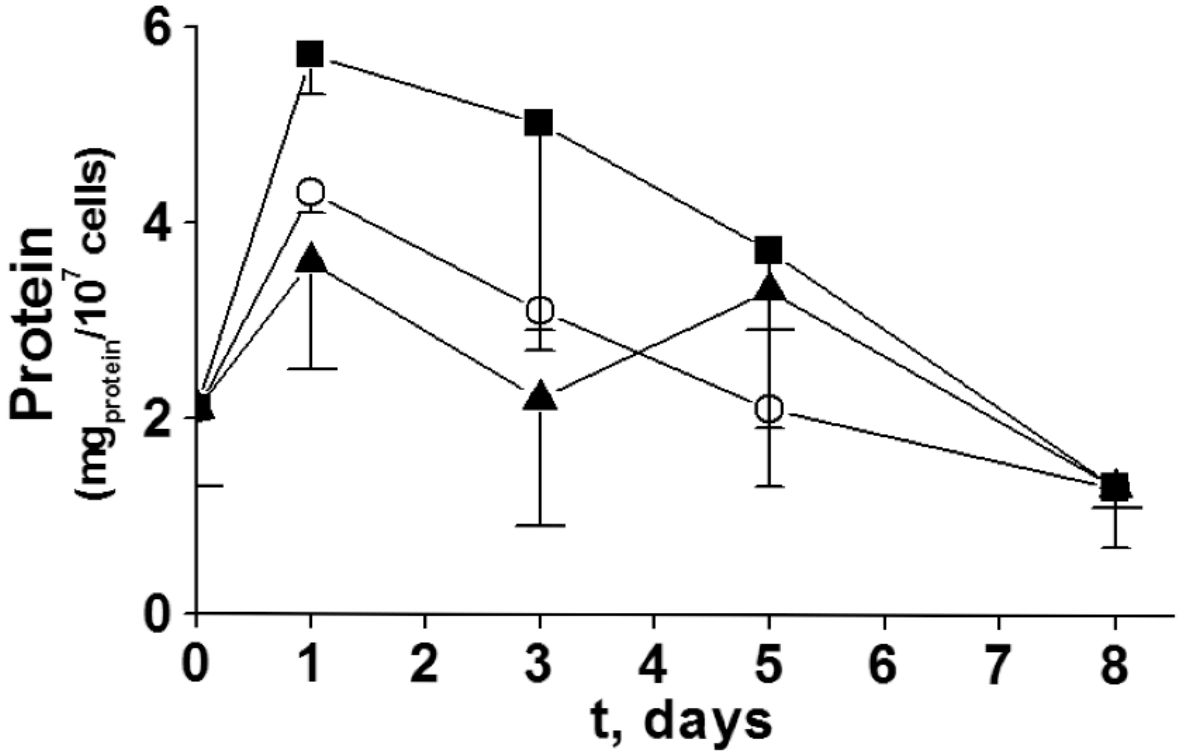

Figure S1. Changes in protein content during Cd^{2+} accumulation in *E. gracilis.* Control conditions (○) or exposed to 50 (■) and 200 (▲) μM Cd^{2+} for 8 days. Vertical lines indicate the SD of at least 3 independent determinations.

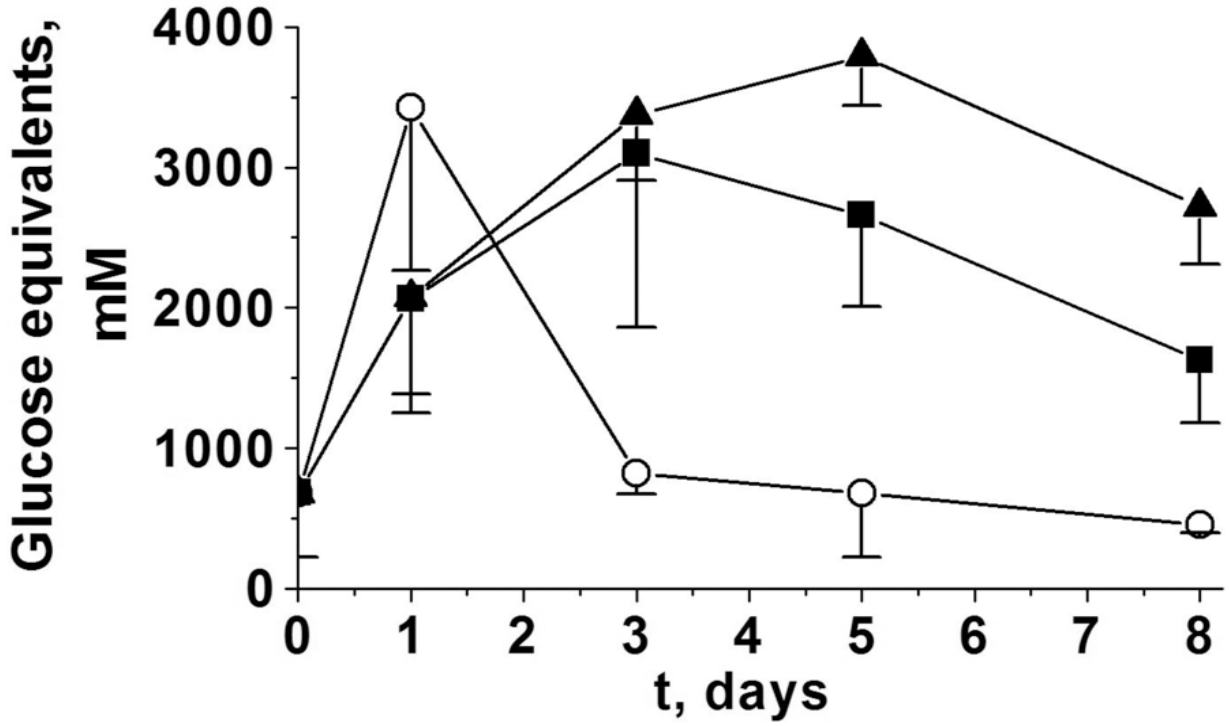

Figure S2. Paramylon content in *E. gracilis* exposed to Cd^{2+}.

Control conditions (○), or in exposure to 50 (■) and 200 (▲) μM Cd^{2+} by 8 days. The results are presented as glucose equivalents. Vertical lines indicate the SD of at least 3 independent determinations.

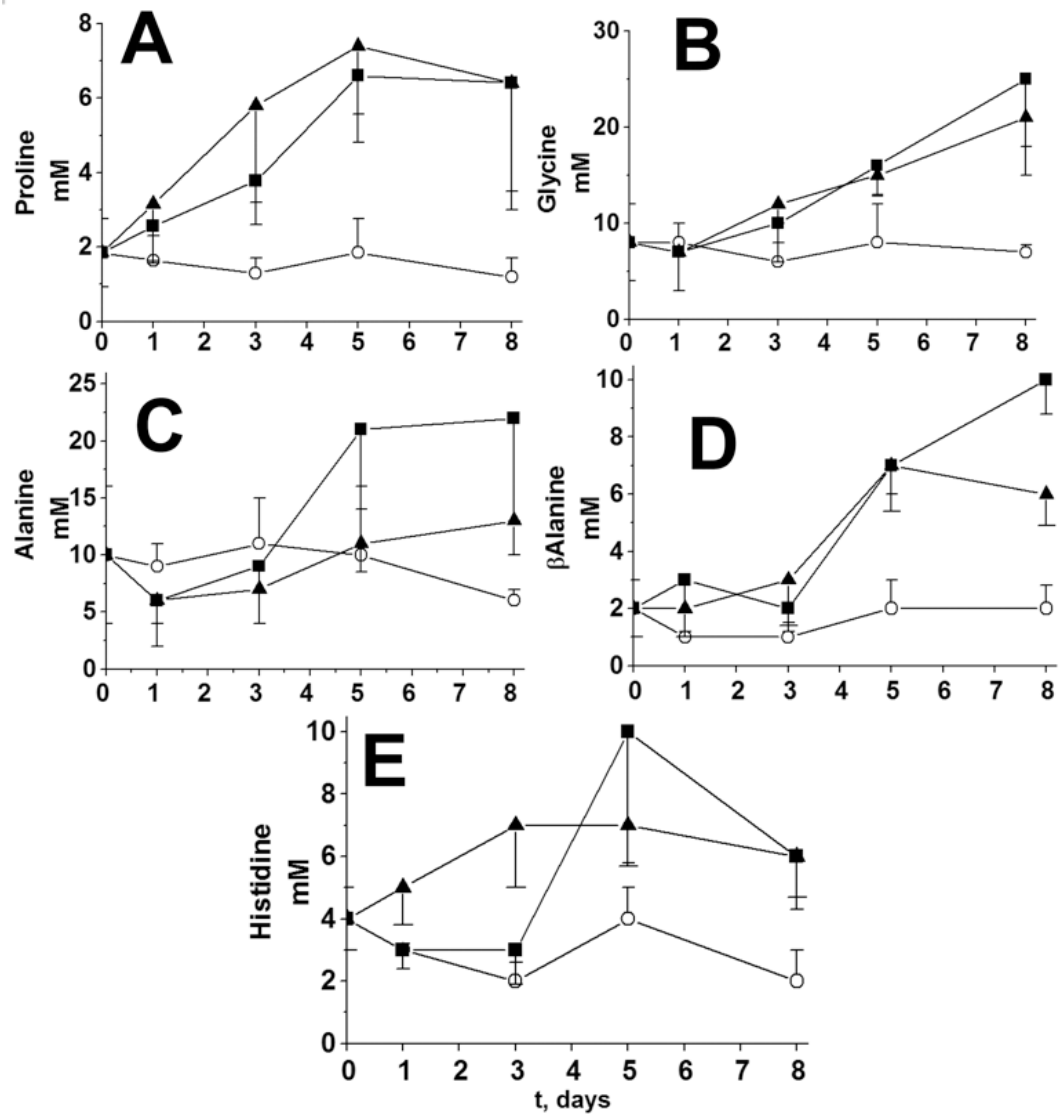

Figure S3. Amino acids content in *E. gracilis* exposed to Cd^{2+}.

The concentration of **(A)** proline, **(B)** glycine, **(C)** alanine, **(D)**β-alanine and **(E)** histidine in cells in control conditions (○), or under exposure to 50 (■) and 200 (▲) μM Cd^{2+} for 8 days. Vertical lines indicate the SD of at least 3 independent determinations.

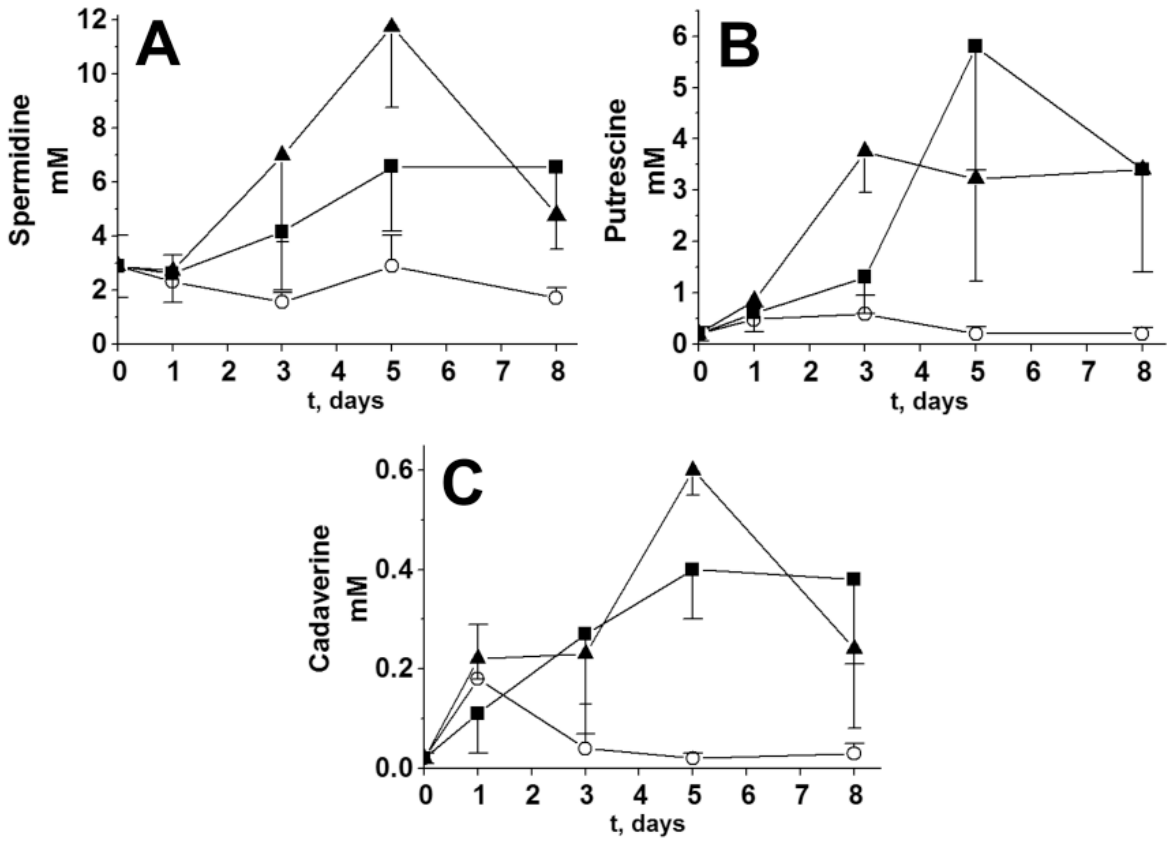

Figure S4. Polyamines in *E. gracilis* exposed to Cd^{2+}.

The concentration of **(A)** spermidine, **(B)** putrescine and **(C)** cadaverine in control cells (○), and in cells exposed to 50 (■) and 200 (▲) μM Cd^{2+} for 8 days. Vertical lines indicate the SD of at least 3 independent determinations.

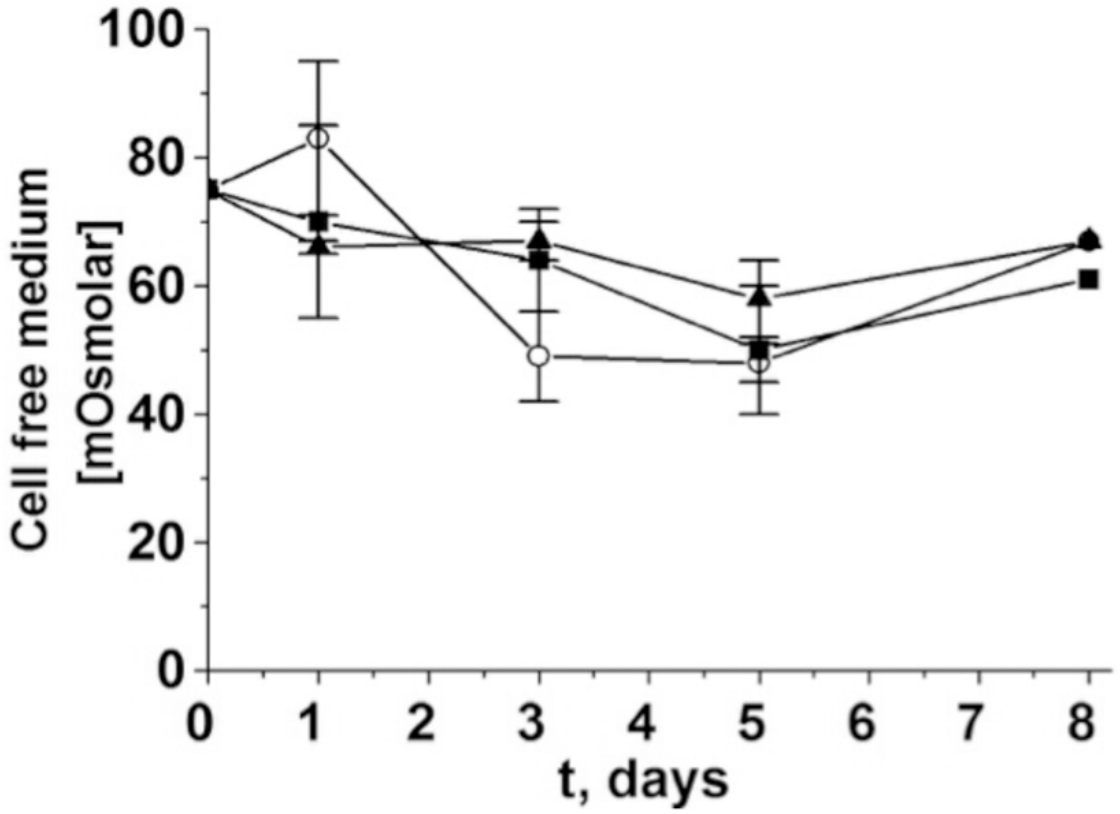

Figure S5. Osmolarity of the culture medium.

Osmolarity of the cell-free medium after 8 days of culture under control conditions (○) or exposed to 50 (■) or 200 (▲) μM. Cd^{2+}. Vertical lines indicate the SD of at least 3 independent determinations.

Response to Reviewers

Reviewer 1:

"It lacks a part statistics in the M&M. I add that for me, the unique use of a Student's t-test in the article is a little bit disappointing."

R: A statistical analysis section in M&M (p. 10) is now shown. In addition to the Student *t*-test, the results were now also analyzed using ANOVA/Scheffé for a more complete and rigorous statistical data analysis (see Tables 1 and 2; figures 1, 4, 5 and 6). The statistically significant differences described before remained unchanged.

"Introduction, Line 209: "E. gracilis is a photosynthetic protist…." As this species can live according three different metabolisms photo-autotrophy, mixotrophy and heterotrophy, according the growth conditions presented in the article: by the use of an organic medium, and a cultivation with light, it should be more exact to write "E. gracilis is a mixotrophic protist……" "

"Introduction, line 239: It was assessed is a photosynthetic E. gracilis. The same remark than with line 209"

R: In agreement with the reviewer, the description of *E. gracilis* as a photosynthetic protist was changed for mixotrophic protist in p. 4, last paragraph and p. 5.

"Material and Methods, line 251: Euglena gracilis (a Z-like strain) No number of strain! Why? "

R: The *Euglena gracili*s strain used was not obtained from a commercial source. These cells originally came from the collection of a Mexican Institution. In a previous paper, we characterized the strain (Plant Sci., 48: 151–157). Detailed information in this regard was now included in Materials and methods section, p. 5, 3rd paragraph.

"Material and Methods: line 373: HCL (0.1% v/v) can't we get a value of pH ! "

R: The pH value was added (p. 7, last paragraph).

"Results Line 683 : cells were exposed to 200 μM Zn2+, as in a precedent paper it was written "This strain was grown in culture media containing 300 μM ZnCl2 for over 20 years" R.Sánchez-Thomas (2016), the addition of a supplementary level of concentration, per ex. 400-500 μM, could be useful."

R: We thank the reviewer for his/her analysis of our previous work. Indeed, 200 μM Zn^{2+} was initially used to resemble the extra- and intracellular concentrations of Cd^{2+}. However, 500 and 1000 μM Zn^{2+} were also tested but no changes in water volume were observed; this information was originally described in section 3.2.1 in p. 13-14.

"A final remark, as I wrote above Euglena gracilis can live under different metabolic modes, it should be interesting to test the accumulation of cadmium per example under pure photo-autotrophic conditions. It should test the robustness/universality of the results."

R: Experiments were now performed to determine the accumulation of cadmium in *E. gracilis* under strict photoautotrophic conditions*, i.e.* in a culture medium with no carbon sources. Under these conditions, the cells grew less and slower in saline medium compared to the cells in complete medium (see Fig. A below), and the accumulation of cadmium was slightly lower (Fig. B below). Under strict phototrophic conditions, it was also evident that Cd^{2+} accumulation increased intracellular volume. These results indicated that *E. gracilis* can accumulate high levels of cadmium even under conditions that are not optimal for growth, but which are closer to the environmental conditions *Euglena* may encounter in polluted water systems. These new data were described in p. 14, last paragraph. However, it should be noted that cadmium accumulation under near phototrophic conditions (low carbon source content in the culture medium) had been already described in Fig. 4 and section 3.2.2 (p. 14-15).

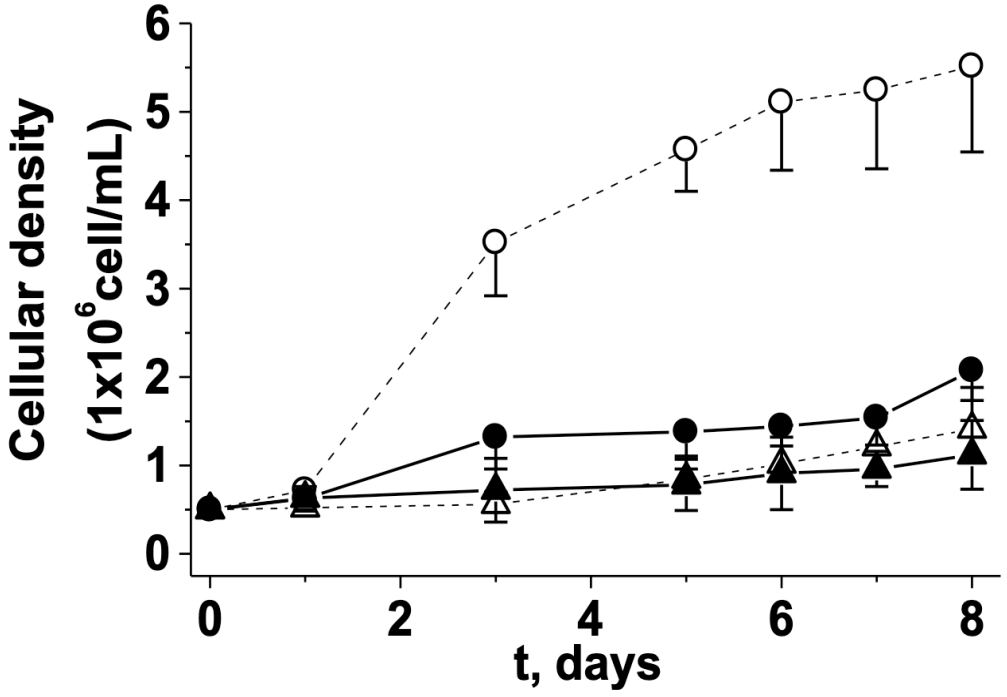

Figure A. *E. gracilis* growth under photoheterotrophic or phototrophic conditions.
Growth of *E. gracilis* with no Cd^{2+} (O,Δ) or 200 μM Cd^{2+} (●,▲) at 20-25ºC. Circles represent cells cultured photo-heterotrophycally on Hutner medium with glutamate and malate (75 mOsM), and triangles show cells grown under phototrophic conditions with saline medium (25 mOsM). The photoperiod for both conditions involved cycles of 12h light (90 μmol quanta m^{-2} .s^{-1})/12h dark. n = 4.

For these experiments, the initial inoculum was 0.5 x 10^6 cells/mL instead of the usual 0.2 x 10^6 cells/mL.

Cd^{2+} (μM)	Intracellular Volume (μL/10^7 cells)	
0	5.9	3.4
200	17.3	6.8

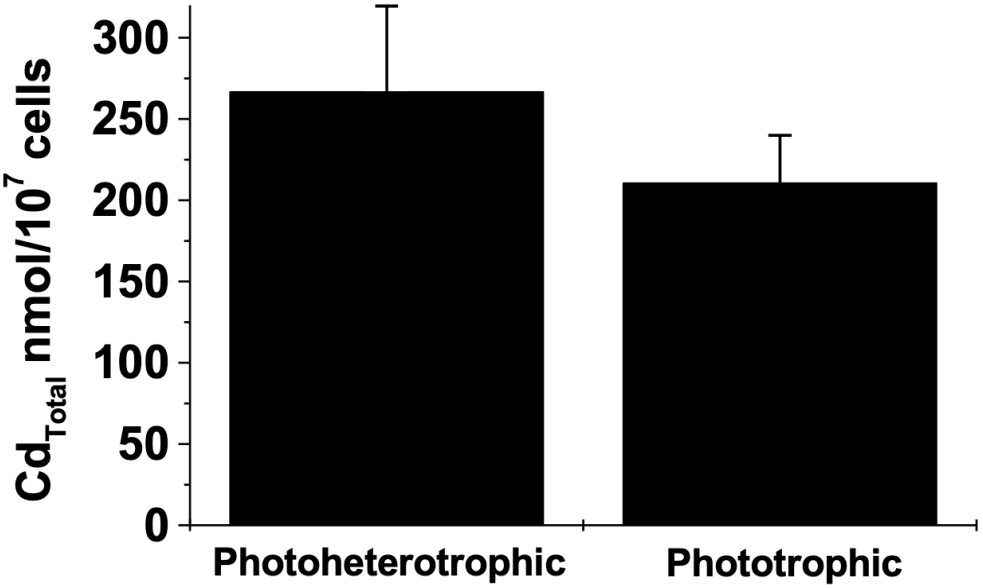

Figure B. Intracellular volumen and Cd^{2+} accumulation in *E. gracilis*.
Cells were cultured with 200 μM Cd^{2+} for 8 days under either photo-heterotrophic or phototrophic conditions. The total intracellular cadmium content in photoheterotrophic cells was very similar to that shown by 75 mOsM cells in Fig. 4, whereas the cadmium content in phototrophic cells was slightly lower than that shown by 35 mOsM cells. The number of independent cell cultures used was 4 for the intracellular cadmium content determinations and 2 for the intracellular water volume assays.

Reviewer 2:

Line 198: „leaf" instead of „leave"

Line 498: "ground" instead of "grinded"

Line 520: "universal gas constant"

Line 524: "problem solution" sound peculiar to me, maybe "given solution"?

Line 554ff: "The volume changes peaking after 1 day did not strictly correlate with the accumulation of cadmium, which reached a maximum by the fifth and eighth day of culture for 50Cd and 200Cd cells

respectively (Fig. 1B), as previously reported [31].” I do not see a maximum at the fifth day. As the curves do not correlate at all, I would delete “strictly”.
Line 765: remove “.” after “Table”
Section titles 3.1 and 3.1.1 end with period, while the others do not. Also 3.2.3

R: These 7 remarks pointed out by the reviewer were amended.

"Table 1: wrong or unexplained index (#) at “Free Phosphate” "
R: The # symbol was a typographical error, therefore it was amended.

"Figure 4: this needs some rework, I think. It contains some redundancies (like the osmolarities) and also some unexplained information, like the numbers in the fields above the bars."
R: Legend to figure 4 now describes in more detail what the numbers above the bars represent and the line redundantly describing the medium osmolarities was erased.

"Section 2.1 Growth conditions: reference 31 does refer to another publication, in which the medium is described. I think reference 31 should take the interested reader right to the information they seek, although it costs a (self-)citation."
R: Reference 31 mentions the details of the culture medium formulation, in addition to referring to an earlier paper of the same group. It should be noted that the original acidotrophic Hutner medium was slightly modified regarding zinc and vitamin B12 contents and hence these changes have to be fully disclosed.

7. Discusión general

La historia de la vida en la Tierra incluye una serie de adaptaciones y procesos evolutivos que dependieron de los cambios ambientales a los que se enfrentaron una gran cantidad de organismos. Debido a esto, existen diferentes organismos que son capaces de sobrevivir a ambientes extremos (extremófilos), como a altas o bajas temperaturas, a diferentes concentraciones de oxígeno, a una exposición luminosa de alta intensidad, en particular a las radiaciones de luz ultravioleta, a la falta o exceso de agua, a la salinidad, a ambientes con pH extremos, a desperdicios tóxicos, y a metales pesados, los cuales se consideran severos o inhóspitos para la vida (Boothby *et al*., 2019; Rampelotto *et al*., 2013). El estudio de estos organismos extremófilos pretende entender los mecanismos que confieren estas resistencias, para así buscar las propiedades genéticas que las codifican y eventualmente utilizarlas con el fin de resolver problemas derivados de la globalización y el impacto ambiental del hombre sobre la tierra (Arora y Panosyan, 2019).

La presión de selección asociada a los retos ambientales permitió la supervivencia de diferentes organismos y con ello la preservación de los mecanismos asociados, lo cual posteriormente contribuyó a resistir otras situaciones de estrés. En este sentido, los organismos que pueden sobrevivir a más de un factor de estrés ambiental se han catalogado como poli-extremófilos (Rampelotto *et al*., 2013), y ha surgido la interrogante de si estos organismos utilizan mecanismos (i) distintos, (ii) superpuestos, o (iii) una combinación de ambos para sobrevivir a diferentes tipos de estrés. Responder esta pregunta ha sido complicado pues se han descubierto ejemplos para los tres mecanismos (Boothby *et al*., 2019). En este contexto, se espera que los organismos como las plantas, levaduras, protistas y bacterias posean mecanismos similares para contender contra diferentes tipos de estrés (Jian-Kang *et al*., 2016; Holzinger *et al*., 2016; Slaveykova *et al*., 2016; Święciło, 2016). El presente trabajo describió el efecto protector que provee el Zn^{2+} sobre la acumulación de otro metal tóxico como el Cd^{2+}, y los efectos de tipo osmótico que aparecen durante la acumulación del Cd^{2+} en condiciones que no constituyen un estrés osmótico. Los estudios que intentan entender el fenómeno cruzado entre diferentes tipos de estrés sugieren que hay dos mecanismos: (1) ciertos organismos requieren un pequeño estímulo o estrés previo que induce la resistencia a un segundo estrés; y que (2) otros organismos poseen mecanismos generales que se encienden para varias situaciones de

estrés (Boothby *et al*., 2019). Estos antecedentes indican que hay ciertos tipos de estrés que tienen una relación estrecha y/o que la respuesta a un primer estrés puede favorecer para contender exitosamente contra otros tipos de estrés.

7.1 El Zn^{2+} y su efecto protector

El Zn^{2+} y el Cd^{2+} coexisten en sitios contaminados aunque el Zn^{2+} comúnmente está en mayores cantidades (>20 veces) con respecto al Cd^{2+} (Augustynowicz *et al*., 2014; Magdaleno *et al*., 2014; Zeng *et al*., 2015), posiblemente por que los minerales de donde se extrae el zinc poseen cadmio. Además, en la búsqueda de estrategias que puedan conferir una protección a la toxicidad por Cd^{2+} se ha propuesto la pre-exposición o co-exposición a metales esenciales como el Se^{3+}, Mn^{2+} y Zn^{2+} (Sandbichler y Höckner, 2016). Por ello, resultaba importante evaluar el comportamiento y la respuesta celular a la presencia simultánea de ambos metales, y sobre todo determinar si la presencia del Zn^{2+} contribuye o no en la acumulación de Cd^{2+} en microorganismos que podrían ser utilizados en procesos de remoción de metales.

E. gracilis resultó ser un microorganismo altamente resistente a Zn^{2+} (IC_{50Zn}= 1.7 mM), con efectos tóxicos claros en el sistema fotosintético solo a concentraciones mayores a1000 μM y exhibiendo capacidad para hiperacumular este metal. Este fenotipo es poco común en microorganismos, ya que las microalgas *C. reinhardtii*, *Chlorella ellipsoidea*, *Cyanidium caldarium*, *Ankistrodesmus fusiformis*, *Monoraphidium contortum* y *Scenedesmus acuminatus* son extremadamente sensibles a Zn^{2+}con una IC_{50Zn}= 0.6-75 μM (Magdaleno *et al*., 2014; Mikulic y Berdall, 2014). En este sentido, se ha descrito que el Zn^{2+} puede reemplazar al Mg^{2+} en el anillo de porfirina que posee la clorofila en su estructura, lo cual genera una menor eficiencia en la transferencia de electrones en la fotosíntesis provocando clorosis (Küpper *et al*., 2002; Gerola *et al*., 2011; Subba *et al*., 2014). Debido a esto, el fenotipo resistente e hiperacumulador de *E. gracilis* podría estar relacionado con su habitat natural, ya que la actividad de la enzima que sintetiza fitoquelatinas (*Eg*FQS) es activa principalmente cuando hay Zn^{2+} en el medio, y no con Cd^{2+} u otros metales (García-García *et al*., 2014). Sin embargo, el pre-acondicionamiento con Zn^{2+} no favoreció sustancialmente la acumulación de Cd^{2+}. Esto puede explicarse con el hecho de que el Zn^{2+} no resultó ser un buen inductor de estrés oxidante en *E. gracilis*, ya

que no se indujo un aumento en la actividad de APx y GPx, y por lo tanto, la cantidad de moléculas con grupos tiol como el GSH (sustrato de la síntesis de FQ) se mantuvo sin cambios en presencia de Zn^{2+}.

En cambio, el pre-acondicionamiento con Zn^{2+} si protegió a las células del estrés por Cd^{2+}. El Zn^{2+} puede proveer de protección en algas, plantas y mamíferos mediante la inducción de enzimas antioxidantes (SOD, CAT y APx) o inhibiendo el transporte de Cd^{2+} (Aravind *et al*., 2009; Aravind y Prasad, 2004, 2005; Lavoie *et al*., 2012a,b; Li y Zhou, 2012; Tsuji *et al*., 2002). No obstante, en *E. gracilis* el Zn^{2+} no indujo estrés oxidante, pero pudo haber ocupado grupos susceptibles a Cd^{2+}, como residuos de cisteína e histidina en proteínas. El Zn^{2+} y el Cd^{2+} pueden compartir transportadores de membrana (Garnham *et al*., 1992; Reid *et al*., 1996) y la presencia de altas concentraciones de Zn^{2+} pueden inhibir el transporte de Cd^{2+} (Lavoie *et al*., 2012a,b; Li y Zhou, 2012); nuestras observaciones sugirieron que en *E. gracilis* el Cd^{2+} utiliza vías de ingreso que no comparte con el Zn^{2+}, ya que en ausencia de Zn^{2+} la IC_{50} de *E. gracilis* por Cd^{2+} sí es menor pero la acumulación de Cd^{2+} no se modifica significativamente. Tampoco se puede descartar que el Zn^{2+} tenga efectos protectores a otros niveles celulares. Entonces, los mecanismos asociados a la resistencia e hiperacumulación de Zn^{2+} en *E. gracilis* todavía requieren de mayor investigación.

7.2 Los mecanismos osmoreguladores en la acumulación de Cd^{2+}

Los mecanismos asociados a un estrés osmótico y/o salino pueden contribuir a la resistencia y la acumulación de metales pesados, ya que se ha propuesto que las plantas halófilas (resistentes a la salinidad y al estrés hídrico) presentan una tolerancia cruzada con la resistencia a metales pesados y viceversa (Ben *et al*., 2013; Nikajle *et al*., 2018). Un análisis de la literatura y de la información genética en las plantas catalogadas como halófilas o hiperacumuladoras de metales pesados propuso que existe una correlación taxonómica estrecha, pero no filogenética, en el establecimiento de dichos fenotipos. Esto sugirió que el desarrollo de un fenotipo de tolerancia a la salinidad o de hiperacumulación de metales pesados depende de los cambios en el ambiente y no es producto de un proceso evolutivo (Moray *et al*., 2016). A pesar de esto, no se ha descrito cómo los mecanismos que protegen ante un estrés osmótico pueden contribuir a la acumulación del

Cd^{2+} y otros metales pesados, aunque está claro que existen respuestas celulares y mecanismos que se activan con ambos tipos de estrés.

7.2.1 Volumen intracelular de agua

La acumulación de metales pesados genera diferentes efectos y respuestas celulares que pueden coincidir con los que se inducen con un estrés osmótico. Se han descrito a las plantas halófilas *Atriplex halimus*, *Mesembryanthemum crystallinum* e *Iris ensata* como hiperacumuladoras de Cd^{2+}, Cu^{2+} y Pb^{2+} (Lutts *et al*., 2004; Thomas *et al*., 1998; Usman *et al*., 2012). Además, la exposición a metales pesados en las plantas induce una disminución en el contenido de agua, mientras que la concentración de osmo-metabolitos y la presión osmótica incrementa o se modifica (Poschenrieder *et al*., 1988; Han *et al*., 2012; Rucińska-Sobkowiak, 2016). En *E. gracilis*, la exposición a Cd^{2+} aumenta el tamaño y el volumen intracelular de agua, lo cual no concuerda con los efectos observados en las plantas, levaduras y microalgas, posiblemente por la ausencia de pared celular en este protista (O´Neill *et al*.,2017). Incluso la exposición a Cd^{2+} en las plantas genera plasmólisis, que sucede cuando la célula pierde tanta agua que la membrana plasmática se separa de la pared celular y las plantas se marchitan y mueren (Daud *et al*., 2009; Ge *et al*., 2012).

La halófita *Kosteletzkya virginica* puede acumular en sus hojas hasta 60 mg Cu/Kg de peso seco al ser expuesta a 10 μM Cu^{2+} durante 4 semanas, lo cual se acompaña de una disminución en el potencial osmótico de la planta (Rui-Ming *et al*., 2012). Los resultados de esta tesis muestran que la osmolaridad interna se incrementa en *E. gracilis* después de 24 horas de estar expuesta al Cd^{2+}, en concentraciones que no se esperan que representen un estrés por osmolaridad (50-200 μM Cd^{2+}). La concentración de Cd^{2+} total intracelular alcanzada fue de ≈39 mM, aunque es muy probable que esta concentración no sea osmóticamente activa, pues el Cd^{2+} se une fuertemente a las moléculas con grupos tiol con una constante de afinidad (asociación) de 2.24×10^{15} M^{-2} para la formación del complejo Cd-$(GS)_2$ (Vatamaniuk *et al*., 2000) y otros complejos de muy alto peso molecular.

7.2.2 Osmo-metabolitos

Los cambios en la presión osmótica son resultado del incremento en la cantidad de osmo-metabolitos y metabolitos quelantes que responden al estrés por Cd^{2+} en *E. gracilis*. Se ha descrito en plantas como *Zea mays, Ulva lactuca, Salvinia natans y Avena sativa* que los osmolitos como la prolina, la glicina-betaína, la sacarosa y las poliaminas aumentan 2-9 veces cuando estas plantas son expuestas a 100-300 μM Cd^{2+} (Weinstein *et al*., 1997; El-Shora *et al*., 2010; Dhir *et al*., 2012; Anjum *et al*., 2016). En *E. gracilis*, se observó que la cantidad de trehalosa aumenta en exposición a Cd^{2+} y su aumento fue similar a lo descrito cuando este microorganismo está sujeto a un estrés con 150-200 mM de NaCl (Takenaka *et al*., 1997; Porchia *et al*., 1999). La trehalosa es un osmolito que aumenta sus niveles en distintos organismos durante un estrés abiótico como temperatura, salinidad, sequía, metales pesados (Sharma *et al*., 2014).

Una de las principales explicaciones que se tiene sobre el papel de la trehalosa y otros osmolitos indica que estas moléculas tienen una elevada capacidad para interactuar y secuestrar una gran cantidad de moléculas de agua (Pagnotta *et al*., 2010), por lo que la trehalosa podría estar disminuyendo el estrés hídrico que genera la hiperacumulación de Cd^{2+}. De manera general, los osmo-metabolitos participan como estabilizadores celulares de la osmolaridad interna al mantener estable la cantidad de agua **libre**, balanceando la osmolaridad intracelular con la del medio circundante. Además, se ha descrito que la síntesis de osmo-metabolitos es una respuesta que no sólo se induce por un estrés osmótico, sino que es una respuesta generalizada por otros tipos de estrés (Slama *et al*., 2015). Por otro lado, los osmo-metabolitos protegen a las proteínas, los ácidos nucleicos, y los orgánulos al mantener estable el arreglo molecular y el contenido del agua, e incluso como atrapadores ó removedores de ERO (Yancey, 2015; Slama *et al*., 2015; Singh *et al*., 2015).

7.2.3 El estrés oxidativo en la respuesta osmoregulatoria

La toxicidad de los metales pesados se debe a tres principales efectos:(i) bloqueo de las actividades de las enzimasal integrarse en los sitios activos que poseen grupos con cisteínas e histidinas; (ii) desplazamiento de cationes esenciales en sitios específicos de

otras proteínas (por ejemplo, proteínas que contienen anillos de zinc) que se requieren para el buen funcionamiento de la célula; y (iii) generación directa de ERO mediante la reacción de Fenton con el Cu^{2+} y el Fe^{2+} (Miazek *et al*., 2015). Los resultados de este trabajo demostraron que el Zn^{2+} no induce cambios de tipo osmótico como el Cd^{2+}, lo que permite concluir que los cambios osmóticos producidos por el Cd^{2+}, y no el Zn^{2+}, estaban mediados por estrés oxidante. Esto se estableció al observar un aumento en la actividad de la APx y/o la GPx, la cantidad de GSSG y porque el Cd^{2+} posee una afinidad mayor (7.76 x 10^9 M^{-1}) por residuos de cisteína con respecto al Zn^{2+} (3.16 x 10^8 M^{-1}) (Sillen y Martell, 1964) y puede secuestrar de manera más eficiente al GSH y a otras moléculas con grupo tiol.

Por lo tanto, se propone que el estrés oxidante es el que promueve efectos de tipo osmótico. Esta relación ya había sido propuesta por otros autores (Nikalje y Suprasanna, 2018), quienes sugirieron que existe un mecanismo tipo “crosstalk” entre el estrés osmótico y el estrés por metales pesados asociado con una elevación en los niveles de ERO (Fig. 11).

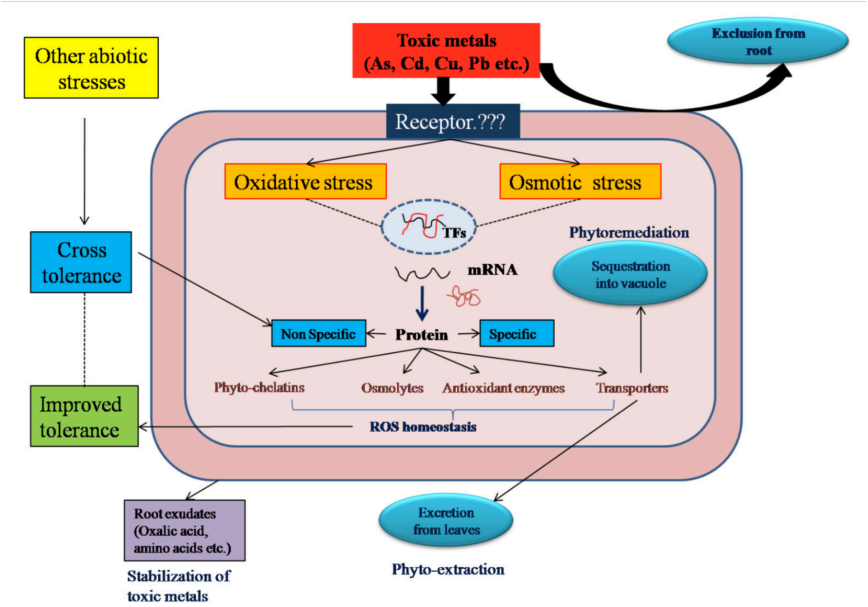

Figura 11. Representación del mecanismo “crosstalk” entre un estrés osmótico y por metales pesados. Tomado de Nikalje y Suprasanna, 2018.

La proteína cinasa MAPK14, que se llama Hog1 en levaduras y p38 en mamíferos, constituye la parte central de la cascada de señalización encargada de percibir un

desbalance osmótico en las células eucariontes (Suescún-Bolívar y Thomé, 2015). La respuesta celular al estrés oxidante en *Candida albicans* depende de esta MAP cinasa (Alonso-Monge *et al*., 2003), y la sobre-expresión de la MAPK14 en tomate incrementa la tolerancia a la sequía y al Cd^{2+} (Muhammad *et al*., 2019). También hay datos que vinculan a estas proteínas con los cambios en el volumen intracelular, ya que la regulación transcripcional de la acuaporina (Fps1) de *Saccharomyces cerevisae* depende de la fosforilación de la MAPK14, afectando su actividad, y que además regula la tolerancia y acumulación de As^{3+} (Thorsen *et al*., 2006). Estas observaciones sugieren que la MAPK14 puede estar involucrada en la inducción de la respuesta osmótica durante la acumulación del Cd^{2+}.

7.3 El volumen intracelular en la acumulación de Cd^{2+}

La exposición a metales pesados genera un desbalance del contenido de agua en las plantas. La mutante *SGECd* en chícharo generada por tratamiento con etilmetanosulfonato (Tsyganov *et al*., 2007), es capaz de tolerar y hiperacumular Cd^{2+} con respecto a plantas silvestres. Este fenotipo se asoció con la capacidad que tiene para transportar agua a través del xilema, con una mejor conducción del agua de los estomas, y con el aumento del potencial osmótico e hídrico, turgencia y transpiración (Belimov *et al*., 2015). De igual forma, *Phytolacca americana* es una planta que se ha caracterizado como hiperacumuladora de Cd^{2+}, lo cual puede estar relacionado con su capacidad para mantener la tonicidad, transpiración y el funcionamiento de los estomas (Liu *et al*., 2010); además, este fenotipo se suprime en presencia de Hg^{2+}, sugiriendo la participación de las acuaporinas (AQP). Las AQP se han relacionado con la capacidad que tienen algunas plantas para resistir diferentes tipos de estrés, como el exceso de agua y de metales pesados (Zhang *et al*., 2008; Deshmukh *et al*., 2017). En esta tesis se demostró que *E. gracilis* aumenta su volumen intracelular en presencia de Cd^{2+}, y la acumulación de este metal también disminuye en presencia de inhibidores de las acuaporinas como el Hg^{2+} y la pentamidina.

El volumen intracelular regula la acumulación de Cd^{2+} en *E. gracilis*, lo cual sugiere que el transporte de Cd^{2+} también podría ser a través de las AQP. Existe información que indica que las AQP de *Pteris vittata*, *Hydrangea macrophylla* y *Hordeum vulgare* son

capaces de transportar As^{3+}, Al^{3+} y B^{3+}(Schnursbusch *et al*., 2010; Negishi *et al*., 2012; He *et al*., 2016), pero no hay reportes que demuestren que el Cd^{2+} puede ser transportado a través de estas proteínas. A pesar de esto, hay información que relaciona a las AQP con otro catión divalente muy similar, el Ca^{2+}. El transporte de Ca^{2+} en plantas es a través de la vía del apoplasto (homeostasis hídrica). El Ca^{2+} es capaz de modificar la actividad y/o expresión de AQP y la inhibición del transporte de agua con $HgCl_2$ sólo se observa en presencia de Ca^{2+} (Wang *et al*., 2016). Por otro lado, la cantidad de Ca^{2+} libre en el citosol de plantas de *Capsicum annuum* puede regular la actividad de las AQP (Cabañero *et al*., 2006), la cual se inhibe en presencia de verapamil un inhibidor de los canales de Ca^{2+}. Incluso, la sobre-expresión de AQP de *Triticum aestivum* en *A. thaliana* aumenta la acumulación de Ca^{2+} (Hu *et al*., 2012). Además, la función y estructura de las AQP se ha relacionado con los canales iónicos y el Cd^{2+} es capaz de permear a través de canales iónicos de Ca^{2+} (Perfus *et al*., 2002). Por lo tanto, sería importante evaluar el papel de las AQP y el Ca^{2+} en el transporte de Cd^{2+} de *E. gracilis*.

8. Conclusiones

1) La acumulación del Cd^{2+} en *E. gracilis*, y no la del Zn^{2+}, induce cambios de tipo osmótico tales como el aumento en el volumen intracelular de agua, el tamaño celular, la presión osmótica y la concentración de osmolitos mediante un proceso que al parecer está mediado por la generación de estrés oxidante.

2) *E. gracilis* es un organismo altamente resistente e hiperacumulador del Zn^{2+}. Sin embargo, los mecanismos asociados a este fenotipo no están relacionados con la síntesis de FQ y/o mecanismos osmoreguladores.

3) Existe una correlación entre la capacidad de acumular Cd^{2+} y el contenido intracelular de agua, *i.e.* un aumento en el volumen intracelular de agua contribuye a una mayor acumulación del Cd^{2+}.

4) El transporte de agua responsable de los cambios en el volumen intracelular durante la acumulación del Cd^{2+} en *E. gracilis* es sensible a el Hg^{2+} y a la pentamidina, lo cual sugiere la participación de acuaporinas como un mecanismo que modula la acumulación de Cd^{2+} en este microorganismo.

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10. Publicaciones adicionales

Publicaciones relacionadas con el proyecto de investigación en las que participó Rosina Sánchez Thomas durante el doctorado.

El trabajo publicado en *Biotech. Adv.* 2016, 34: 859-873 (FI= **10.744**; número de citas = 37, septiembre de 2020) fue una revisión publicada por el grupo de trabajo en el que realicé mi tesis doctoral. Este trabajo resalta las capacidades que poseen microorganismos como *E. gracilis* en la remoción de metales pesados tóxicos y en la recuperación de metales de interés comercial e industrial.

Biotechnology Advances 34 (2016) 859–873

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Biotechnology Advances

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Research review paper

Bio-recovery of non-essential heavy metals by intra- and extracellular mechanisms in free-living microorganisms

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ARTICLE INFO

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Recovery of heavy metals
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Heavy metal bioaccumulation
Phytochelatin metabolism
Bioleaching
Cyanide biosynthesis
Gold
Cadmium

ABSTRACT

Free-living microorganisms may become suitable models for recovery of non-essential and essential heavy metals from wastewater bodies and soils by using and enhancing their accumulating and/or leaching abilities. This review analyzes the variety of different mechanisms developed mainly in bacteria, protists and microalgae to accumulate heavy metals, being the most relevant those involving phytochelatin and metallothionein biosyntheses; phosphate/polyphosphate metabolism; compartmentalization of heavy metal-complexes into vacuoles, chloroplasts and mitochondria; and secretion of malate and other organic acids. Cyanide biosynthesis for extra-cellular heavy metal bioleaching is also examined. These metabolic/cellular processes are herein analyzed at the transcriptional, kinetic and metabolic levels to provide mechanistic basis for developing genetically engineered microorganisms with greater capacities and efficiencies for heavy metal recovery, recycling of heavy metals, biosensing of metal ions, and engineering of metalloenzymes.

Contents

Abbreviations: APSR, adenosine 5′-phosphosulfate reductase; CS, cyanide synthase; CPCB, Computer Printed Circuit Boards; dw, dry weight; γ-ECS, γ-glutamylcysteine synthetase; IC_{50}, half-maximal inhibition of growth; PCS, phytochelatin synthase; PCs, phytochelatins; polyPs, poly-phosphates; PMST, plasma membrane sulfate transporters.

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El artículo aparecido en *J. Hazard. Mat.* 2018. 343: 10-18 (FI=**9.038**; número de citas = 14, septiembre de 2020) fue derivado de la tesis de licenciatura de la Biól. Karla Adriana Peña Sanabria. El Ni^{2+} es un metal pesado que también contamina suelos y cuerpos de agua. En este trabajo yo participé como asesora técnica de la estudiante, realicé experimentos y participé en la discusión para caracterizar los mecanismos de resistencia y acumulación de Ni^{2+} en *E. gracilis*.

Journal of Hazardous Materials 343 (2018) 10–18

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Nickel accumulation by the green algae-like *Euglena gracilis*

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HIGHLIGHTS

- *Euglena gracilis* accumulates high levels of nickel.
- Extracellular magnesium and copper limit the nickel uptake.
- Histidine synthesis is activated by nickel.
- Thiol-molecule pools are depressed by nickel.
- Nickel is more noxious for energetic pathways of *E. gracilis* than cadmium.

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Ascorbate peroxidase
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Nickel expelling
Oxygen consumption
Photosynthesis
Polyphosphates

ABSTRACT

Nickel accumulation and nickel effects on cellular growth, respiration, photosynthesis, ascorbate peroxidase (APX) activity, and levels of thiols, histidine and phosphate-molecules were determined in *Euglena gracilis*. Cells incubated with 0.5–1 mM $NiCl_2$ showed impairment of O_2 consumption, photosynthesis, Chl a + b content and APX activity whereas cellular integrity and viability were unaltered. Nickel accumulation was depressed by Mg^{2+} and Cu^{2+}, while Ca^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} were innocuous. The growth half-inhibitory concentrations for Ni^{2+} in the culture medium supplemented with 2 or 0.2 mM Mg^{2+} were 0.43 or 0.03 mM Ni^{2+}, respectively. Maximal nickel accumulation (1362 mg nickel/Kg DW) was achieved in cells exposed to 1 mM Ni^{2+} for 24 h in the absence of Mg^{2+} and Cu^{2+}; accumulated nickel was partially released after 72 h. GSH polymers content increased or remained unchanged in cells exposed to 0.05–1 mM Ni^{2+}; however, GSH, cysteine, γ-glutamylcysteine, and phosphate-molecules all decreased after 72 h. Histidine content increased in cells stressed with 0.05 and 0.5 mM Ni^{2+} for 24 h but not at longer times. It was concluded that *E. gracilis* can accumulate high nickel levels depending on the external Mg^{2+} and Cu^{2+} concentrations, in a process in which thiols, histidine and phosphate-molecules have a moderate contribution.

1. Introduction

Nickel-containing compounds derived from cigarettes, mining sites and other polluted environments are linked to development of human cancer depending on dosage, time and way of exposure [1–4]. Waste mismanagement of Ni-Cd batteries, stainless steel, paints and alloys to manufacture coins, jewelry and electronic equipment, cigarettes, among other anthropogenic activities, generates nickel pollution [2,3,5]. In polluted sites, nickel reaches concentrations of 2.2–118 mg/L (equivalent to 0.037–2 mM) in water [5–8]; 8–93 mg/Kg in sediments [6,9]); and 278–2200 mg/Kg in soils [10,11]. Therefore, the pollution by nickel is becoming another environmental problem that has to be solved.

The bioremediation of nickel, and other toxic heavy metals, has the aim to be an alternative ecologically-friendly technology for cleaning polluted sites [12–14]. In this regard, several plants mainly of the genesus *Alyssum* and *Thlaspi* [15] have been classified as nickel hyperaccumulators because they may achieve high intracellular levels (>1000 mg nickel/Kg DW). This phenotype is supported by the generation of elevated levels of different intracellular chelating-metabolites such as thiol-molecules and histidine [16–19].

For instance, *Thlaspi goesingense*, *T. oxyceras* and *T. rosulare* exposed to 100 ppm of nickel show a strong correlation between nickel accumulated and levels of cysteine (Cys) and glu-

Abbreviations: APX, ascorbate peroxidase; Chl a + b, chlorophyll a + b; DW, dry weight; γ-EC, gamma-glutamylcysteine; poly-GSH, total polymers of glutathione; polyP, polyphosphate molecules.

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El trabajo publicado en *Toxicol. Appl. Pharmacol.* 2019. 370: 65-77 (FI=**3.347**; número de citas = 9, septiembre de 2020) pertenece al grupo de estudio en cáncer del departamento de Bioquímica. La experiencia y conocimiento desarrollados durante el análisis del metabolismo del GSH en *E. gracilis* me permitió contribuir en este trabajo experimentalmente con la determinación de metabolitos con grupos tiol y en la discusión del papel pro-oxidante que posee el resveratrol en células cancerosas.

Toxicology and Applied Pharmacology 370 (2019) 65–77

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Toxicology and Applied Pharmacology

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

Resveratrol inhibits cancer cell proliferation by impairing oxidative phosphorylation and inducing oxidative stress

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ARTICLE INFO

Keywords:
Antioxidant response
Cancer
Oxidative phosphorylation
ROS production
Mitophagy

ABSTRACT

The resveratrol (RSV) efficacy to affect the proliferation of several cancer cell lines was initially examined. RSV showed higher potency to decrease growth of metastatic HeLa and MDA-MB-231 (IC_{50} = 200–250 μM) cells than of low metastatic MCF-7, SiHa and A549 (IC_{50} = 400–500 μM) and non-cancer HUVEC and 3T3 ($IC_{50} \geq 600$ μM) cells after 48 h exposure. In order to elucidate the biochemical mechanisms underlying RSV anti-cancer effects, the energy metabolic pathways and the oxidative stress metabolism were analyzed in HeLa cells as metastatic-type cell model. RSV (200 μM/48 h) significantly decreased both glycolysis and oxidative phosphorylation (OxPhos) protein contents (30–90%) and fluxes (40–70%) *vs.* non-treated cells. RSV (100 μM/1–5 min) also decreased at a greater extent OxPhos flux (net ADP-stimulated respiration) of isolated tumor mitochondria (> 50%) than of non-tumor mitochondria (< 50%), particularly with succinate as oxidizable substrate. In addition, RSV promoted an excessive cellular ROS (2–3 times) production corresponding with a significant decrement in the SOD activity (but not in its content) and GSH levels; whereas the catalase, glutahione reductase, glutathione peroxidase and glutathione-S-transferase activities (but not their contents) remained unchanged. RSV (200 μM/48 h) also induced cellular death although not by apoptosis but rather by promoting a strong mitophagy activation (65%). In conclusion, RSV impaired OxPhos by inducing mitophagy and ROS over-production, which in turn halted metastatic HeLa cancer cell growth.

1. Introduction

The beneficial role of several phytochemicals for multiple illnesses (cardiovascular events, obesity, diabetes and cancer) has been widely documented (Szkudelska and Szkudelski, 2010; Jiang et al., 2017). Particularly, resveratrol (*trans*-3,5,4′-tryhydroxystilbene, RSV) a natural polyphenol found in large quantities in grapes, berries and peanuts has shown multiple positive effects on normal cells (Mukherjee et al., 2010; Baarine et al., 2011). At 0.1–50 μM doses, RSV (a) activates signaling and transcription factors involved in cell-cycle regulation, apoptosis, angiogenesis, antioxidant mechanism; and down-regulates cyclooxygenase 2 and NF-kB in immune cells (Gao et al., 2001; Švajger and Jeras, 2012); (b) shows ROS scavenger capacity (increasing NOS expression and SIRT1 activation) improving mitochondrial function in heart (Turan et al., 2012) whereas in neurons, RSV shows a protective role against Alzheimer's and Parkinson's diseases in aging models (Richard et al., 2011); and (c) inhibits initiation, promotion and progression of cancer development with apparent low toxicity for normal cells (Kueck et al., 2007; Gwak et al., 2015).

In cancer cells, RSV (15–50 μM for 24–72 h) arrests the cellular cycle, induces apoptosis and promotes a massive reactive oxygen species (ROS) production (García-Zepeda et al., 2013). RSV also decreases

Abbreviations: ATPS, ATP synthase; ANT, adenine nucleotide translocase; CAT, catalase; Cys, cysteine; CS, citrate synthase; COX, cytochrome *c* oxidase; CHP, cumene hydroperoxide; DHE, dihydroethidium; GA, glutaminase; GLUT1, glucose transporter 1; GLUT2, glucose transporter 2; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GDH, glutamate dehydrogenase; GST, glutathione S-transferase; HKI, hexokinase I; HKII, hexokinase II; HPI, hexose phosphate isomerase; MCTS, multi-cellular tumor spheroids; NBT, nitroblue tetrazolium; ND1, NADH dehydrogenase subunit 1; RSV, resveratrol; SOD, superoxide dismutase; *t*-BHP, *tert*-butylhydroperoxide; 2OGDH, 2-oxoglutarate dehydrogenase; γ-EC, gamma-glutamylcysteine.

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0041-008X/

El trabajo publicado en *MicrobiologyOpen*. 2020. 00:e1006 (FI= **3.142**) pertenece al programa de Ingeniería Genómica del Centro de Ciencias Genómicas, UNAM. La re-implementación de la técnica para determinar aminoácidos durante mi proyecto de doctorado me permitió contribuir en este trabajo experimentalmente con la determinación de β-Ala en reacciones *in vitro* de la β-alanina sintasade la bacteria *Rhizobiu metli*.

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ORIGINAL ARTICLE

MicrobiologyOpen Open Access WILEY

A novel way to synthesize pantothenate in bacteria involves β-alanine synthase present in uracil degradation pathway

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Abstract

Pantothenate is an indispensable vitamin precursor of the synthesis of coenzyme A (CoA), a key metabolite required in over 100 metabolic reactions. β-Alanine (β-ala) is an indispensable component of pantothenate. Due to the metabolic relevance of this pathway, we assumed that orthologous genes for ß-alanine synthesis would be present in the genomes of bacteria, archaea, and eukaryotes. However, comparative genomic studies revealed that orthologous gene replacement and loss of synteny occur at high frequency in *panD* genes. We have previously reported the atypical plasmid-encoded location of the pantothenate pathway genes *panC* and *panB* (two copies) in *R. etli* CFN42. This study also revealed the unexpected absence of a *panD* gene encoding the aspartate decarboxylase enzyme (ADC), required for the synthesis of β-ala. The aim of this study was to identify the source of β-alanine in *Rhizobium etli* CFN42. In this study, we present a bioinformatic analysis and an experimental validation demonstrating that the source of β-ala in this *R. etli* comes from β-alanine synthase, the last enzyme of the uracil degradation pathway.

KEYWORDS
β-alanine, pantothenate, CoA, comparative genomics, uracil degradation, pantothenate, vitamin

1 | INTRODUCTION

β-Alanine is a nonproteinogenic β-amino acid that occurs in all living organisms. In prokaryotes, β-ala is indispensable for the synthesis of pantothenate, the precursor of the essential cofactor coenzyme A (CoA). CoA is the source of 4'-phosphopantetheine for fatty acid and polyketide synthesis (Leonardi & Jackowski, 2007). In eukaryotes, β-amino acids and β-peptides play important roles in the regulation of nutritional metabolism, immunity, and the central nervous system (Naveed Riaz, Rehman M, & Mahboob Ahmad, 2017).

The major pathway for β-ala synthesis in *Escherichia coli* is the decarboxylation of aspartate by aspartate decarboxylase (ADC; Cronan, 1980). The ADC protein is a pyruvoyl-dependent enzyme that is initially synthesized as a zymogen (pro-ADC). A cleavage of pro-ADC occurs between Gly24 and Ser25, creating the active-site pyruvoyl moiety. Stuecker (Stuecker, Bramhacharya,

El trabajo publicado en *Algal Research* 2020. 47:101890 (FI= **4.008**) es un artículo experimental publicado recientemente por el grupo de trabajo en el que realicé mi tesis doctoral. Este trabajo está enfocado en caracterizar mediante el uso de biología molecular y cinética enzimática, el sitio de unión a los metales pesados en la enzima encargada de sintetizar a las fitoquelatinas en *E. gracilis* (*Eg*FQS).

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Algal Research

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Mapping the metal-catalytic site of a zinc-activated phytochelatin synthase

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ARTICLE INFO

Keywords:
Phytochelatins
Euglena
Zinc
Cadmium

ABSTRACT

Phytochelatins are small enzyme-synthesized peptides that mediate tolerance to several heavy metals. To gain insight into the unusual metal catalytic site of the *Euglena gracilis* phytochelatin synthase (*Eg*PCS), site-directed mutagenesis was performed on the C-terminal Cys/His (C/H) rich region 421-C-C-X-H-X-H-X-H-H-H-430. *Eg*PCS mutants were analyzed at the cellular, kinetic and structural levels. Cd^{2+}-resistance was conferred to Cd^{2+}-sensitive yeast cells by *Eg*PCS_wt (476aa) and truncated *Eg*PCS_435 (435aa), but not by *Eg*PCS_415 (415aa), suggesting that the resistance phenotype was linked to the enzyme C/H rich region. Moreover, full-length mutants (in which C and H residues in the C/H rich region were replaced by Ala) *Eg*PCS_C2 (C421A/C422A) and *Eg*PCS_C2H5 (C421A/C422A/H424A/H426A/H428A/H429A/H430A) did not provide Cd^{2+}-resistance; in contrast, *Eg*PCS_H2 (H424A/H426A), *Eg*PCS_H3 (H428A/H429A/H430A), and *Eg*PCS_H5 (H424A/H426A/H428A/H429A/H430A) mutants conferred similar Cd^{2+}-tolerance like *Eg*PCS_wt. Kinetic analysis showed that maximal rate (V_{max}) for PC_2 synthesis, affinity constants ($K_{m\ Zn\text{-}GS2}$ or $K_{m\ Cd\text{-}GS2}$) and catalytic efficiencies (V_{max}/K_m) were differentially impaired in the mutants, as compared to *Eg*PCS_wt, with *Eg*PCS_C2 being the most perturbed enzyme; however, the $K_{0.5}$ values for GSH were not affected. All *Eg*PCS mutants were predominantly monomeric. Far UV circular dichroism spectra and differential scanning calorimetry endotherms, indicated that alterations of the catalytic properties of *Eg*PCS_C2 were not due to partial unfolding or destabilization of the native state of this mutant. The results indicated that C421/C422 and H424A/H426A/H428/H429/H430 are essential components of *Eg*PCS for catalysis and activation by metal-substrate complexes.

1. Introduction

Phytochelatin synthase (PCS) provides resistance against heavy metal toxicity by synthesizing metal-chelating polymers called phytochelatins (PCs), commonly from PC_2 ([Glu-Cys]$_2$-Gly) to PC_6 ([Glu-Cys]$_6$-Gly) units. PCS uses GSH and different metal-*bis*-glutathionate (Me-GS_2) complexes as substrates to produce PCs, which in turn bind and inactivate metals intracellularly; the metal-PCs complexes may be further compartmentalized into vacuoles, chloroplasts and mitochondria [1]. PCs synthesis is mainly induced in plants and other organisms by cadmium, although PC synthesis can also be triggered to lower extents by arsenite, mercury, lead, zinc, copper, nickel, silver and chromium [2–8]. The molecular and kinetic mechanisms that determine the metal-dependence of phytochelatin synthases (PCSs) have not been yet elucidated. Understanding these mechanisms is important to engineer PCSs efficiency to thus improving their performance and consequently the accumulation/traffic/storage processes of essential- and non-essential metals in plants and microorganisms [1,9,10].

The *Arabidopsis thaliana* PCS1 (*At*PCS1) is perhaps one of the best characterized PC synthases. For instance, the activity (V_{max}) of *At*PCS1 in presence of GSH and cadmium-*bis*-glutathionate (Cd-GS_2) is higher than with zinc-*bis*-glutathionate (Zn-GS_2) as co-substrate, despite

Abbreviations: *At*PCS, phytochelatin synthase from *Arabidopsis thaliana*; CD, circular dichroism; Cd-GS_2, Cd-*bis*-glutathionate; DSC, differential scanning calorimetry; *Eg*PCS, phytochelatin synthase from *Euglena gracilis*; γ-EC, gamma-glutamylcysteine; GSH, glutathione; HPLC, high-performance liquid chromatography; Me-GS_2, metal-*bis*-glutathionate; MCS, metal-catalytic site; PCs, phytochelatins; PCSs, phytochelatin synthases; T_m, thermal midpoints; *ycf1*, *yeast cadmium factor protein* cells; Zn-GS_2, Zn-*bis*-glutathionate

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El trabajo derivado de los estudios de licenciatura de la estudiante Mariel Hernández Garnica dirigidos por Rosina Sánchez Thomas están por ser publicados en la revista *Environmental Pollution* (FI=**6.792**), el el cual la sustante de esta tesis de doctorado es el **autor de correspondencia.** A continuación, se muestra la portada de dicho manuscrito y la carta de respuesta con las revisiones recibida el 08 de septiembre del 2020.

Manuscript File

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Lead accumulation mechanisms in photosynthetic *Euglena gracilis*

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Abbreviations: Cys, cysteine; γEC, gammaglutamyl cysteine; *Eg*-Ca, cells under calcium deficiency; *Eg*-P, cells under phosphate deficiency; *Eg*-S, cells under sulfate deficiency; GSH, glutathione; Pi, inorganic phosphate; PolyP, polyphosphates; Poly-GSH, glutathione polymers; DHE, dihydroethidium.

Key Words: Lead, *Euglena*, Calcium, Polyphosphates, Bioremediation.

Manuscript Number: ENVPOL-D-20-00840

Lead accumulation mechanisms in photosynthetic Euglena gracilis.

Dear Dr. Sánchez-Thomas,

Thank you for submitting your manuscript to Environmental Pollution.

I have completed my evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Oct 23, 2020.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

To submit your revised manuscript, please log in as an author at https://www.editorialmanager.com/envpol/, and navigate to the "Submissions Needing Revision" folder.

Environmental Pollution values your contribution and I look forward to receiving your revised manuscript.

Kind regards,
Joerg Rinklebe
Editor

Environmental Pollution

Editor and Reviewer comments:

Reviewer #1: This manuscript evaluated the effects of Euglena gracilis on cellular growth, respiration, photosynthesis, chlorophyll, calcium, and levels of thiol- and phosphate-molecules to understand the biochemical mechanisms that modulate lead accumulation. This study set a complex experimental design, and obtained many valuable results. The results obtained seems to be of interest to journal readers. Yet, there are some major issues before the recommendation for accept. The following specific comments are intended to improve the quality of manuscript.

1) Many parts in introduction section seems to be redundant, in particular L48-71. These parts are very voluminous (more than half of introduction section) although the contents are general.
2) In contrast, the motivation parts of this study (L92-) is vague. The authors should clarify the need for the purpose referring the previous knowledge. We cannot understand the biochemical mechanisms of toxic metal and metalloid including lead.
3) In discussion, the authors should discuss the amount of lead accumulated compared with

other toxic metal(loid)s and the differences of biochemical mechanisms between lead and other toxic metal(loid)s.

Reviewer #2: This manuscript reports on accumulation of Pb2+ in the alga Euglena gracilis. Some questions arise due to the high concentrations which were used in the experiments. Detailed comments:
- Introduction: a large part of the introduction reports on toxicity of lead to humans, whereas the topic of this manuscript is on Pb in algae. Some references to Pb in algae may be added.
- p.4, lines 72-73: the cited concentrations are extremely high and occur only in very polluted environments. They should be compared with unpolluted waters, in which Pb is typically in the range 0.1 - 1 microg/L.
- p. 5, lines 110-112: why is an acidic medium used ? What is the composition of this medium with respect to complexing ligands for Pb2+ ? What is the Pb(II) speciation in this medium ?
- p. 13, lines 210-211: very high Pb concentrations in cells are mentioned here. Is it possible that there is some precipitationn of Pb solid phases ?
- p. 14, lines 221-229: is it really Pb release or a dilution effect caused by the higher number of cells ? how does the Pb concentration in the medium change ?

Reviewer #3: Hernández-Garnica et al. presented a study of lead accumulation mechanisms in photosynthetic Euglena gracilis and analyzed its effects on cellular growth, respiration, photosynthesis, chlorophyll, calcium, and levels of thiol- and phosphate-molecules. The topic and the findings were interesting. In general, the structure of this paper is well designed and conducted. However, it still needs some improvements to get published. I have some questions to discuss with authors and some shortcomings.
1. P1: The title is too ambitious in the Ms science it relates to many very complex mechanisms for lead accumulation in microorganism. Also, there living environment is recommended to be included in the title.
2. P1 L42: Change "whereas," with ",whereas".
3. P3 L51-54: I don't think the information referenced from 2000 and 2001, circa 20 years ago, should gave effective information on "currently".
4. L55-L78: detailly addressed the lead toxicity to human. I don't think it needs so many details relative to the other contents relating to the topic.
5. L80-81: Fabaceae and Brassicaceae in italics?
6. L82-83: Here do the "lead hyperaccumulators" refer plants or microorganism? Also are the documents in L84-86 related to plants or microorganism?
7. L84-87: Such "lead accumulation" in plants or microorganism?
8. L96 Are "carboxylic acids and histidine" in Euglena gracilis sensitive to lead? Why didn't the authors measure the two indexes?
9. L119: What are the concentrations of the exposure Pb?
10. L129: Here the experiments were well conducted to detect cell responses. Well in the actual water systems, the phosphate contents are generally higher than the designed concentration, especially in the eutrophic water systems, are there any differences? Should the authors discuss these differences in the discussion part?
11. L206: Delete "or lower".
12. Table 1 and 2 have different formats.
13. Fig.1A and B and Fig. 4 lines are hard to read or to distinguish.

14. Do the variations in "Lead accumulation and toxic effects" have significant differences? I did not find any information in the paragraphs or Fig.1.
15. The responses, mechanisms and applications in the actual environments should be further discussed.
16. Too many earlier findings were references. More latest references should be added.
17. When you compare your date with different species, are the environments or the disparities in species diversity? I think you could suggest for future works ecotoxicological tests in the tissue.

Reviewer #4: In manuscript "Lead accumulation mechanisms in photosynthetic Euglena gracilis" Authors analyzed the biochemical mechanisms that modulate lead accumulation in E. gracilis and its effects on cellular growth, respiration, photosynthesis, chlorophyll, calcium, and levels of thiol- and phosphate-molecules. Interesting work but needs minor revision to improve it.

The entire text of the manuscript should be justified.
Standardize abbreviations throughout the text and remove commas and replace them with dots next to numbers.
- Highlights - need reworking, expand the abbreviations used for the first time, or we use Pb or lead ions
- Abstract -
line 50 - wrong citation order in parentheses
line 80 - plant family names in italics
- Materials and methods
Please describe in detail the media and buffers used, e.g. Hunter medium
line 113 - correct citation, date in brackets
lines 119-122 - Add what concentrations of Pb and what salt were used for the tests
line 139 - remove italic 'plus' and from line 150 'versus'
- Results
lines 211-217 - replace commas with dots in numerical results
Figures - in all graphics the font on the X and Y axes should be reduced,
- Discussion
line 388 - replace '&' with 'and' in citation
Supplement Materials - Some graphs in Figures S2 and S3 are cut off at the bottom and not visible

Reviewer #5: The manuscript entitled as "Lead accumulation mechanisms in photosynthetic Euglena gracilis" reported high accumulation of Pb by Euglena gracillis and demonstrated the role of polyphosphates and Ca2+ in mediating the Pb resistance of the microorganism. The study was well designed with the data clearly presented. I suggested that the manuscript could be accepted for publication for which the discussion part may need to be reorganised to meet the requirement for publication. For comments, please explain why the LD50 of Pb was not determined.

Reviewer #6: Heavy metal toleratnce of Euglena is a quite interesting observation, with some already detailed studies reoprted. In this ms, lead accumulation by Euglena gracilis and its effects on cellular growth, respiration, photosynthesis, chlorophyll, calcium, and levels of thiol- and phosphate-molecules were analyzed. However, there is a very important

reference missing, Microorganisms. 2020 Jan 14;8(1):115. doi: 10.3390/microorganisms8010115, Probing the Role of the Chloroplasts in Heavy Metal Tolerance and Accumulation in Euglena gracilis, published in Jan, 2020.
In the previous report, the Minimum Inhibitory Concentration (MIC) for Pb in the Zm-strain was 9000 ppm, which is about the same as that of the Z-strain from another previous study [Khatiwada B., Hasan M.T., Sun A., Kamath K.S., Mirzaei M., Sunna A., Nevalainen H. Proteomic response of Euglena gracilis to heavy metal exposure-Identification of key proteins involved in heavy metal tolerance and accumulation. Algal Res. 2020;45:101764. doi: 10.1016/j.algal.2019.101764]. MIC 9,000ppm Pb^{2+} showed growth inhibition in the previous reports, however, in this ms, 200 uM (~41.4 ppm Pb^{2+}) for up to 8 days did not modify growth, viability, chlorophyll content and oxygen consumption/production. Even in pre-test, for the testing concentration, we should target one concentration under which cells could be inhibited. Please clarify this before further discussion.

Reviewer #7: This manuscript described a highly lead resistant and hyper-accumulating microorganism, E. gracilis. authors found that Pb^{2+} accumulation in cell depends on PolyP content but not on thiol molecules. The absence of Ca^{2+} promotes increased lead accumulation. the results is interesting.
Minior comments is as follows.
1. the title should be more specific.
2. introduction part. From line 4 to line 71, the authors used three paragraph to describe the risk of Pb. It is better to let them more concise.
3. Materials and methods part. authors should explain or have brief introduction the origin of the strain
4. the unit "Kg" should be "kg"