Universidad de Huelva

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Efectos de los metales pesados a lo largo del gradiente mareal en cinco especies de plantas halófitas en el Paraje Natural Marismas del Odiel, Huelva

Memoria para optar al grado de doctor presentada por:

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A mi familia

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CAPITULO 1: Capitulo Introductorio

1. Introducción.

1.1. La contaminación por metales pesados.

Según cita el Ministerio de Transición Ecológica en su página web (2022) "*existen varias maneras de definir el término metal pesado*". Algunas de estas definiciones se fundamentan en la naturaleza de los elementos, como ocurre con la formulada por la International Union of Pure and Applied Chemistry (2002), una de las más completas, que indica que *"son aquellos elementos químicos con densidad superior a 5 g/cm^3, una masa mayor a 22,99 g/mol y un numero atómico mayor a 20*". Sin embargo, otras inciden en mayor medida en sus efectos, como la de Lucho-Constantino et al. (2005), quienes los definen como "*cualquier elemento químico que tenga una densidad relativa alta y sea tóxico incluso a bajas concentraciones*". Ante esta disparidad de definiciones ocurre que se incluyen entre los metales pesados algunos elementos tóxicos ligeros como el berilio o el aluminio, o algún semimetal como el arsénico.

De forma natural los metales pesados se encuentran en la naturaleza en la composición de distintos minerales (Salomons et al., 1995), los cuales pueden aflorar a la superficie por procesos geológicos como los volcanes, formándose zonas metalíferas enriquecidas en estos metales. En estas zonas los minerales sulfurosos, muy insolubles en condiciones reductoras, son expuestos a condiciones aerobias, y al entrar en contacto con el O_2 y la humedad se oxidan, aumentando la acidez y liberando parte de su contenido al medio. Por otra parte, el hombre es responsable del enriquecimiento del medio en metales pesados a través de actividades como la industria, actividades de procesado de residuos, utilización de combustibles fósiles, la minería o el tráfico (Castillo et al., 2017; Van-Camp et al., 2004), entre otras. Entre los grandes generadores de metales pesados, como se recoge anteriormente, podemos encontrar la actividad minera, siendo las escombreras, las excavaciones subterráneas y los procesos de trituración del mineral las actividades que liberan mayor cantidad de metales pesados al medio mediante el llamado drenaje acido de minas (Sainz et al., 2004). Cuando las redes fluviales son afectadas por el drenaje ácido de minas se producen aguas de carácter ácido, con alto contenido en metales y sulfatos que afectan a toda la biodiversidad de la zona por donde discurre.

Por su situación estratégica los estuarios suelen ser medios donde la contaminación de metales pesados es muy frecuente, debido a la presencia de puertos que generan una elevada actividad de transporte, industrial, así como el asentamiento de núcleos urbanos muy poblados (Cerón et al., 2000; Curado et al., 2010).

En los estuarios los metales disueltos precipitan a medida que aumenta la salinidad, por lo que algunos de estos metales quedan atrapados en los sedimentos, mientras que otros, más móviles, permanecen en solución, como el Cu, Mn y Zn (Van Geen et al., 1997; Elbaz-Poulichet et al., 2001; Achterberg et al., 2003; Braungardt et al., 2003; López-González et al., 2006). De esta manera, las marismas formadas en estos estuarios suelen estar expuestas a altos niveles de metales pesados que se acumulan en los sedimentos de los distintos hábitats intermareales, que actúan como sumideros. Pero cuando las condiciones ambientales como el pH, conductividad, condiciones de anoxia, etc. cambian, pueden actuar como fuente de contaminación liberando esos metales al medio (Harter, 1983; Förstner, 1989; Izquierdo et al., 1997; Zoumis et al., 2001), haciéndolos biodisponibles y transformándolos en componentes solubles que pueden ser absorbidos por las raíces de las plantas (Blaylock, 2000).

En general, los metales pesados son una amenaza para los ecosistemas, considerándose la contaminación por metales como un problema global (Baker, 1981; Liao et al., 2006; Seneviratne et al., 2017; Ali & Khan, 2019; Agrawal et al., 2007). Esto es debido a su alta toxicidad, ya que afectan a los procesos bioquímicos, lo que se traduce en problemas de desarrollo, crecimiento y reproducción para los seres vivos (Roy et al., 2005), y a su persistencia en el medio por no ser biodegradables y por su comportamiento bioacumulativo, ya que se acumulan en los organismos más longevos y se concentran en cada salto de nivel trófico (Williams et al., 1994; Buccolieri et al., 2006).

La entrada de los metales pesados en la cadena trófica se realiza fundamentalmente a través de su absorción por los vegetales, y a través de éstos pasa por ingestión a los distintos niveles de consumidores y, por supuesto, al hombre (Khan et al. 2013; Pandey et al., 2016 ; Yang et al. 2017; Ali & Khan., 2019), donde sus efectos toxicológicos, particularmente del Cd, Zn, Hg y Pb y de metaloides como el As, han sido bien documentados (Adriano, 2001; Rubio et al., 2007). En este sentido, la seguridad alimentaria es una de las principales preocupaciones actuales a nivel mundial, dando lugar a una creciente demanda de investigación sobre los riesgos asociados al consumo de alimentos contaminados por metales pesados (Khan et al., 2015; Kananke et al., 2016; Chen et al., 2018; Chabchoubi et al., 2021).

Los peligros asociados a la ingesta de metales pesados en el cuerpo humano han llevado a muchos países y agencias a establecer niveles de tolerancia para los alimentos y los piensos a través de la utilización de parámetros que cuantifican estos peligros, el más común es el Índice de Riesgo para la Salud (HIR), que se basa en la Ingesta Diaria Estimada (EDI) y en las Dosis de Referencia (RD), todos ellos utilizados por la Agencia de Protección Ambiental de los Estados Unidos (EPA) en los vegetales de consumo.

Debido a lo mencionado anteriormente, las investigaciones sobre contaminación por metales pesados son de gran importancia en zonas sensibles y altamente productivas como son las zonas costeras, humedales, marismas y esteros (Arellano et al., 1999; Cohen et al., 2001; Blasco Moreno et al., 2010).

1.2. Tolerancia de las plantas a los metales pesados.

En las plantas, los metales pesados pueden alterar diversos procesos bioquímicos, pueden tener efectos en la homeostasis de los nutrientes, el intercambio de gases, la producción de enzimas y antioxidantes, la movilización de proteínas y la fotosíntesis (Seneviratne et al., 2017). Sin embargo, no todos los metales poseen el mismo comportamiento sobre las plantas, así, entre los metales pesados hay elementos esenciales y no esenciales, aunque el límite entre estos dos grupos no está claramente definido y la lista de elementos biológicamente importantes aumenta según aumenta la investigación sobre ellos. Se reconocen como elementos esenciales al Fe, Mn, Zn, Cu, Co y Mo, los cuales son necesarios para las plantas en bajas concentraciones, aunque pueden resultar tóxicos a concentraciones superiores; como elementos beneficiosos el Ni y Cr; y se considera que no tienen ninguna función biológica elementos como el Cd, Hg, Pb y As, por lo que resultan tóxicos a bajas concentraciones (Brady & Weil, 2008).

Tampoco todas las especies de plantas poseen la misma respuesta ante la presencia de los metales pesados en el medio. Existen determinadas especies de plantas que poseen la capacidad de sobrevivir en un ambiente contaminado por metales pesados donde otras plantas no podrían establecerse, para ello presentan distintos mecanismos basados en la exclusión de estos metales a nivel de raíz, evitando su absorción, o basados en la reducción de su toxicidad una vez absorbidos, mediante adaptaciones fisiológicas y bioquímicas que les permiten incorporar los metales pesados y acumularlos en sus tejidos (Baker, 1981; Rotkittikhun et al., 2006). La capacidad de acumular metales en los diferentes tejidos vegetales es variable, de forma que pueden encontrarse niveles contrastados de concentración si comparamos raíces, tallos y hojas (Kloke et al., 1994). En este sentido, las plantas halófitas, que presentan mecanismos adaptativos para sobrevivir en ambientes salobres, con frecuencia muestran una alta tolerancia a la contaminación por metales pesados (Thomas et al., 1998; Manousaki & Kalogerakis, 2011; Van Oosten & Maggio, 2015).

Dentro del ciclo de vida de una planta, la germinación de las semillas y el crecimiento de las plántulas son etapas muy sensibles a la contaminación por metales pesados, y por tanto nos dan una información valiosa sobre la tolerancia de la especie a la contaminación (Munzuroglu & Geckil, 2002; Ahsana et al., 2007). Los efectos de los metales pesados en el proceso de germinación se basan en su capacidad para llegar a los tejidos embrionarios a través de las cubiertas de las semillas, una barrera fisiológica, y en las propiedades físicas y químicas de los propios iones metálicos (Ko et al., 2012). Diferentes especies de plantas poseen diferente anatomía y estructura de la cubierta de la semilla y, por lo tanto, la misma concentración de metal puede tener efectos diferentes en diferentes especies (Munzuroglu & Geckil, 2002). Además, se ha descrito que algunos metales como Cu y Cd inhiben la absorción de agua, y por lo tanto no ocurre la germinación (Kranner & Colville, 2011).

Los metales pesados también pueden causar estrés oxidativo, dañar los sistemas fotosintéticos y provocar daños estructurales en las plántulas. Estos daños suelen centrarse en las raíces, ya que son los principales objetivos de los aniones metálicos y su crecimiento suele verse más gravemente afectado que el de las partes aéreas, por lo que las pruebas de raíces se utilizan ampliamente para evaluar los niveles de toxicidad de las sustancias tóxicas, incluidos los metales pesados (Seneviratne et al, 2017).

1.3. Usos de plantas halófitas en suelos contaminados por metales pesados: fitorremediación y/o consumo.

El aumento de la contaminación de suelos con metales pesados por parte del hombre ha llevado a un aumento de la preocupación por su recuperación, llevando a diversos organismos y administraciones públicas a establecer límites máximos permitidos para determinadas concentraciones de metales, con el fin de proteger el suelo y el medio ambiente. Es el caso de la Unión Europea y del Estado Español que fijan límites para Cd, Cu, Ni, Pb, Zn, Hg y Cr (EUCD, 1986/278; Real Decreto 1310/1990), y más localmente en nuestro caso, la Comunidad Autónoma de Andalucía que fijó niveles para As, Cd, Co, Cr, Cu, Ni, Pb, Tl y Zn (Consejería de Medio Ambiente, 1999). Otro impulso normativo se produjo tras la publicación de la comunicación de la Comisión de las Comunidades Europeas *"Hacia una estrategia temática para la protección del suelo"* (Comisión Europea, 2002), que obligó a los países miembros a realizar un inventario de las áreas contaminadas de su territorio y a promover estrategias para su recuperación.

En la actualidad se dispone de muchas tecnologías de recuperación de suelos contaminados con metales pesados, basadas en procesos físico-químicos y biológicos. Usualmente las técnicas basadas en tratamientos físico-químicos tienen un coste económico elevado, tienen unos largos tiempos de vigilancia (Glass et al., 1999), y dejan el suelo poco apto para el crecimiento de vegetación, mermando su productividad (Marques et al., 2009).

Los métodos biológicos o biorremediación son un conjunto de técnicas que reducen la concentración de determinados contaminantes, realizando procesos bioquímicos a través de las plantas y microorganismos asociados a ellas, para eliminar, reducir, trasformar, mineralizar, degradar, volatilizar o estabilizar contaminantes (Kelley et al., 2000; Barceló & Poschenrieder, 2003; Pilon-Smits, 2005; Eapen et al., 2007). Estos métodos, además de menos costosos, son más respetuosos con el medio ambiente al realizarse mediante métodos naturales y fomentar el establecimiento de las plantas en estos suelos.

La fitorremediación de suelos contaminados mediante el empleo de plantas se basa en su capacidad para absorber y extraer los contaminantes del suelo, y acumularlos en sus tallos y hojas, de manera que estas partes donde se acumula el contaminante pueden ser extraídas y destruidas o recicladas, retirando el metal del suelo (Kumar et al., 1995). Esta técnica es más viable cuanto mayor es la capacidad de la especie para acumular metales pesados en sus tejidos (Brooks, 1998), denominándose hiperacumuladoras a las plantas capaces de acumular al menos 100 µg/g (0,01 % peso seco) de Cd y As; 1000 µg/g (0,1 % peso seco) de Co, Cu, Cr, Ni y Pb; y 10000 µg/g (1,0 % peso seco) de Mn (Watanabe, 1997; Reeves et al., 1999; Kamal et al., 2004; Reeves, 2006). Actualmente se han identificado más de 400 especies hiperacumuladoras de Ni, Co, Cu, Se, Pb, Cd, Zn y Mn, y los niveles de acumulación de estos metales están siendo revisados (Reeves et al., 1999; Van der Ent et al., 2013).

Por otro lado, la degradación de las tierras agrícolas por salinización se produce en todo el mundo debido a una disminución del agua dulce y las aguas subterráneas (Daliakopoulos et al., 2016), y muchas especies halófitas adaptadas a ese medio salino han sido estudiadas como acumuladores potenciales de metales (Oyuela-Leguizamo et al., 2017), ejemplo de ello son: *Halimione portulacoides* Aellen, un acumulador adecuado para Hg, Cr, Cu, Cd y Pb, (Sleimi et al., 2014; Castro et al., 2006) que tiene además potencial de estabilización para Cr y Cu (Almeida et al., 2009); *Sarcocornia fruticosa* (L.) A.J. Scott, que acumula As, Cd, Cu, Pb y Zn en la biomasa subterránea en concentraciones varias veces superiores a las concentraciones de los metales en el suelo (Duarte et al., 2010; Santos-Echeandía et al., 2010; Caetano et al., 2008); o *Atriplex halimus* L. que ha demostrado ser adecuado para la fitoextracción de Cd, Cu y Zn en suelos salinos (Bankaji et al., 2014; González-Alcaraz et al., 2011). Debido a esa capacidad de acumulación el uso de estas plantas para consumo humano debe estar vigilado, y la Unión Europea está considerando la posibilidad de establecer niveles máximos de metales pesados para estos alimentos.

1.4. Descripción de la zona de estudio.

El estuario de la ría de Huelva se localiza en el sudoeste de la Península Ibérica, en el sector noroccidental del Golfo de Cádiz, en la desembocadura de los ríos Tinto y Odiel (Figura 1). Estos ríos nacen en la sierra de Huelva y atraviesan la provincia de norte a sur hasta desembocar en el Océano Atlántico. Este estuario tiene un régimen mesomareal con un rango mareal medio en su sector central de 2,69 m llegando a un rango medio de 3,06 m en marea viva y de 1,7 m en marea muerta (Morales & Borrego, 2000).

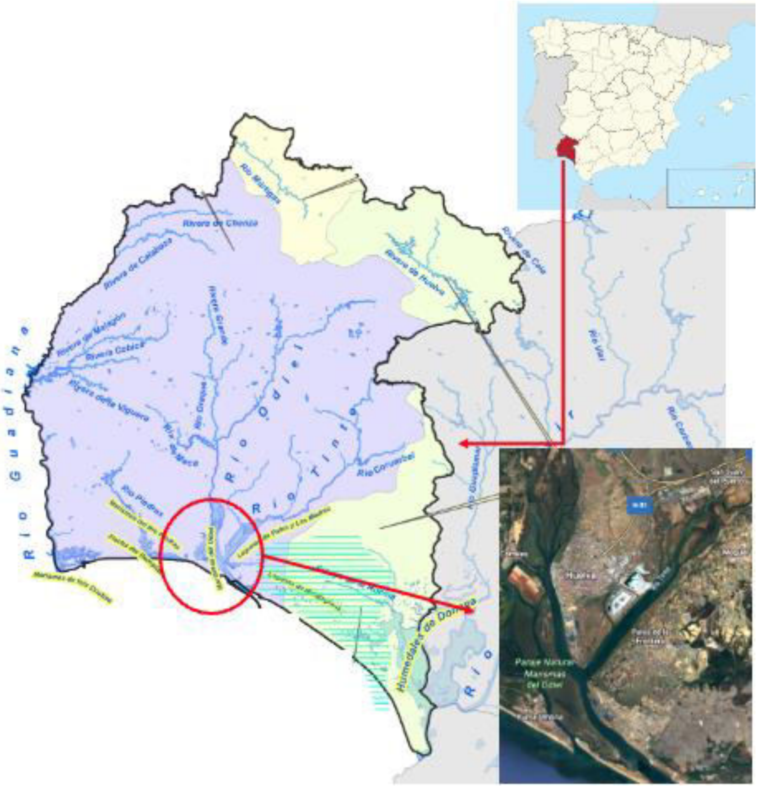

Figura 1: Localización de la ría de Huelva (modificado de Wikipedia.es y Google earth@, 2022)

El estuario tiene una extensión aproximada de 15 km^2 y está conectado con el mar a través de dos amplios canales mareales: el Canal de Punta Umbría y el Canal del Padre Santo. Este último es la vía de navegación principal por la que tiene lugar el tránsito hacia el mar abierto, y donde se unen los cauces bajos de los ríos Tinto y Odiel en un único canal. En el interior del estuario se encuentra el Paraje Natural Marismas del Odiel (Figura 2), con una superficie de 7185 ha, calificado como Reserva de la Biosfera por la UNESCO en 1989, y que contiene las Reservas Naturales de Marismas del Burro e Isla de Enmedio (Junta de Andalucía, 2022).

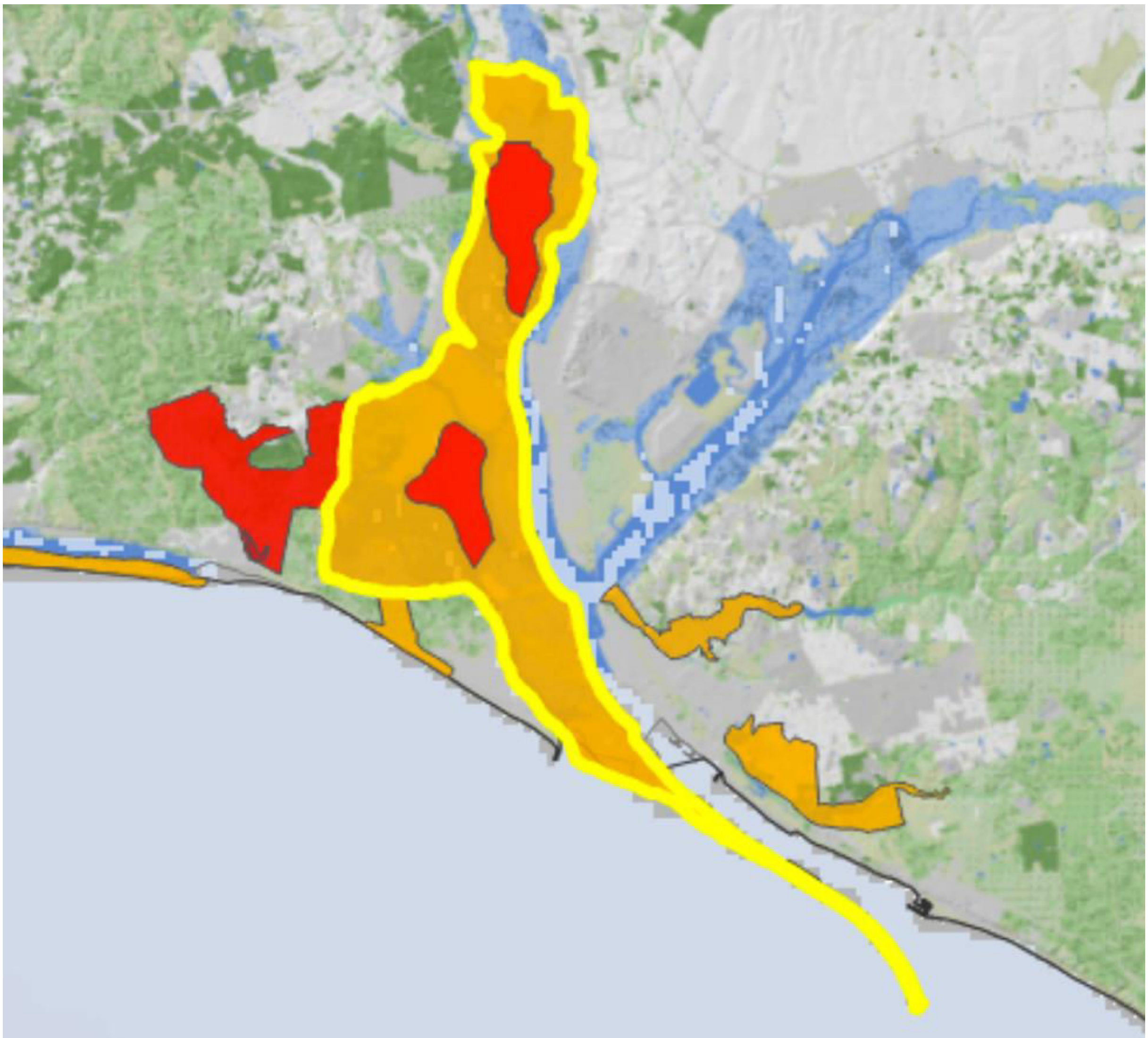

Figura 2: Límites del Paraje Natural Marismas del Odiel (línea amarilla) y situación de las Reservas de Marismas del Burro e Isla de Enmedio (Junta de Andalucía, 2022)

El estuario es un sistema muy complejo, formado por una serie de canales de drenaje que separan las diferentes zonas de marismas; este sistema está controlado por el régimen de mareas y las entradas fluviales de los ríos Tinto y Odiel. El estuario presenta una división longitudinal en tres sectores (Figura 3). En el dominio fluvial y parte del dominio central (estuario alto) existe un control de los ríos en el aporte de sedimentos, con una descarga fluvial interanual muy irregular y con una marcada estacionalidad. Sus aguas presentan un acusado carácter ácido, con una media de pH 2 en el Tinto y pH 3,5 en el Odiel, lo que le permite contener altas concentraciones iónicas. La parte baja del dominio central (el estuario bajo) está dominado por el régimen mareal que controlan directamente las condiciones de sedimentación. Por último, el dominio marino está controlado por la acción conjunta de las mareas y el oleaje (González et al., 2002).

Figura 3: Identificación de los sectores del estuarino (González et al. 2002). Modificada de Google earth@2022.

La ría de Huelva es uno de los sistemas con mayor contaminación por metales pesados a nivel mundial, en parte por la actividad portuaria e industrial, y sobre todo por el aporte de metales pesados arrastrados en disolución por los ríos Tinto y Odiel tras su paso por la franja pirítica (Cerón et al, 2000; Sainz et al., 2004; Blasco Moreno et al., 2010;), siendo la actividad minera la responsable del 99% de la contaminación por metales pesados del estuario (Pérez-López et al., 2011).

Dentro de la marisma, existe una zonación en función de los distintos niveles topográficos, estableciéndose así diferentes zonas en función del grado de inundación, que da lugar a los diferentes hábitats que presentan distintas comunidades de especies de plantas en función de su tolerancia a las características edáficas y al régimen de inundación de cada zona (Contreras-Cruzado et al., 2017) (Figura 4).

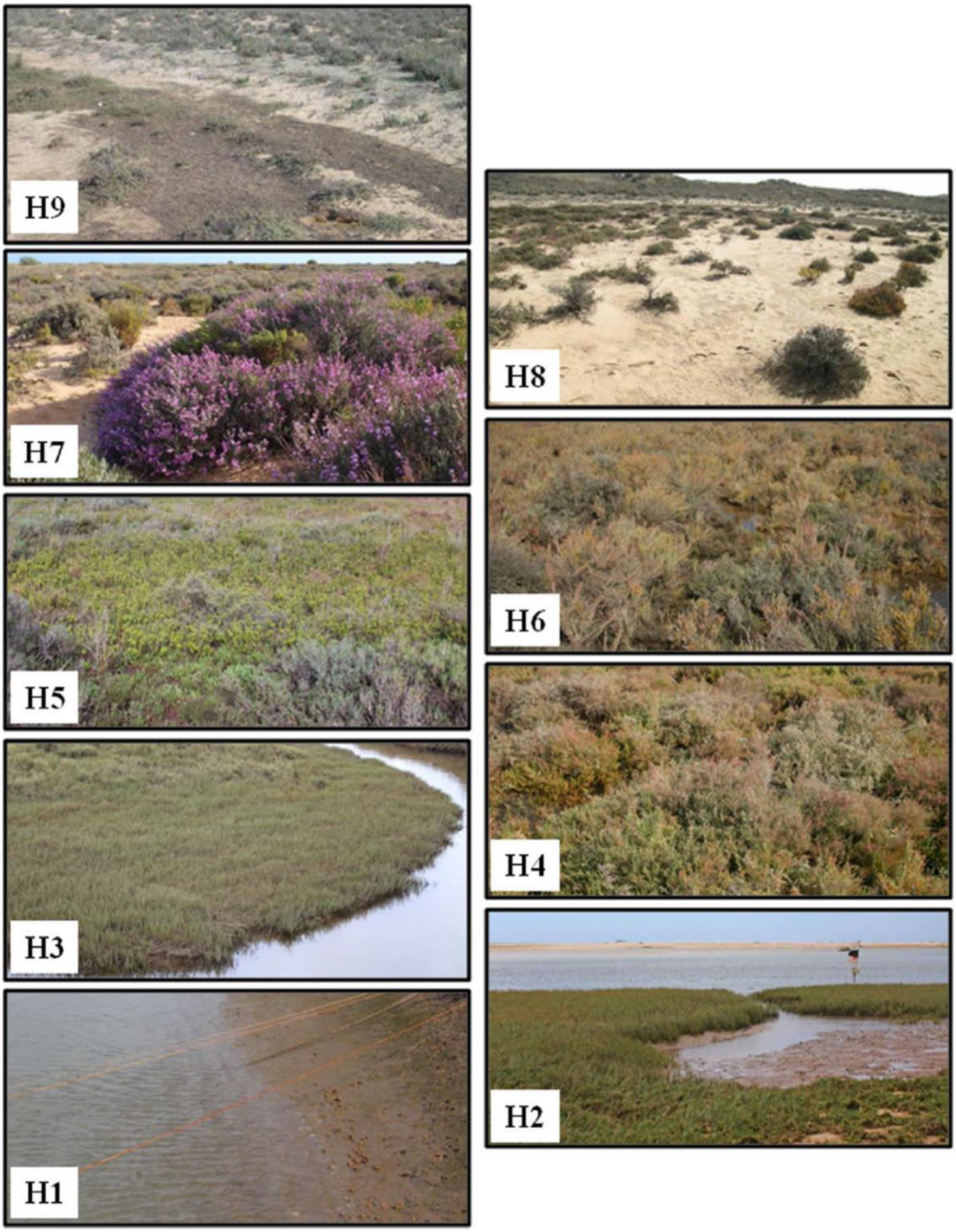

Figura 4: Diferentes comunidades vegetales en función de su situación topográfica, de H1 en las zonas más bajas a H9 en el límite superior de la pleamar (Contreras-Cruzado et al., 2017).

Tradicionalmente la marisma se divide en tres zonas: marisma alta, marisma media y marisma baja. En la marisma alta dominan las condiciones de exposición subaérea, ya que solo se encharca durante grandes crecidas fluviales y/o mareas altas vivas extremas, y su límite inferior lo marca la altura media de la pleamar en mareas vivas (MHWS), mientras que el límite superior de la marisma baja es el nivel medio alcanzado por las pleamares en mareas muertas (MHW), la marisma media es el espacio entre los dos limites descritos anteriormente (Figura 5).

Las marismas media y baja son zonas intermareales caracterizadas por una vegetación halófila con predominio de los géneros *Spartina*, *Salicornia* y *Sarcocornia*, donde suele aparecer una zonación topográfica definida por la presencia de diferentes especies vegetales en función de su tolerancia a los diferentes grados de exposición/sumersión y, por tanto, de salinidad (López González, 2008), Debemos tener en cuenta que esta clasificación de la marisma no es estricta y existen otros factores derivados del grado de inundación que pueden condicionar el establecimiento de unas u otras especies, como pueden ser los valores de pH o el contenido en materia orgánica entre otros (Contreras-Cruzado et al., 2017).

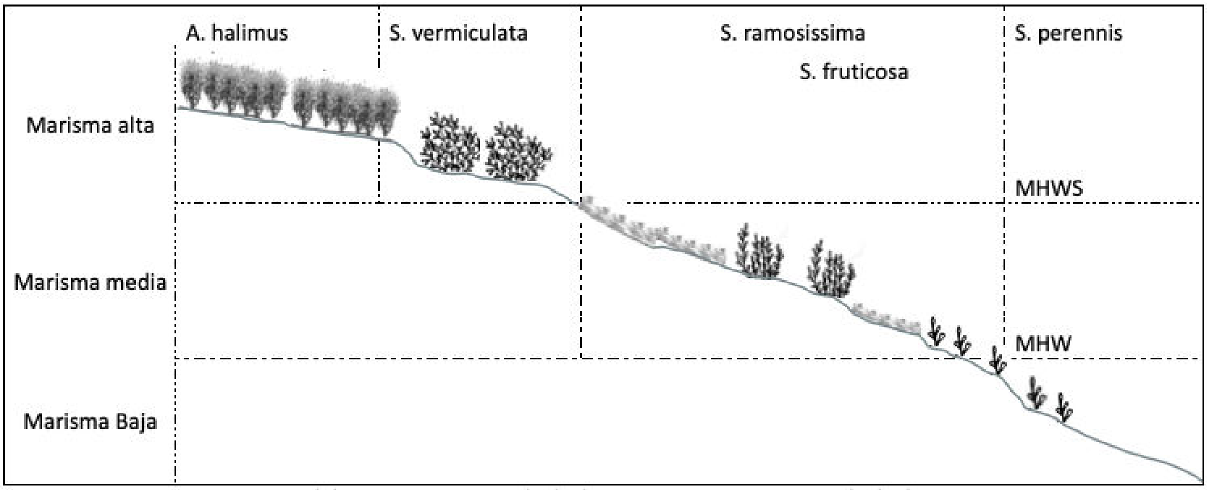

Figura 5: Zonas de la marisma; MHWS: Media de pleamar en marea viva; MHW: Media de pleamar en marea muerta . Elaboración propia.

1.5. Especies estudiadas.

Muchas especies halófitas han sido estudiadas como acumuladores potenciales de metales en suelos salinos, y pueden ser consideradas especies valiosas para la fitorremediación de suelos contaminados por metales (Oyuela-Leguizamo et al., 2017).

La familia Quenopodiácea presenta un gran número de especies y son dominantes en la vegetación de las marismas mediterráneas (Fernández-Illescas et al., 2010; Contreras-Cruzado et al., 2017); muchas de sus especies han sido estudiadas desde el punto de vista de su relación con los metales pesados como se ha expuesto en apartados anteriores.

Para la realización de esta Tesis Doctoral se han seleccionado varias especies presentes en la zona de estudio que pueden tener diversos usos, ya sea como fitorremediadoras o para consumo. A continuación, se hace una breve descripción de las especies incluidas en este estudio:

***Atriplex halimus* L.**: Se trata de un arbusto de hasta 2,5 m (Figura 6) que se desarrolla sobre suelos arcillosos, limosos o arenosos, siempre con cierto grado de salinidad, que se distribuye en la región mediterránea y el sur de África, y que habita en las zonas costeras de la Península Ibérica, el valle del Ebro, la Mancha y las Baleares. (Castroviejo et al. 1993)

Es una especie halo-nitrófila dominante en los terrenos emergidos de las marismas donde la salinidad es alta. Ha sido descrita como una especie importante para la rehabilitación de suelos afectados por exceso de salinidad y baja humedad (Abbad et al., 2004).

Figura 6: *Atriplex halimus*, aspecto de la planta, frutos y hojas.

Salsola vermiculata **L.***:* Subarbusto de hasta 1m de altura (Figura 7), muy irregularmente ramificado. Es un arbusto de amplia distribución y amplitud ecológica, y constituye un elemento estructural importante en la vegetación de las zonas áridas y costeras del sur de Europa, norte de África, Macaronesia y suroeste de Asia (Creager, R.A., 1988). En las marismas del suroeste de la Península Ibérica habita en sedimentos arenosos en zonas de marismas altas que solo se inundan durante las mareas astronómicas (Contreras-Cruzado et al., 2017).

Figura 7: *Salsola vermiculata*, aspecto de la planta, flores y frutos

***Sarcocornia fruticosa* (L.) A.J. Scott:** Arbusto de hasta 150 cm de altura, muy ramificado (Figura 8), propio de marjales salinos, marismas y saladares con abundante humedad todo el año. Se distribuye por Europa, oeste de Asia, norte y sur de África, centro y sur de América y la Polinesia, y se presenta en toda la costa de la Península Ibérica e Islas Baleares, así como en algunos puntos aislados del interior peninsular (Castroviejo et al. 1993). Es uno de los principales componentes de la marisma media en las marismas del Golfo de Cádiz (Contreras-Cruzado et al., 2017).

Figura 8: *Sarcocornia fruticosa*, aspecto de la planta e inflorescencia en antesis.

***Sarcocornia perennis* (Mill.) A.J. Scott**: Planta con tallos postrados y radicantes que forma céspedes con tallos erguidos de hasta 20 cm (Figura 9), que se distribuye por Europa y la región mediterránea, habitando todas las costas de la Península Ibérica (Castroviejo et al., 1993). En las marismas mareales ocupa las zonas bajas de la marisma, y a veces como especie colonizadora de sustratos arenosos (Davy et al., 2006).

Figura 9: *Sarcocornia perennis,* césped y aspecto de la planta

***Salicornia ramosissima* J. Woods**: Planta anual erecta con tallos de entre 3 y 40 cm, en general bastante ramificados (Figura 10) y muy polimórfica, que habita en el oeste de Europa y noroeste de África, encontrándose en suelos salinos de toda la Península Ibérica e Islas Baleares, siendo característica de salinas y saladares temporalmente encharcados del litoral, lagunas salobres y marismas (Castroviejo et al., 1993). En las marismas mareales coloniza suelos desnudos de distintos niveles, siendo frecuente en las zonas de marisma baja y en las cubetas hipersalinas. Esta especie juega un papel importante en el reciclaje de nutrientes en los estuarios (Figueroa et al., 1987).

Figura 10: *Salicornia ramosissima. Aspecto de la planta e inflorescencia*

1.6. Justificación.

Existen muchos estudios sobre la contaminación por metales pesados en los sedimentos de la costa y marismas de Huelva (Caliani et al., 1997; Ruiz et al., 1998; Grande et al., 2000; Borrego et al., 2002; Morillo et al., 2002; Bermejo et al., 2003; Blasco Moreno et al., 2010), pero pocos donde se analicen los efectos de estos contaminantes sobre la fauna y flora existente.

Conocer cómo se comportan las especies de los diferentes hábitats de las marismas nos ayudaría a conocer los efectos de esta contaminación y a diseñar y planificar estrategias fitorremediadoras de marismas contaminadas. Además, la salinidad de los suelos a nivel mundial está aumentando por lo que encontrar plantas adaptadas a los medios salinos será una prioridad para la alimentación futura.

Por ello, esta tesis por compendio de artículos pretende comprobar cómo afecta la presencia de metales pesados a la germinación y al crecimiento de las plántulas de cinco especies halófitas de marismas que habitan en distintos hábitats del Paraje Natural Marismas del Odiel, Huelva. Además, se estudia cómo se acumulan los metales pesados en dos de ellas, y si este tipo de plantas, concretamente *S. ramosissima* puede ser una alternativa de cultivo con fines alimentarios en estas marismas.

1.7. Objetivos y metodología.

I. Objetivos.

Los objetivos de la presente tesis doctoral son:

- Analizar el efecto de los metales pesados sobre la germinación de especies halófilas usando los siguientes parámetros: Tasa de germinación, velocidad de germinación y desarrollo de las plántulas (evaluado en raíz, hipocótilo y cotiledones).

  Para ello, se usaron especies características de distintos hábitats de las marismas. Las especies estudiadas han sido: *Sarcocornia perennis* (marisma baja), *Sarcocornia fruticosa* (marisma media), *Salicornia ramosissima* (marisma baja marisma media y suelos hipersalinos), *Salsola vermiculata* (marisma alta) y *Atriplex halimus* (suelos salinos por encima de la pleamar).

- Analizar la capacidad de acumulación de metales pesados en los tejidos de las especies halófitas de cara a su utilización como especies fitorremediadoras. Este estudio se ha realizado en *Salsola vermiculata* registrando la acumulación de distintos metales en plantas expuestas a distintas concentraciones de éstos.

- Analizar el transporte y la acumulación de metales pesados entre diferentes partes de las especies halófitas. El estudio se ha realizado en *Salicornia ramosissima*, especie anual comestible, analizando en distintas poblaciones las concentraciones de diversos metales en suelo, raíz, tallos desnudos y tallos con hojas frescas. Los resultados permiten además conocer los límites, sin riesgos para el consumo humano, para el establecimiento de esta especie en lugares contaminados con los metales estudiados.

II. Metodología.

Recolección de material.

Todo el material vegetal necesario para estos estudios se recolectó en poblaciones naturales del Paraje Natural Marismas del Odiel. Las plantas fueron recolectadas y almacenadas en bolsas de papel hasta su utilización.

Ensayos de germinación.

Para las pruebas de germinación, en primer lugar, se procedió a la extracción y limpieza de las semillas, que fueron esterilizadas superficialmente justo antes de su uso mediante inmersión en hipoclorito de sodio al 5% (v/v) durante 10 min y enjuagadas en agua desionizada. Posteriormente, se sembraron en placas Petri (9 cm de diámetro) con dos capas de papel filtro autoclavado, regadas con 5 ml de las diferentes soluciones de tratamiento (agua desionizada o las soluciones metálicas) y selladas con cinta adhesiva (Parafilm™) para evitar la desecación. Para cada tratamiento se utilizaron tres réplicas de 25 semillas.

Las placas fueron mantenidas en condiciones controladas con un fotoperiodo de 12 horas de luz y 12 horas de oscuridad, con luz proporcionada por lámparas fluorescentes, y a una alternancia de temperaturas de 25ºC/20ºC. La germinación fue monitoreada durante 30 días comprobando cada placa cada dos o tres días. Una semilla se consideró germinada después de emerger la radícula.

Los metales utilizados en estos trabajos (Zn, Cu, Mn y Ni) fueron elegidos en base a estudios previos que describen la presencia de metales en el agua y suelos de estas marismas. Se seleccionaron concentraciones entre 0 y 2000 µM, añadidos como sulfatos, y en el caso de *Salsola vermiculata* se utilizó una concentración de 4000 µM para poder comprobar su capacidad de acumulación en los tejidos.

La dinámica de germinación se analizó tomando el porcentaje de germinación final a los 30 días, el tiempo en que germinó la primera semilla (T_0) y el número de días necesarios para alcanzar el 50% del porcentaje final de germinación (T_{50}) para cada placa.

Desarrollo de las plántulas

Las plántulas se dejaron crecer en la placa durante 15 días después de la germinación y luego se midieron utilizando una lupa. La longitud de los cotiledones, hipocótilos y raíces se utilizó para estudiar los efectos de los diferentes metales y concentraciones en el desarrollo inicial de las plántulas, observando si existían diferencias significativas en el desarrollo de estos órganos frente a las plántulas control, cultivadas en agua. En el caso de *Salsola vermiculata*, para evaluar la tolerancia de las plántulas a los metales, se calculó el índice de tolerancia, aplicado a la longitud de los cotiledones, hipocótilos y radículas (TI% = 100 × (longitud media de órgano en el tratamiento/longitud media de órgano longitud en el control).

Análisis de la acumulación de metales en los tejidos.

Las plántulas de *S. vermiculata*, después haber crecido durante 15 días en los distintos tratamientos, se extrajeron de las placas y se lavaron cuidadosamente con agua ultrapura, se secaron completamente, se pulverizaron con un mortero y se almacenaron en tubos de polipropileno sellado herméticamente a 4°C hasta el momento de su análisis. En el caso de las plantas de *S. ramosissima* recolectadas en el campo, éstas se diseccionaron en el laboratorio y se separaron en raíz, tallos desnudos y tallos con hojas, almacenándose igualmente en tubos de polipropileno herméticamente sellados a 4 ° C para su análisis posterior.

Las muestras se analizan mediante espectrofotómetro de masas (análisis por triplicado) para conocer el contenido de metales en su interior.

Análisis del contenido en metales en el suelo.

En las poblaciones de *Salicornia ramosissima* estudiadas, se tomaron muestras de sedimentos con un anillo de acero de 50 mm de diámetro y de profundidad, en las mismas zonas donde se recolectaron las plantas, y se almacenaron a -20 °C hasta su análisis posterior en el laboratorio.

Para la cuantificación de metales biodisponibles, las muestras fueron pretratadas mediante el protocolo Alan & Kara (2019) y posteriormente se analizaron en espectrofotómetro de masas de plasma.

Cálculo del factor de translocación y bioconcentración.

Las concentraciones de metales pesados registradas se utilizaron para estimar los factores de translocación y bioconcentración. Los factores de translocación (TF) se calcularon dividiendo las concentraciones de metales pesados en las diferentes partes de las plantas: tallos/raíces y hojas/tallos; los factores de bioconcentración (BCF) se calcularon dividiendo las concentraciones de metales pesados en las diferentes partes de las plantas por la concentración del suelo.

Análisis estadístico.

Los análisis estadísticos de los datos se realizaron utilizando los programas Statistica 8.0 y SPSS. Los datos se sometieron a pruebas de normalidad y homogeneidad de la varianza mediante las pruebas de Kolmogórov-Smirnov y Levene, respectivamente. Si los datos se ajustaban a una distribución normal y homogénea se realizaron test de ANOVA de una vía para detectar diferencias significativas entre medias ($p<0,05$). Si los datos no se ajustaban a una distribución normal y/o homogénea, se analizaron utilizando las pruebas U de Kruskal-Wallis y Mann-Whitney para detectar diferencias significativas ($p<0,05$).

Del mismo modo, para los análisis de correlación entre parámetros, hemos utilizado el coeficiente de correlación de Pearson, cuando la serie de medias tenía distribución normal, o de lo contrario, el coeficiente de correlación de Spearman, aplicando la corrección de Bonferroni a cada serie de análisis.

1.8. Discusión de resultados.

I. Efectos sobre la germinación.

En Atriplex halimus, prácticamente todos los porcentajes de germinación observados en los metales testados (Cu, Mn, Ni, Zn) y en todas las concentraciones (hasta 2000 µM) fueron del 100% o cercanos. Además, la germinación fue muy rápida, con valores de T_0 y T_{50} de 1 en todos los tratamientos, estos resultados están de acuerdo con los resultados publicados anteriormente por Muñoz-Rodríguez et al. (2012).

En *Salsola vermiculata*, tras testar el efecto de Cu, Mn, Ni y Zn en concentraciones hasta 4000 µM, sólo se observó reducción en la germinación en 4000 µM para el Cu y Zn, y el tiempo de germinación sólo se vio afectado por un aumento del T_{50} por la exposición a Zn 4000 µM.

En el caso de *Sarcocornia fruticosa* y *S. perennis*, la tasa de germinación y los tiempos de germinación no se vieron afectados por su exposición a Cu, Zn y Ni en concentraciones de hasta 2000 µM.

Por último, en *Salicornia ramosissima* no hubo diferencias en cuanto a la tasa de germinación ante Cu, Mn, o Zn en concentraciones de hasta 2000 µM, pero esta tasa se redujo en todas las concentraciones de Ni, y además no se observaron retrasos en la germinación en las distintas concentraciones de estos metales.

En general, se admite que las altas concentraciones de metales inhiben la germinación (Kranner y Colville, 2011), pero nuestros resultados muestran lo contrario, y están en la línea de diferentes autores que indican que las especies halófitas muestran mayor tolerancia a los metales pesados (Thomas et al., 1998; Van Osten y Maggio, 2015).

II. Efectos sobre las plántulas.

Los efectos sobre las plántulas fueron diversos según el metal, concentración y especie, por lo que se describe a continuación el efecto de cada metal de forma individualizada:

El Cu es un micronutriente esencial que se encuentra en las proteínas y contribuye al transporte de electrones fotosintéticos o la respiración mitocondrial (Marschner, 1995;

Yruela, 2005). Un exceso de Cu reduce la germinación y afecta a raíces y brotes en determinadas especies (Peralta et al., 2001; Singh et al., 2007; Ahsana et al., 2007). En nuestros trabajos hemos observado que el cobre afecta de forma diversa a las diferentes especies. En *Atriplex halimus* obtuvimos un aumento de la longitud de la raíz a 25 µM y una reducción a concentración superiores a 250 µM, mientras que los hipocotilos y cotiledones se vieron reducidos a concentraciones superiores a 1000 µM. En *Salsola vermiculata* afectó al crecimiento de hipocótilos y cotiledones a 4000 µM, y redujo la longitud de la radícula en concentraciones por encima de 250 µM, con una reducción significativa por encima de 1000 µM. La longitud de cotiledones, hipocótilos y radículas de *S. fruticosa* disminuyó gradual y significativamente a concentraciones superiores a 100 µM. Para *S. ramosissima* obtuvimos reducciones significativas de la radícula a concentraciones superiores a 1000 µM, sin observar efectos en cotiledones e hipocótilos. Finalmente, en *Sarcocornia perennis* no se vieron afectados ni cotiledón ni hipocótilo, pero sí se observó una disminución de la radícula en concentraciones superiores a 250 µM.

Según los datos registrados por diferentes autores que estudian el contenido de metales pesados en sedimentos en el estuario de la ría de Huelva citados anteriormente, las concentraciones máximas de este metal en los sedimentos de las marismas del Odiel oscilan entre 500 y 1000 µM, por lo que todas las especies estudiadas podrían verse afectadas a la hora de colonizar determinados lugares con concentraciones elevadas de Cu.

El Mn es importante para el desarrollo de las plantas interviniendo en procesos de oxido-reducción y metabólicos, entre otros. Su toxicidad varía con las especies y se han descrito efectos en la germinación y crecimiento de las plántulas (Marschner, 1995; Kitao et al., 2001; Mumthas et al., 2010). En nuestros trabajos no obtuvimos ningún efecto negativo en las plántulas de las especies estudiadas con ninguna de las concentraciones testadas.

El Ni es esencial para distintas funciones, contribuyendo al metabolismo del nitrógeno y desempeñando un papel funcional y estructural (Marschner, 1995). Sin embargo, el exceso de este metal inhibe la tasa de actividad metabólica y disminuye la absorción de agua y nutrientes (Gajewaska et al., 2006), y se ha descrito que el Ni afecta a la germinación y crecimiento de las plántulas a diferentes concentraciones (Yadav et al., 2009; Peralta et al., 2001; Ahmad & Ashraf, 2011). En nuestros estudios hemos observado que el Ni afecta a la raíz de *A. halimus* a concentraciones superiores a 100 µM, al hipocótilo a partir de 250 µM y al cotiledón a partir de 1000 µM. En el caso de *S. vermiculata*, el níquel reduce los cotiledones a 4000 µM, disminuye los hipocótilos a 2000 µM y afecta las radículas a partir de 2000 µM, provocando una reducción drástica a 4000 µM. En *Sarcocornia fruticosa*, la longitud del cotiledón disminuyó gradualmente en las concentraciones más altas de Ni, mientras que la longitud del hipocótilo y la radícula disminuyeron en concentraciones superiores a 100 µM. En el caso de *S. ramosissima* solo afectó a la raíz a partir de 250 µM. Por último, en *S. perennis* se vieron afectados los cotiledones a concentraciones de 2000 µM, los hipocótilos a concentraciones superiores a 500 µM y la radícula disminuyó en concentraciones superiores a 250 µM.

En el caso del Ni se encontraron máximos de 50 µM en los sedimentos de la zona de estudio, por lo que no se espera que este metal pueda tener efectos adversos en las plántulas en ninguna de las especies estudiadas en las marismas del Odiel.

El Zn provoca la inhibición de la germinación de las semillas, del crecimiento de plantas y del desarrollo radicular (Marschner, 1995; Wang et al., 2009; Lingua et al., 2008). En nuestros estudios hemos observado que el Zn provoca diversos efectos. En *A. halimus* no se vieron afectados en su desarrollo ni el cotiledón ni el hipocótilo, y las raíces mostraron una disminución en concentraciones superiores a 250 µM. En *Salsola vermiculata* tampoco se vio efecto en cotiledones ni hipocótilo, pero la raíz fue significativamente más larga a 50 µM, este efecto positivo también ha sido observado por Peralta et al. (2001) en *Medicago sativa* L., sin embargo, a 4000 µM se observó una reducción de este órgano. En *S. fruticosa*, los cotiledones fueron más cortos en concentraciones superiores a 500 µM, mientras que la reducción del crecimiento de hipocótilo y radícula se registró en concentraciones superiores a 100 µM. *S. ramosissima* solo vio afectadas sus raíces por encima de 1000 µM; y en *S. perennis* se registró una reducción del tamaño de la raíz a 2000 µM.

Las concentraciones máximas de este metal registradas en las marismas del Odiel oscilaron entre 500 µM y 1000 µM, esto determina que algunas de estas especies puedan tener problemas para establecerse en determinados lugares.

III. Acumulación de metales en Salsola vermiculata.

Analizamos el contenido de Cu, Mn, Ni y Zn en *S. vermiculata* cultivada en concentraciones de hasta 4000 µM, con el objetivo de comprobar la capacidad de acumulación de estos metales que tiene la planta sin sufrir daños en sus estructuras, y comprobar si *S. vermiculata* es una candidata para utilizarse en estrategias de biorremediación.

En general, se considera que el Cu puede ser tóxico cuando se acumula en el tejido vegetal en niveles superiores a 20 mg kg^{-1} peso seco (Marschner, 1995), aunque estos valores difieren según las especies y las condiciones de crecimiento. Nuestros resultados muestran que las plantas presentaron los primeros efectos negativos, que se registraron en la radícula, a concentraciones de 250 µM, mientras que las plantas no se vieron afectadas al ser cultivadas en solución 100 µM, acumulando 154 mg kg^{-1} peso seco. Estos resultados coinciden con los de Boularbah et al. (2006) quienes encontraron un contenido de Cu de 69,5 mg kg^{-1} peso seco en sitios mineros en Marruecos, sin presentar síntomas de toxicidad.

La dinámica de acumulación de Cu frente a la concentración del medio de cultivo muestra una ecuación con un alto valor positivo para el componente “a”, lo que significa que la acumulación aumenta exponencialmente con el aumento del metal en el medio, lo cual coincide con las observaciones de Kabatas-Pendias & Pendias (2000). Esta acumulación exponencial podría estar relacionada con daños en las raíces producidos por el metal.

En cuanto al Mn, el contenido normal de este metal varía mucho entre especies (30–500 mg kg^{-1} peso seco) (Clarkson, 1988), así como su umbral de daño. En general, las plantas se ven afectadas negativamente por concentraciones de Mn superiores a 500 ppm, aunque se han descrito especies tolerantes a concentraciones superiores a 1000 ppm (Marschner, 1995). *S. vermiculata* alcanzó niveles de hasta 4676 mg kg^{-1} peso seco, cultivada en soluciones de hasta 4000 µM, sin presentar efectos negativos. Sin embargo, Zhang et al. (2018) informaron de daños en *Suaeda glauca* Bunge en concentraciones a partir de 1000 µM.

La acumulación de Mn en plántulas fue de 4373 mg kg^{-1} peso seco cuando fueron expuestas a 2000 µM, manteniéndose este nivel en concentraciones más altas, alcanzándose el máximo citado anteriormente a 4000 µM. Este comportamiento contrasta con las observaciones de Zhang et al. (2018), quienes encontraron aumentos aritméticos en el contenido de Zn en *Suaeda glauca*. Estos datos nos revelan que *S. vermiculata* podría ser adecuada para acumular este metal.

La concentración de Ni requerida para el crecimiento normal en la mayoría de las plantas es muy baja, varía entre 0,05 y 30 mg Kg^{-1} peso seco según el autor. En la mayoría de las especies estudiadas el Ni en los tejidos es tóxico a concentraciones de entre 10 y 100 mg Kg^{-1} peso seco (Chen et al., 2009; Kabata-Pendias & Pendias, 2010).

En nuestro estudio, *S. vermiculata* alcanzó niveles de 7130 mg kg^{-1} peso seco cuando se cultivó en soluciones de 4000 µM, pero la concentración más alta a la que no se observaron efectos tóxicos en las plántulas fue 1000 µM, acumulándose en las plántulas hasta 1537 mg kg^{-1} peso seco. En la ría del Odiel se ha encontrado este metal en concentraciones inferiores a 50 µM, y su acumulación en las plantas estudiadas osciló entre los 13,0 mg kg^{-1} peso seco en *Salicornia ramosissima* y los 45,7 mg kg^{-1} peso seco en *Spartina maritima* (Curtis) Fernald (Luque et al., 1999).

A los niveles de concentración ensayados, la acumulación de este metal aumenta aritméticamente a medida que aumentaban las concentraciones en el medio, lo cual coincide con Lu et al. (2017), quienes establecieron que las concentraciones de Ni en muchas especies se correlacionan positivamente con las concentraciones en el medio hasta un cierto umbral, posiblemente por la inexistencia de mecanismos de control sobre la absorción del Ni.

Con respecto al Zn, las plantas presentan niveles críticos de toxicidad en sus tejidos de 100 a 500 mg kg^{-1} peso seco (Kabata-Pendias & Pendias, 2010). En nuestro estudio, en *S. vermiculata* el Zn alcanzó acumulaciones máximas de 3990 mg kg^{-1} peso seco cuando se cultivó a 2000 µM, sin mostrar daños en las plántulas a esta concentración, no aumentando cuando se cultivó en concentraciones mayores. Nuestros resultados son consistentes con Boularbah et al. (2006), quienes encontraron un contenido de Zn de

819 mg kg^{-1} peso seco en zonas mineras de Marruecos, sin síntomas de toxicidad. Sin embargo, este comportamiento contrasta con lo establecido por otros autores, quienes determinan que la concentración de Zn (al igual que el Mn) en las plantas es proporcional a su presencia en el suelo (Kabatas-Pendia & Pendias, 2010).

IV. Traslocación y Bioconcentración de metales en S. ramosissima.

Los rangos de concentraciones de As, Cr, Fe, Mn y Ni en las diferentes partes de *S. ramosissima* registrados en las poblaciones estudiadas incluyen las medias obtenidas por Luque et al. (1999) para toda la planta, en la misma especie y en el mismo estuario, pero, por el contrario, los valores de Cu, Pb y Zn fueron mayores a los observados en nuestro estudio. Los valores para Mn, Fe, Ni, Cu, Zn, As y Pb coincidieron con los de Sánchez-Gavilán et al. (2021) para plantas enteras de *Salicornia patula* Duval-Jouve, en una ubicación cercana a la zona de nuestro estudio, así como los resultados obtenidos por Mesa-Marín et al. (2020) para Cd y Ni. Sin embargo, estos autores registraron valores más altos que los nuestros para As, Cu, Pb y Zn.

A nivel de población o hábitat, las plantas procedentes de la marisma media mostraron las concentraciones de metales en raíces más altas para 11 de los 14 metales pesados estudiados.

No encontramos correlaciones significativas en ninguno de los metales estudiados entre la concentración disponible en el suelo y las concentraciones en las raíces, como observaron Sánchez-Gavilán et al. (2021) para las concentraciones de Fe en plantas enteras de *S. patula*.

Esto no es inusual, Greger (1999) ya lo describió estableciendo que la fitodisponibilidad de los metales para una especie vegetal no se correlaciona linealmente con la concentración de metal biodisponible en el suelo o con su concentración total. De este modo, *S. ramosissima* no se consideraría una especie indicadora, según lo definido por este autor, ya que en éstas las concentraciones en los tejidos reflejan las concentraciones del metal en el medio. De esta forma, parece que la absorción de metales por parte de *S. ramosissima* podría estar afectada por diferentes factores como las propiedades del suelo, la salinidad, la exclusión de la absorción de iones metálicos a nivel radicular, la saturación en los tejidos radiculares a altos niveles de concentración en el suelo (Greger, 1999; Nikalje & Suprasanna, 2018; Ali & Khan., 2019), la tasa de transpiración (Tani & Barrington, 2005) o las interacciones entre metales de cara a su absorción (Agrawal et al., 2007), además, como indica Greger (1999),es posible que hubiera diferencias en el mecanismo de absorción de metales entre poblaciones de la misma especie debido a las diferencias genotípicas entre ellas.

Un alto valor del factor de bioconcentración (BCF) indica un transporte eficiente de metales desde el suelo hasta la raíz, mientras que un valor alto del factor de translocación (TF) indica una translocación eficiente de la raíz a la parte aérea (McGrath & Zhao, 2003; Van der Ent et al., 2013; Ali & Khan, 2019).

En las raíces, nuestros resultados indican que el Cr, Mn, Co, Ni, Cu, Zn, As y Cd presentaron un BCF inferior a 1 en la mayoría de sus poblaciones, por lo tanto, *S. ramosissima* podría considerarse un excluyente de estos metales (Baker, 1981; Nikalje & Suprasanna, 2018). Por el contrario, para V, Tl, Pb y U, los valores de BCF oscilaron entre 1 y 3, y la mayoría de sus poblaciones tenían valores superiores a 1, lo que indica una acumulación de estos metales. Estos resultados coinciden con los observados por De la Fuente et al. (2010) y Sánchez-Gavilan et al. (2021) para *S. patula*, y los obtenidos por Khalilzadeh et al. (2021) para *S. europea.* Los valores de BCF en las raíces superaron 1 en todas las poblaciones para Al y Fe. Parece que *S. ramosissima* podría acumular estos metales en la raíz en concentraciones superiores a las del suelo, lo que indica que debe haber mecanismos que favorezcan su absorción.

El BCF en tallos y hojas fue menor que 1 para V, Cr, Mn, Co, Ni, Cu, Zn, As, Cd, Pb y U, lo que coincide con los resultados obtenidos por Sánchez-Martínez et al. (2017) en *Arthrocnemum subterminale* en presencia de Zn, Cu, Cd, Pb y As. Para Al, Fe y Tl, los valores de BCF en tallos y hojas fueron cercanos a 1, superando el valor 1 para los tallos en la mayoría de las poblaciones, mientras que para las hojas se registraron valores inferiores a 1 en la mayoría de las poblaciones, coincidiendo con Sánchez-Gavilán et al. (2021) y Fuente et al. (2010) para *S. patula.* Por lo tanto, podemos suponer que *S. ramosissima* no es una planta acumuladora en términos de la descripción de Nikalje y Suprasanna (2018), ni por lo indicado por Baker (1994), Watanabe (1997), Kamal et al. (2004) y Reeves et al. (2018)

Como se esperaba, en vista de los resultados de BCF, los valores de TF fueron inferiores a 1 o cercanos a 1 para todos los metales estudiados en el transporte raíz-tallo y tallo-hoja, lo que indica una tendencia a reducir la concentración de metales de raíces a tallos y de tallos a hojas, lo cual es un comportamiento común, ya que durante su transporte a través de la planta los metales se unen en gran medida a las paredes celulares (Greger, 1999).

V. Evaluación del riesgo alimentario para la salud humana.

Los resultados obtenidos determinan que no se consuman las plantas de *S. ramosissima* que crecen en la mayoría de los suelos de este estuario, pues en 3 de las 14 poblaciones estudiadas las concentraciones de Cd en la hoja superaron el límite de contenido de metales pesados para hortalizas de hoja, 11 poblaciones superaron el límite de Pb en la hoja, y algunas poblaciones excedieron el límite para As (European Union Commission Regulation 2021/1323; European Union Commission Regulation 2021/1317).

En el caso del Cd, se ha encontrado en bajas concentraciones en el suelo y en las diferentes partes de la planta, con valores de BCF y TF inferiores a 1, lo que indica que la planta no acumula este metal. Además, el resultado del Índice de riesgo para la salud (HRI) para Cd muestra que no existiría ningún riesgo potencial por la ingesta de esta planta como alimento para el ser humano. Esta discrepancia entre el límite establecido en el contenido de alimentos y el riesgo calculado por su ingestión también ha sido observada por Chabchoubi et al. (2021) en *Sarcocornia fruticosa.* Pero este metal había sido clasificado como cancerígeno en humanos por la Agencia Internacional para la Investigación del Cáncer, y tiene numerosos efectos tóxicos (Agencia Española de Seguridad Alimentaria y Nutrición, 2021) por lo que los límites máximos en verduras comestibles son bastante bajos. Algo similar ocurre con el Pb, el cual se acumula en la hoja en la mayoría de las poblaciones estudiadas por encima del límite establecido por la normativa europea, aunque su Índice de riesgo para la salud (HRI) es inferior a 1 en todas ellas.

Las concentraciones de Tl en el suelo fueron bajas, lo que lo convierte en el segundo metal pesado más escaso en términos de concentración, pero presentó un valor de BCF en la hoja superior a 1 en 9 de las poblaciones estudiadas, lo que lo convierte en el tercer metal con mayor BCF en la hoja, por detrás de Al y Fe, por lo que su concentración en las hojas era mayor que para otros metales que eran más abundantes en el suelo.

Al y Fe fueron los metales pesados con mayores niveles de concentración en las hojas de *S. ramosissima*, a pesar de no ser los metales más abundantes en el suelo, y mostraron los valores más altos de BCF en raíces, tallos y hojas. El Fe es un elemento esencial en la nutrición humana y, al igual que ocurre con el Al, hay pocos indicios de que sea tóxico para los humanos cuando se ingiere por vía oral. No obstante, algunos medios plantean que el Al es un factor de riesgo para el desarrollo o la aceleración de la enfermedad de Alzheimer en humanos (World Health Organization, 2017). En ambos metales el HRI muestra bajo riesgo para la ingestión de las plantas procedentes de la zona de estudio.

1.9. Conclusiones

En concentraciones hasta 2000 µM de Mn, Cu, Zn y Ni no se observaron efectos sobre la tasa de germinación en *Atriplex halimus*, *Salsola vermiculata*, *Sarcocornia fruticosa* y *Sarcocornia perennis*, mientras que en el caso de *Salicornia ramosissima* la germinación se redujo ante la presencia de Ni. Cu y Zn redujeron la tasa de germinación en *Salsola vermiculata* en concentraciones de 4000 µM.

El tiempo medio de germinación (T_{50}) no se vio afectado a las concentraciones usadas de los metales usados en este estudio en *Atriplex halimus*, *Sarcocornia fruticosa*, *Sarcocornia perennis* y *Salicornia ramosissima*, pero sí se observó incidencia en *Salsola vermiculata* expuesta a concentraciones de Zn de 4000 µM.

En cuanto a los efectos sobre las plántulas:

- El Mn no produjo efectos negativos en ningún caso.
- El Cu y el Ni redujeron el crecimiento de la raíz, el hipocótilo y los cotiledones, observándose el efecto sobre la raíz a concentraciones más bajas. La especie más sensible fue *Sarcocornia fruticosa* y las más resistentes fueron *Salicornia ramosissima* y *Salsola vermiculata*.
- El Zn sólo afectó a la radícula, excepto en *Sarcocornia fruticosa* en la que afectó también a los cotiledones. La especie más sensible fue *Sarcocornia fruticosa* y la más resistente fue *Salsola vermiculata*, en la que incluso se observó un efecto positivo en la raíz a bajas concentraciones.
- La raíz es el órgano donde primero se observan los efectos de los metales en las plántulas.

En *Salsola vermiculata* se observaron los siguientes comportamientos de acumulación:

- La acumulación de Cu en los tejidos aumenta exponencialmente con el aumento del metal en el medio, y las plantas llegan a acumular 154 mg kg^{-1} peso seco sin presentar daños.

- La acumulación de Mn aumenta aritméticamente hasta los 2000 µM en el medio y a partir de esta concentración se estabiliza. Se acumularon niveles de Mn de hasta 4676 mg kg^{-1} peso seco sin presentar las plantas efectos negativos.
- La acumulación de Ni en los tejidos aumenta aritméticamente con el aumento del metal en el medio, y las plantas llegan a acumular 1537 mg kg^{-1} peso seco sin presentar daños.
- La acumulación de Zn aumenta aritméticamente hasta los 2000 µM en el medio, estabilizándose a partir de esta concentración. Se acumularon niveles de Zn de hasta 3990 mg kg^{-1} peso seco sin presentar las plantas efectos negativos.

Por ello se propone a *Salsola vermiculata* como una buena candidata para proyectos de fitorremediación de suelos contaminados principalmente por Mn y Zn.

En cuanto al contenido en metales pesados en plantas de *Salicornia ramosissima*, se observó una gran heterogeneidad en las concentraciones de metales tanto en los suelos como en diferentes partes de la planta en las 14 ubicaciones estudiadas, y no se observaron correlaciones significativas entre la concentración de metales en las diferentes partes de la planta y la concentración de metales en el suelo.

Las raíces de *S. ramosissima* parecen excluir la absorción de Cr, Mn, Co, Ni, Cu, Zn, As y Cd, pero actúa como acumulador de Al y Fe.

Con respecto a las plantas de *S. ramosissima* procedentes de poblaciones de la ría de Huelva, se desaconseja su consumo ya que en muchos casos se superan los límites establecidos en la normativa vigente respecto a las concentraciones de Cd, Pb y Tl en hojas.

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CAPITULO 2: Artículos publicados.

1. *The effects of heavy metals on germination and seedling characteristics in two halophyte species in Mediterranean marshes.*

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Abstract

The influence of different concentrations (10–2000 µM) of heavy metals (Cu, Mn, Ni, Zn) was analysed in *Atriplex halimus* and *Salicornia ramosissima* germination pattern and seedling size. They are two halophyte species that grow in the Estuary of Huelva (Southwest Iberian Peninsula, Spain), one of the most heavy metal-polluted environments in the world.

All of the metals tested affected the final germination percentage in *A. halimus* and only Ni reduced germination in *S. ramosissima*. The germination rate was unaffected in both species.

The study of seedling development shows that *S. ramosissima*, an intertidal annual species, has a higher tolerance of metals than *A. halimus*, a bush that inhabits the upper part of the marshes.

Taking into account the metal concentrations in the estuary and the effects of these on the seedling development of the species analysed, we conclude that metals might limit plant colonization in some parts of the marshes.

1.1. Introduction

The Estuary of Huelva (SW Spain) is located in the southwest of the Iberian Peninsula and is formed by the convergence of the rivers Tinto and Odiel. In view of its ecological importance, it is protected by law in Andalusia as a Natural Park and is internationally recognized as a Biosphere Reserve by UNESCO. The estuary is characterized as being one of the most heavy metal-polluted systems in the world (Nelson and Lamothe, 1993; Sainz et al., 2004). The heavy metals are the result of the industrial activity that takes place on the banks of the estuary itself and up-stream where there are mines belonging to the Iberian Pyrite Belt (IPB), an important metal-rich sulphide deposit. This latter source is responsible for more than 99% of the total metal content in the estuary (Pérez-López et al., 2011).

The metals dissolved in the estuary precipitate as the salinity increases, resulting in some of these metals being trapped in the sediments while others are more mobile and remain in the solution, such as Cu, Mn and Zn (van Geen et al., 1997; Elbaz-Poulichet et al., 2001; Achterberg et al., 2003; Braungardt et al., 2003; López-González et al., 2006). Studies that have analysed the heavy metals in the estuary (Achterberg et al., 2003; Borrego et al., 2002; Braun-gardt et al., 2003; Elbaz-Poulichet et al., 1999, 2001; Caliani et al., 1997; Galán et al., 2003; González-Pérez et al., 2008) refer to a classification according to maximum concentrations registered in solution: >1000 μM (Fe, Al); >500 μM (Zn, Cu); 50–500 μM (Mn); <50 μM (As, Co, Pb, Ni, Cd, Hg). The metals groups in the estuary sediments are: Fe2O3 up to 32%, Al2O3 up to 18%, MnO up to 0.3%, >1000 ppm (Zn, Pb, Cu, Ba, As), <200 ppm (Cr, Ni, Cd, Hg).

The consequences of heavy metals in the marshes of Huelva could have possible adverse effects on the wildlife there as the heavy metals accumulate in halophyte species tissues that grow in the soils (Stenner and Nicless, 1975), with the species in the lower part of the marshes generally showing higher levels of metals than those in the higher marsh areas. The metal accumulation in order of mean metal weight in the halophytes studied is Fe > Zn > Mn > - Cu > Ti > Pb > As > Ni > Cr (Luque et al., 1999). Despite the numerous studies that have analysed the heavy metal composition in the Estuary of Huelva, there is no information on the effects of the metals on the development of these species, yet neither are there studies that reveal any negative effects; for example, re-search by Fernández-Illescas et al. (2010a) found no link between heavy metal pollution and the high abortion rates in the pollen of some of these marsh species. However Curado et al. (2010) using soils collected at five different sites distributed along the Tinto River channel, from its mouth to 28.9 km landward, found that final germination of seeds of the alien species Spartina densiflora decreased linearly with concentrations of Cr and Al, and germination was slowest in the most polluted sediments, and the observed that aerial and subterranean growth rate of seedlings decreased in more acidic sediments highly polluted with heavy metals.

Seed germination is the first step of the life of a plant and it is one of the most sensitive physiological process in plants, affected by hormonal interactions and environmental factors, both biotic and abiotic (Ahsana et al., 2007; Moosavi et al., 2012), and therefore to the presence of excess of metals. The effects of metals on the germination process rely on their ability to reach embryonic tissues across the seed coats, a physiological barrier, and in the physical and chemical properties of the metal ions themselves (Ko et al., 2012 and references therein). Different plant species possess different seed's coat anatomy and structure (Munzuroglu and Geckil, 2002) and therefore the same concentration of metal can have different effect in different species. In addition, some metals like Cu and Cd has been described to inhibit the uptake of water and therefore the germination does not occur (Kranner and Colville, 2011). The most widely used test for metal toxicity is the seed germination test (Kapustka and Reporter, 1993; Munzuroglu and Geckil, 2002). After permeation through the seed coat, the germination relies on the seed reserves for the supply of metabolites for respiration and metals can cause oxidative stress and disrupt the process, likewise interfere with the enzymes involved in the germination process (Ko et al., 2012 and references therein; Van Assche and Clijsters, 1990).

The present work analyzes and compares the effects of heavy metals on the germination and initial seedling development of two common species in the Mediterranean marshes, *Atriplex halimus* L. and *Salicornia ramosissima* J. Woods (Davy et al., 2001; Fernández-Illescas et al., 2010b). These two halophyte species survive in highly polluted soils but live in different habitats: *A. halimus* is a dominant halo-nitrophylum bush species that grows on emerged land from the marshes where salinity concentration is high; *S. ramosissima* is an annual species that colonises bare ground in intertidal communities. Both species are exposed to the effects of heavy metals in the Estuary marshes but in different ways: *A. halimus* is exposed to the metals that remain in the soils while *S. ramosissima* is exposed to the metals in the marsh mud and in the water (Fernández-Illescas et al., 2010b). The importance of the present study is that it has a focus on the possible use of *A. halimus* in marsh ecosystem restoration, which has been described as a crucial species for the rehabilitation of soils affected by excessive salinity and low moisture (Abbad et al., 2004). *S. ramosissima* is a tidal herbaceous species that plays an important role 50eionint recycling in estuaries (figueroa et al., 1987).

1.2. Materials and methods

I. Plant material

Seeds were collected in the Odiel Marsh Natural Park from 10 individuals then mixed and stored in paper bags at room temperature until use. *A. halimus* seeds were collected from Bacuta Island (37º13´48´´N 6º57´53´´W) in September 2010, and *S. ramosissima* seeds were collected from a low marsh located at the margin of Acebuchal Island (37º12´16´´N 6º57´04´´W) in October 2011. The seeds were surface sterilized just before use by immersion in 5% $^{(v/v)}$ sodium hypochlorite for 10 min and rinsed three times in sterile water, a treatment that does not affect germination parameters or seedling characteristics (Muñoz-Rodríguez et al., 2012).

II. Experimental conditions and germination assays

Germination tests were carried out in petri dishes (9 cm diameter) with two layers of autoclaved filter paper, watered with 5 mL of the different treatment solutions (51eionized water or the metal solutions) and sealed with adhesive tape (Parafilm™) to avoid desiccation. Three replicates of 25 seeds were used for each treatment. A seed was considered germinated after radicle emergence.

The seeds were germinated under controlled-environment conditions with 12/12h of day/night at 24/20 LC, respectively, that have been proved to be appropriate conditions to enhance high levels of germination in *A. halimus* (Muñoz-Rodríguez et al., 2012). The light was provided by fluorescent lamps that produce a photosynthetic photon flux density of 60 μmol m^2 s^1. Germination was monitored for 30 days with the germination in each plate recorded every 2 or 3 days (Keiffer and Ungar, 1997).

The metals used in this experiment were chosen on the basis of previous studies that describe the metal composition in the water and soils of the marshes where *A. halimus* and *S. ramosissima* grow (Achterberg et al., 2003; Borrego et al., 2002; Braungardt et al., 2003; Elbaz-Poulichet et al., 1999, 2001; Caliani et al., 1997; Galán et al., 2003; González-Pérez et al., 2008). Iron is the most abundant metal found in both solution and soils, but it was not included in the study, firstly because it is an essential element and secondly because it has been described as beneficial for plants that grow in heavy metal-polluted soils, as is the case of Erica andevalensis (Márquez-García et al., 2009). The following metals, Zn, Cu and Mn, abound in the marshes and are included in the present study. Ni was also selected due to its presence in the area and the little information available on its effects on plants.

Seeds from both species were sown in different concentrations (10, 25, 50, 100, 250, 1000 and 2000 μM) of these metals, added as the sulphates $CuSO_4 5H2O$, $MnSO_4 H2O$, $NiSO_4 6H2O$ and $ZnSO_4 7H2O$, and dissolved in 51eionized water. The chemical form of the metals added, the sulphate, was determined according to the most abundant form in the environment the plant usually inhabits (Barba-Brioso et al., 2010 and references therein). The use of sulphate was also justified by the negative effects that chloride might have on the germination process and seedling development (León et al., 2005). The concentrations applied were determined by the levels found (in water or soil) in the area where the seeds were collected. A control treatment with 52eionized water was used for each species.

The germination dynamic was analysed by taking the final germination percentage after 30 days, the time for the first seed germinated (T_0) and the number of days necessary to reach 50% of the final germination percentage (T_{50}) for each plate.

III. Seedling measurements

Seedlings were let to growth in the plate for 15 days after germination, and then they were measured under the magnified glass using a ruler. The length of the cotyledons, hypocotyls and roots was used to study the effects of the different metals and concentrations on initial seedling development.

IV. Statistical analysis

The statistical analyses of the data were performed using SPSS. For statistical analysis data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov test and Levene's test, respectively. The germination rate pattern and the size of the seedlings were analysed by the one-way ANOVA technique (due to the normality and homogeneity of the data), while the T_0 and T_{50} were analysed by non-parametric tests (Kruskal Wallis and Mann Whitney U tests) due to the no normality of the data.

1.3. Results

I. Effects of metals on seed germination

A. halimus germination was not affected by any of the concentrations of the metals tested (Cu, Mn, Ni or Zn), with final germination percentages equal or very close to 100% together with non-significant differences compared to the control plants (p>0.05) (Table 1). Neither did the metals affect the germination rate of this species, which was fast with T0 and T50 values equal to 1 day for all the treatments (Table 1).

Treatment	Concentration (mM)	Atriplex halimus			Salicornia ramosissima		
		Final germination (%)	T_0	T_{50}	Final germination (%)	T_0	T_{50}
Control	0	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	81.6 ± 6.8 a	1.0 ± 0.0 a	3.3 ± 1.4 a
$CuSO_45H_2O$	10	97.3± 1.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	70.1 ± 15.0 a	1.0 ± 0.0 a	4.5 ± 1.8 a
	25	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	77.3 ± 8.3 a	1.5 ± 0.9 ab	4.7 ± 0.6 a
	50	98.6± 1.4 a	1.0 ± 0.0 a	1.0 ± 0.0 a	68.0 ± 0.0 a	2.0 ± 0.9 ab	3.3 ± 1.4 a
	100	98.7± 1.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	73.4 ± 6.0 a	2.0 ± 0.9 ab	3.3 ± 1.4 a
	250	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	73.3 ± 8.3 a	2.5 ± 0.0 b	3.8 ± 1.3 a
	1000	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	64.4 ± 2.1 a	2.0 ± 0.9 ab	5.7 ± 1.2 a
	2000	98.7± 1.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	77.3 ± 0.6 a	1.5 ± 0.9 ab	4.8 ± 2.3 a
$MnSO_4H_2O$	10	98.6± 1.4 a	1.0 ± 0.0 a	1.0 ± 0.0 a	64.3 ± 17.0 a	1.0 ± 0.0 a	4.0 ± 2.6 a
	25	97.3± 1.4 a	1.0 ± 0.0 a	1.0 ± 0.0 a	75.3 ± 12.5 a	2.0 ± 0.9 ab	4.2 ± 1.4 a
	50	98.7± 1.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	75.9 ± 14.2 a	1.5 ± 0.9 ab	4.2 ± 1.4 a
	100	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	73.9 ± 6.7 a	2.0 ± 0.9 ab	4.7 ± 0.6 a
	250	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	62.7 ± 4.6 a	2.5 ± 0.0 b	3.3 ± 1.4 a
	1000	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	67.1 ± 10.7 a	2.0 ± 0.9 ab	3.3 ± 1.4 a
	2000	98.7± 1.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	66.2 ± 5.1 a	2.0 ± 0.9 ab	3.0 ± 0.9 a
$NiSO_46H_2O$	10	98.7± 1.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	58.0 ± 12.2 b	1.5 ± 0.9 ab	3.3 ± 1.4 a
	25	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	56.8 ± 7.8 b	1.5 ± 0.9 ab	4.0 ± 2.6 a
	50	98.6± 1.4 a	1.0 ± 0.0 a	1.0 ± 0.0 a	61.5 ± 11.0 b	2.0 ± 0.9 ab	4.8 ± 2.3 a
	100	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	55.6 ± 13.8 b	2.5 ± 0.0 b	5.7 ± 1.2 a
	250	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	54.5 ± 12.9 b	1.5 ± 0.9 ab	2.5 ± 0.0 a
	1000	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	45.8 ± 7.5 b	2.5 ± 0.0 b	3.3 ± 1.4 a
	2000	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	53.3 ± 9.2 b	2.0 ± 0.9 ab	4.2 ± 1.4 a
$ZnSO_47H_2O$	10	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	69.3 ± 11.5 a	2.0 ± 0.9 ab	3.3 ± 1.4 a
	25	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	66.7 ± 15.1 a	2.5 ± 0.0 b	4.7 ± 0.6 a
	50	98.7± 1.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	64.6 ± 16.4 a	1.5 ± 0.9 ab	4.2 ± 1.4 a
	100	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	69.3 ± 11.8 a	1.0 ± 0.0 a	4.2 ± 1.4 a
	250	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	79.4 ± 4.1 a	1.0 ± 0.0 a	3.3 ± 1.4 a
	1000	100.0± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	68.5 ± 6.3 a	2.5 ± 0.0 b	5.7 ± 3.5 a
	2000	97.3± 1.3 a	1.0 ± 0.0 a	1.0 ± 0.0 a	62.7 ± 10.1 a	2.5 ± 0.0 b	4.2 ± 1.4 a

Table 1: Final germination percentage after 30 days, T_0 and T_{50} of *Atriplex halimus* and *Salicornia ramosissima* seeds sown in different concentrations of $CuSO_45H_2O$, $MnSO_4H_2O$, $NiSO_46H_2O$ and $ZnSO_47H_2O$. The data show mean ± SD of three different replicates (n = 3). Different letters indicate significant differences between each metal and the control for each species, following Duncan post hoc test ($p < 0.05$) for final germination percentages and Kruskal Wallis and Mann Whitney U tests ($p < 0.05$) for the T_0 and T_{50} values.

S. ramosissima reached a final germination of 81.6% under control conditions (Table 1), similar to that obtained by Rubio-Casal et al. (2003) with seeds collected from Odiel salt marshes. The final germination percentages in Cu, Mn and Zn showed no significant differences compared to the control (p>0.05) (Table 1). However, the presence of Ni, with the exception of 50 µM, significantly reduced the germination levels compared to the control (Table 1). The germination rate of S. ramosissima, expressed as T_{50} was not affected in any of the treatments applied, but delays were observed in the initial germination (T_0) for Mn, Ni and Zn (Table 1).

II. Seedling measures

The metals tested affected the cotyledons, hypocotyls and roots of both species in different ways.

In *A. halimus* Cu reduced the size of the cotyledons and hypocotyls at concentrations of 1000 and 2000 µM compared to the control plants; it also produced longer roots at 25 µM, and stopped root growth at concentrations over 250 µM (Fig. 1A). Mn did not have any effect on the length of the cotyledons and hypocotyls at any concentration, but had a positive effect on root length at 10 µM; at concentrations of 250 and 2000 µM this length was significantly reduced (Fig. 1B). Ni had a significant positive effect on cotyledon size at 10 µM, and reduced cotyledon size at concentrations above 1000 µM; hypocotyls were not affected at concentrations below 100 µM but their length was significantly and progressively reduced at concentrations over 250 µM, and the growth of the roots was significantly affected at concentrations starting at 100 µM (Fig. 1C). Zn only reduced the size of the cotyledons compared to the control at 2000 µM, but the hypocotyl length was reduced at 250 and 2000 µM, and the roots were reduced drastically from 250 µM (Fig. 1D).

In *S. ramosissima* Cu had no effect on the size of the cotyledons while the length of the hypocotyls was reduced at concentrations over 25 µM (25, 50, 100 and 2000 µM), and from 1000 lM up-wards the root length was significantly reduced (Fig. 2A). Mn had no effect on cotyledon size, with a significant reduction in the length of the hypocotyls only occurring at 50 µM; and only at 10 µM was the root length significantly and positively affected (Fig. 2B). Ni reduced cotyledons as from 1000 µM, the length of the hypocotyls seemed to be affected from 25 µM, and was drastically reduced from 1000 µM. Ni had a positive effect on the root growth at 10 µM but the effect became negative at concentrations over 100 µM; (Fig. 2C). Zn did not have any effect on the cotyledons and hypocotyls, but reduced the root length at the highest concentrations (1000 and 2000 µM) (Fig. 2D).

1.4. Discussion

We have observed that under control conditions both species reached high levels of germination, and in the case of *A. halimus* the results obtained here are in agreement with the data previously published by Muñoz-Rodríguez et al. (2012) with seeds from the same location and in the same conditions.

The metals used in the present study (Cu, Mn, Ni and Zn) are essential for plants (Kranner and Colville, 2011) but are also toxic when found in high concentrations.

Cu is an essential micronutrient found in proteins, and contributes to photosynthetic electron transport, mitochondrial respiration, oxidative stress response, cell wall metabolism and hormone signalling (Marschner, 1995; Yruela, 2005). When exceeding optimal concentration, it causes growth inhibition and dysfunctions in photosynthesis and respiration (Marschner, 1995; Yruela, 2005). Its toxic effect mainly felt in the root tissue where it accumulates with little translocation to the shoots (Marschner, 1995; Sheldon and Menzies, 2005). Cu has been reported to reduce the germination in alfalfa at concentrations higher than 300 mM (Peralta et al., 2001), in wheat at concentrations over 80 mM (Singh et al., 2007) or rice at levels over 1000 mM (Ahsana et al., 2007). However, in our study no effect was found on the germination. In addition, negative effects on roots have been described in alfalfa and wheat at levels higher than 80 mM (Peralta et al., 2001; Singh et al., 2007) and in shoots at concentrations above 200 mM in rice (Ahsana et al., 2007). In the present study Cu affected negatively the roots of both species *A. halimus* and S. ramosissima, at levels of 250 and 1000 μM respectively. The maximum concentrations of this metal registered in the Estuary of Huelva range from 500 to 1000 μM, indicating that it can reduce the development of *A. halimus* plantlets anywhere and can affect the establishment of *S. ramosissima* by stopping root growth in certain locations.

Mn plays an important role in the redox processes, activates a large number of enzymes, forms part of the superoxide dismutase (MnSOD) and manganese-protein in photosystem II (PSII) and is in-volved in lipid metabolism (Marschner, 1995). Its toxicity varies among species since manganese induces deficiency in other mineral nutrients such as iron, magnesium or calcium (Marschner, 1995; Kitao et al., 2001). Different effects have been described about the effect of Mn in seed germination. No effect of Mn has been described on the germination of Lolium perenne at concentrations up to 200 μM, but the roots and the shoots are affected from 13 and 27 μM respectively (Wong and Bradshaw, 1982), and germination is reduced at concentrations of 200 μM and roots are affected at levels of 100 μM in Vigna radiata (Mumthas et al., 2010). In *A. halimus* we have observed the slight influence of this element on the root length, while in *S. ramosissima* beneficial effects have only been noted at low concentrations. As this metal is present in the Estuary of Huelva at maximum concentrations between 50 and 500 μM, presumably it would have no effect on the establishment of the studied species throughout this estuary.

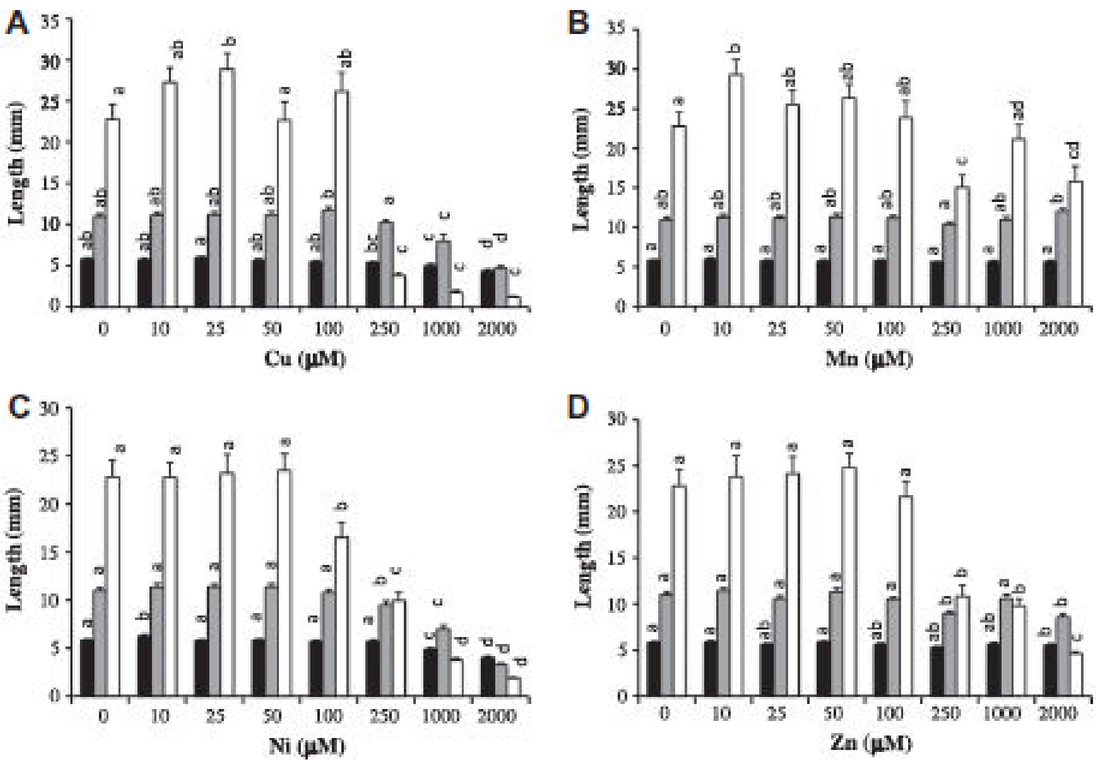

Fig. 1. Length of the cotyledon (black bar), hypocotyl (grey bar) and root (white bar) in *Atriplex halimus* exposed to Cu (A), Mn (B), Ni (C) and Zn (D). The bars shown mean ± SE (n=30, from 3 independent replicates). Different letters indicate significant differences among the concentrations by the Duncan post hoc test ($p < 0.05$).

Ni is contained in enzymes such as urease, where the metal is essential for its structural and catalytic functions; it contributes to nitrogen metabolism and performs a functional and structural role (Marschner, 1995). The excess of this metal inhibits the rate of metabolic activity and decreases water and nutrient uptake (Gajewaska et al., 2006). Ni has been described as beneficial to seed germination at very low concentrations (Homer et al., 1991; Peral-ta et al., 2001), which might explain the positive effects observed on both species in our study, but largely toxic effect has been observed. The toxicity of this metal has been observed in germination at concentrations over 30 μM in Lolium perenne (Wong and Bradshaw, 1982), 100 mM in radish (Yadav et al., 2009) and 300 mM in alfalfa (Peralta et al., 2001). In addition, roots and shoots are also affected in different extent, e.g. from 30 μM in Lolium perenne (Wong and Bradshaw, 1982), 100 mM in the case of radish roots (Yadav et al., 2009) or 700 mM published for alfalfa (Peralta et al., 2001). Ni is toxic for *S. ramosissima* as the germination is inhibited even at the lower concentrations tested, results that are in accordance with studies previously commented. The main effect of this metal in the present study is found in the roots, with length reductions in both species at concentrations above 100 μM. In the Estuary of Huelva this metal has been found in concentrations below 50 μM, so a reduction in the germination of *S. ramosissima* is only to be expected in places with high concentrations of this metal.

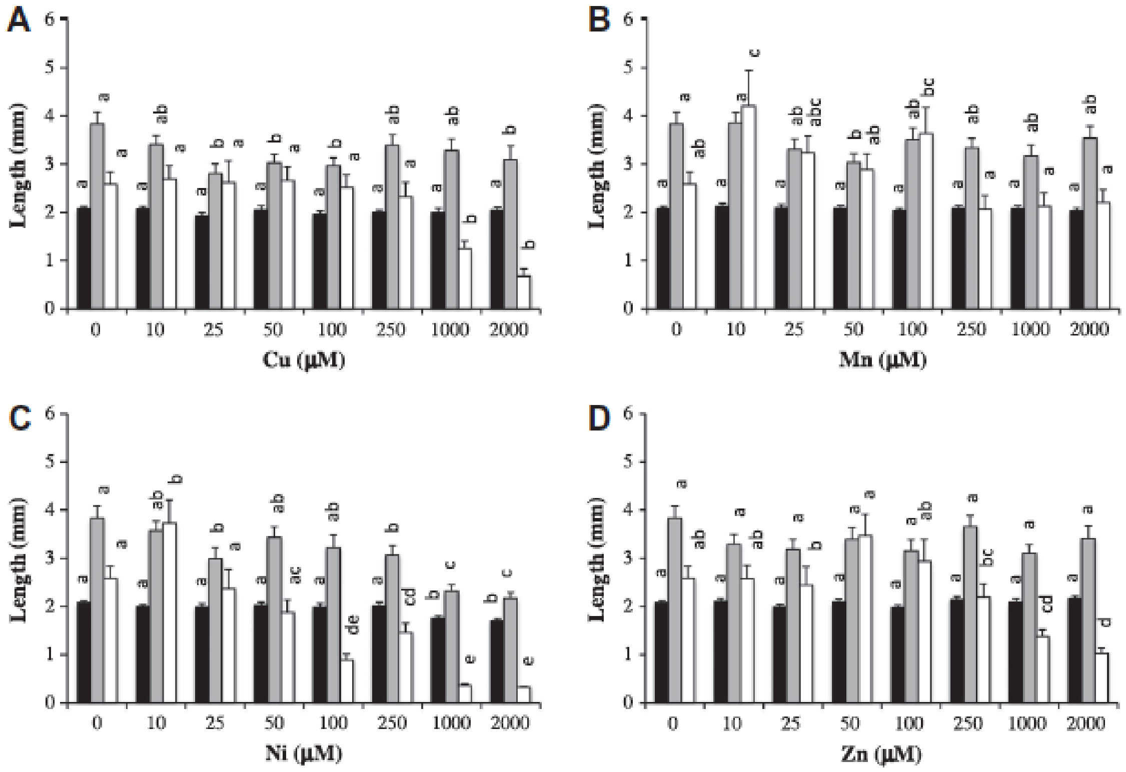

Fig. 2. Length of the cotyledon (black bar), hypocotyl (grey bar) and root (white bar) in *Salicornia ramosissima* exposed to Cu (A), Mn (B), Ni (C) and Zn (D). The bars shown mean ± SE (n = 30, from 3 independent replicates). Different letters indicate significant differences among the concentrations by the Duncan post hoc test ($p < 0.05$).

The excess of Zn causes chlorosis in young leaves and can inhibit photosynthesis (Marschner, 1995), and affect the uptake of other nutrients (Wang et al., 2009), inhibiting seed germination, plant growth (Mrozek and Funicelli, 1982) and root development (Lingua et al., 2008). Studies carried out with Bituminaria bituminosa did not find any effect of Zn in final germination or germination speed using concentrations from 1.5 to 150 mM (Martínez-Fernández et al., 2011) or on Lolium perenne at 450 µM (Wong and Brad-shaw, 1982). We have also registered no effect of this metal on the germination rates of any of the species studied. Regarding the seedling growth, no effect was detected in alfalfa at concentrations up to 600 mM, with a positive effect reflected in the increase of the length of the roots (Peralta et al., 2001), and no effect has been de-scribed for this metal in *Bituminaria bituminosa* (Martínez-Fernández et al., 2011). In our study, the roots of *A. halimus* and *S. ramosissima* seedlings are affected at concentrations over 250 µM and 1000 µM respectively. The maximum concentrations of this metal recorded in the Estuary of Huelva range from 500 µM to 1000 µM so it can affect the establishment of *A. halimus*.

Roots are the primary targets of metal anions and their growth is usually more severely affected than that of the aerial parts, hence root testing is widely used for evaluating the toxicity levels of toxicants, including heavy metals (Yusuf et al., 2011). This is confirmed by the results presented here, as the roots were affected before the hypocotyls or cotyledons in all the metals tested on the two species.

Our results clearly show how these species are able to tolerate the presence of metals and germinate without problem. However, when the levels of these metals are too high seedling development is compromised. We have observed that seedling of *S. ramosissima* are negatively affected at higher concentrations of Cu, Mn and Zn than *A. halimus*. *S. ramosissima* is found in the lower levels of the marshes where the influence of the tides makes for variable conditions in the soils. Plants growing in these soils should have a wider range of tolerance compared with species growing in soils with more stable conditions, as is the case of *A. halimus*, where the metals are accumulated in the soil and the salinity levels are higher.

The marshes are a highly heterogeneous environment concerning the heavy metal distribution. The levels of metals in some locations can be higher than the viable levels for germination and therefore the colonisation and establishment of populations of some species can be limited. However, it would be very interesting to test if the results obtained in the present work regarding the effect of metals on germination and initial seedling growth are also found in the natural conditions, and to analyse if there are variability among populations regarding metal tolerance, as in some cases, genetic differences have been described between individuals of the same species, expressed both in morphological and biochemical/ physiological characteristics (Adam, 1990). Future studies carried out in the field or under greenhouse conditions will explore these aspects.

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1.5. References

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2. Accumulation and Effect of Heavy Metals on the Germination and Growth of *Salsola vermiculata* L. Seedlings.

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Abstract: The influence of different concentrations of heavy metals (Cu, Mn, Ni, Zn) was analyzed in the *Salsola vermiculata* germination pattern, seedling development, and accumulation in seedlings. The responses to different metals were dissimilar. Germination was only significantly reduced at Cu and Zn 4000 µM but Zn induced radicle growth at lower concentrations. Without damage, the species acted as a good accumulator and tolerant for Mn, Ni, and Cu. In seedlings, accumulation increased following two patterns: Mn and Ni, induced an arithmetic increase in content in tissue, to the point where the content reached a maximum; with Cu and Ni, the pattern was linear, in which the accumulation in tissue was directly related to the metal concentration in the medium. Compared to other Chenopodiaceae halophyte species, *S. vermiculata* seems to be more tolerant of metals and is proposed for the phytoremediation of soils contaminated by heavy metals.

Keywords: Chenopodiaceae; metal accumulation; metal tolerance; salt marshes

2.1. Introduction

Heavy metals, due to their high toxicity, persistence, and bioaccumulative behavior, could represent a threat to natural ecosystems, especially in estuary zones with salt marshes [1,2]. They can impact biochemical processes in plants, including nutrient homeostasis, gas exchange characteristics, enzyme and antioxidant production, protein mobilization, and photosynthesis [3].

Soil-restoration technologies are available such as chemical/physical remediation, animal remediation, phytoremediation, and microremediation with microbes [4]. Phytoremediation, or green remediation, uses plants that absorb the heavy metal by the roots, absorbing or removing environmental contaminants; this technology has gained in importance in the last two decades for being cost-efficient, environmentally friendly, producing fewer side-effects, and with no negative impact on landscaping methods [4–11]. In the phytoremediation processes, plants assume various roles in diminishing the effects of metals: phytoextraction by the harvest of aboveground organs where metals concentrate; phytovolatilization by water-soluble metals during transpiration; phytostabilization by immobilization through accumulation by roots or precipitation, or by changing their chemistry; phytodegradation by degradation into insoluble or nontoxic compounds; or phytoaccumulation by accumulation in plant biomass [12–16].

In green remediation strategies, it is necessary to analyze the different properties of species to determine tolerance to contaminants and accumulative behavior [10]; phytotolerance studies are required to determine metal tolerance in plant species and to understand the negative effect of metals on metabolism and the processes in those species [17]. In this regard, seed germination and the seedling stage are more vulnerable to metal stress than later vegetative stages; therefore, testing the effects of metal stress on these processes is useful for assessing a species' establishment potential in metal-contaminated soils [18,19]. The effects of heavy metals on seed germination have been widely studied [20,21], with some of the most common effects being germination rate reduction or damage to seedlings, including a reduction in the elongation and growth of roots, shoots, or leaves, which could kill the seedlings [22,23].

Although the seed coat can act as a barrier to limit the effects of heavy metals [24], most seeds and seedlings show a decline in germination and vigor in response to the presence of heavy metals; processes affected include imbibition [25], or the activity of certain enzymes involved in reserve mobilization, such as acid phosphatases, proteases, amylases, and proteolytic enzymes [26]. Heavy metals can also cause oxidative stress and damage the seedlings' photosynthetic systems [3].

In saline soils, some halophytic plants are now widely accepted as solutions for cleaning up coastal environments [12,27], acting as bioindicators or biomonitors to assess the extent of heavy metal contamination on sediments, due to the linear correlation coefficients between the concentration of metals in their tissue and concentration in the soil, or by contributing to the phytoremediation process by accumulating metals at higher concentrations [1,2,14,16,28].

The Halophytes' accumulative capacity could be the result of different mechanisms. The amount of salt in the soil affects the accumulation of metals in tissue [29,30], and its presence can alleviate the effects of metal toxicity [14,31]. Furthermore, halophytic plant species can reduce the effects of metals in other ways: by retaining the ion intake in structures used to accumulate salts, such as the cell wall, vacuole, or trichome; by using substances for metal chelation; or possessing antioxidant defense systems [14,16,32,33].

The Chenopodiaceae family contributes the largest number of halophyte species [34], and they are dominant in Mediterranean tidal marsh vegetation [35,36]. The Chenopodiaceae family is not included in the predominant families in heavy metal accumulation processes [11]; however, many halophyte species have been studied as potential accumulators of metals in saline soils, and they may be considered a valuable species for the phytoremediation of metal-polluted saline soils.

The most widely studied species are Halimione portulacoides Aellen, *Sarcocornia fruticosa* (L.) A.J. Scott, and *Atriplex halimus* L. [16]. *Halimione portulacoides* is considered a suitable accumulator for Hg, Cr, Cu, Cd, and Pb, contributing to their phytostabilization [37–42]; this species also uses surfactants that affect the mobility of metals in the rhizosphere and has stabilization potential for Cr and Cu [43]. *Sarcocornia* *fruticosa* accumulates As, Cd, Cu, Pb, and Zn in belowground biomass in concentrations several times higher than concentrations of the metals in soil [40,44,45]. This has been demonstrated in phytoremediation of a metal-contaminated saline soil project, using liming to stimulate plant growth and enhance its capacity to stabilize metals [46]. *Atriplex halimus* has proved to be well-suited for the phytoextraction of Cd, Cu, and Zn found in saline soils [14,47–51].

Other species of Chenopodiaceae considered as accumulators for phytoaccumulation or phytostabilization are: Arthrocnemum macrostachyum (Moric.) K. Koch [42,52]; *Atriplex hortensis* and *A. rosea* L. [53]; *A. lentiformis* (Torr.) S. Watson and *A. undulata* (Moq.) D. Dietr. [54]; A. atacamensis Phil. [55]; Hammada scoparia Iljin [56]; *Salicornia europaea* L. [29]; *Salicornia ramosissima* J. Woods [57]; *Salsola fruticosa* Forssk. [42,58]; S*. glauca* M. Bieb. [59]; *S. passerina* Bunge [9]; S. soda L. [29]; *Sarcocornia perennis* (Mill.) A.J. Scott [37,40]; *Suaeda glauca* (Bunge) Bunge [60]; *S. maritima* (L.) Dumort. [61]; *S. salsa* (l.) Pall. [62]; *Salicornia arabica* L. [42]. *Salicornia bigelovii* Torr. has been tested for phytovolatilization of Se [63].

Salsola vermiculata is a shrub of wide distribution and ecological amplitude and is an important structural element in the vegetation of the arid and coastal zones of southern Europe, northern Africa, Macaronesia, and southwestern Asia [64]. This species disperses its seeds by wind during autumn and winter and is covered by a permanent calyx; its seeds have a high germination rate at low-medium salinities (to 0.3 M), and high recovery potential when exposed to fresh water following high salinity stress (0.6–0.9 M) [65]. In marshes in the southwestern Iberian Peninsula, it inhabits sandy sediments in high marsh areas only flooded during astronomic tides [36]. This species is widely dis-tributed in the Odiel Natural Park Marshes (SW Spain) [35,66], which is one of the most heavily metal-polluted systems in the world, mainly as a result of the upstream presence of the Iberian Pyrite Belt (IPB), an important metal rich sulfide deposit and, secondly, due to industrial activity on the estuary [67–69]. These findings would bolster its importance in restoration by phytoremediation in such habitats.

Salsola vermiculata was tested at mining sites in Morocco by Boularbah et al. [70], who analyzed its Cd, Cu, Pb, and Zn content in shoots and its toxicity. They concluded that the species is hypertolerant, accumulating 3.14, 69.5, 283.9, and 819 mg kg^{-1} DW of Cd, Cu, Pb, and Zn, respectively, with no toxic effects; thus, it can be used for phytostabilization in metal contaminated sites.

For the first time, this work investigates the germination of *Salsola vermiculata* under exposure to metals, including Ni, in marsh environments. The analysis is compared to the effects of four heavy metals, Cu, Mn, Zn, and Ni, on the germination and initial seedling development of *S. vermiculata* to determine their accumulation in seedlings in order to evaluate its possible use in marsh ecosystem restoration by phytoremediation. The results are compared to those obtained for other Chenopodiaceae halophytes tested for heavy metal accumulation or tolerance.

Although the study of other metals such as Cd, Cr, and Co may be of great interest, the metals analyzed in this study are the most representative of those present in the Odiel marshes and, therefore, those which can have the greatest effect on its flora.

2.2. Materials and Methods

I. Plant Material

Seeds were collected from the Odiel Marshes (37°08′–37°20′ N, 6°45′–7°02′ W; Spain, southwestern Iberian Peninsula) on 5 October 2016, from more than 10 different individual plants randomly selected, cleaned under a magnifying glass and separated from the floral parts. Seeds from different plants were mixed and stored for 12 days in paper bags at 25 °C in dark conditions prior to the germination experiments.

II. Germination Experiments

The seeds were surface-sterilized by immersion in 5% $^{(v/v)}$ sodium hypochlorite for 10 min and rinsed three times in sterile water [65,71]. Next, the seeds were placed in Petri dishes (9 cm in diameter) with three layers of autoclaved filter paper, watered with 5 mL of different treatment solutions, and sealed with adhesive tape (Parafilm TM) to avoid desiccation. Three Petri dishes with 25 seeds per dish were used in each treatment. Although other methods exist, a seed germination test with a heavy metal solution in a Petri dish with moistened filter paper is the most common methodology for assessing metal phytotoxicity in plant species [72].

The seeds were exposed to eight different concentrations (10, 25, 50, 100, 250, 1000, 2000, 4000 µM) of Cu, Mn, Ni, and Zn added in the form of sulfates $CuSO_4\ 5H_2O$, $MnSO_4\ H_2O$, $NiSO_4\ 6H_2O$, and $ZnSO_4\ 7H_2O$ and dissolved in deionized water. For the control treatment (0 µM) only deionized water was used. The sulfate form of the metals was selected according to the most abundant form found in the Odiel Natural Park Marshes [73] and references therein.

These metals are essential for plants that contribute to plant metabolic function, but excessive amounts are toxic and lead to growth inhibition [21,25]. They were chosen based on previous studies that describe the metal composition in the water and soils of the Odiel Natural Park Marshes [21] and references therein.

The seeds were germinated under controlled environmental conditions with 12/12 h of day/night at 24/20 °C, respectively; it has been demonstrated that such conditions are appropriate for stimulating high levels of germination in *Atriplex halimus* [71] and other Chenopodiaceae, including *Salsola vermiculata* [65]. Light was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 µmol m^{-2} s^{-1}. Germination was monitored for 30 days, with germination in each plate recorded daily in the first week and every 2 or 3 days thereafter. A seed was considered germinated when the radicle emerged.

The germination dynamic was analyzed by noting the final germination percentage after 30 days, and the number of days necessary to reach 50% of the final germination percentage (t_{50}) for each Petri dish.

III. Morphological Analysis of the Seedlings

To analyze the influence of the different treatments on seedling development, the length of the cotyledons, hypocotyls, and radicles were measured in 15-day-old seedlings, using 10 seedlings per dish. The measurements were taken under a magnifying glass [21,71,74].

To assess the tolerance of the seedlings to metals, the tolerance index was calculated according to Wilkins [75], applied to the length of the cotyledons, hypocotyls, and radicles (TI%=100×(mean organ length in the treatment/mean organ length in the control) [76,77].

IV. Metal Content Analysis

To better understand whether the metals were taken up by the seedlings, whole 15-day-old seedlings (approximately 45 per metal and treatment) were carefully washed with ultrapure water, thoroughly dried, pulverized with mortar and pestle, and stored in hermetically sealed polypropylene tubes at 4$^{\circ C}$ until analysis. Mass ratios between different parts could have changed as a result of treatments; therefore, accumulation in the whole seedling was only used as indicative of increased accumulation when the seedlings were exposed to increasing concentrations.

For the metal analysis, 50 mg of a powdered sample were mixed with 640 µL HNO3 and 160 µL of H2O2 in polytetrafluoroethylene vessels and incubated for 10 min. Mineralization (CEM Matthews microwave oven, NC, USA, model MARS) was carried out at 800 W at room temperature, ramped to 180 °C for 10 min, and then maintained for 20 min at that temperature. Then, the solutions were prepared with up to 5 mL of ultrapure water, and the metals were analyzed with an inductively coupled plasma mass spectrometer (ICP-MS) Thermo XSeries2 (Thermo Scientific, Bremen, Germany) equipped with a MicroMist nebulizer, Ni cones, and a Cetac ASX-500 autosampler (Agilent, Wilmington, DE, USA). Rh was added as an internal standard (100 ppb) from Sigma-Aldrich (Steinheim, Germany). All analyses were performed in triplicate.

V. Statistical Analyzes

The statistical analyses of the data were performed using Statistica 8.0. The data were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests, respectively. As data did not follow a normal distribution, Kruskal–Wallis and Mann–Whitney U tests were used to detect significant differences ($p < 0.05$).

The metal accumulations in seedlings at each metal concentration medium were fitted to polynomial curves type y = ax2 + bx + c, and the threshold value of the model was based on R2 and p values ($p < 0.05$).

2.3. Results

The final germination and the germination dynamics of *S. vermiculata* were minimally affected by the presence of metals in the germination media, with some differences depending on the metal (Table 1). Copper and zinc significantly reduced the final germination compared to the control when present at the highest concentration, 4000 µM. However, only zinc had a significant effect on the germination dynamics, with a considerable increase in the time-lapse to reach 50% of germination when present at 4000 µM, rising from 1.43 days in the control to 1.77 days at this concentration.

Table 1. Germination percentage after 30 days, t_{50} and tolerance index for cotyledons, hypocotyls, and radicles.

Concentration (µM)	Germination (%)	t_{50} (Days)	IT Cotyledon	IT Hypocotyl	IT Roots
Cu (µM)					
0	94.65 ± 0.04 a	1.43 ± 0.12 a	100	100	100
10	94.66 ± 0.04 ab	1.35 ± 0.14 a	102	104	113
25	92.00 ± 0.08 ab	1.53 ± 0.14 a	94	91	97
50	96.00 ± 0.04 ab	1.47 ± 0.1 a	93	94	91
100	92.00 ± 0.06 ab	1.28 ± 0.24 a	101	100	113
250	93.33 ± 0.04 ab	1.30 ± 0.19 a	97	96	71 *
1000	98.66 ± 0.02 a	1.30 ± 0.05 a	95	88	38 *
2000	93.33 ± 0.08 a	1.43 ± 0.16 a	95	80	36 *
4000	80.00 ± 0.06 b	1.35 ± 0.10 a	73	46 *	28 *
Mn (µM)					
0	94.65 ± 0.04 a	1.43 ± 0.12 a	100	100	100
10	93.33 ± 0.02 a	1.33 ± 0.37 a	92	96	88
25	91.83 ± 0.04 a	1.44 ± 0.10 a	104	112	104
50	94.66 ± 0.02 a	1.35 ± 0.12 a	98	107	105
100	89.33 ± 0.06 a	1.36 ± 0.23 a	106	102	93
250	96.00 ± 0.04 a	1.55 ± 0.03 a	96	92	113
1000	89.11 ± 0.09 a	1.40 ± 0.09 a	103	106	113
2000	98.66 ± 0.02 a	1.40 ± 0.24 a	104	104	133
4000	90.50 ± 0.04 a	1.33 ± 0.18 a	95	100	116
Ni (µM)					
0	94.65 ± 0.04 a	1.43 ± 0.12 a	100	100	100
10	97.33 ± 0.02 a	1.38 ± 0.08 a	99	103	103
25	98.66 ± 0.02 a	1.41 ± 0.10 a	104	113	108
50	94.66 ± 0.06 a	1.30 ± 0.22 a	96	100	97
100	100.00 ± 0.00 a	1.31 ± 0.17 a	94	96	83
250	89.22 ± 0.04 a	1.36 ± 0.06 a	100	94	98
1000	94.55 ± 0.06 a	1.34 ± 0.08 a	111	96	78
2000	94.55 ± 0.02 a	1.53 ± 0.09 a	95	70 *	69 *
4000	90.66 ± 0.02 a	1.31 ± 0.23 a	85 *	74 *	31 *
Zn (µM)					
0	94.65 ± 0.04 a	1.43 ± 0.12 a	100	100	100
10	93.33 ± 0.02 ab	1.31 ± 0.16 a	96	103	101
25	97.33 ± 0.02 a	1.66 ± 0.32 ab	99	104	102
50	98.61 ± 0.02 a	1.48 ± 0.14 ab	101	103	130 *
100	92.00 ± 0 ab	1.67 ± 0.16 ab	102	107	95
250	96.00 ± 0.04 a	1.61 ± 0.12 ab	96	95	102
1000	96.00 ± 0.04 a	1.56 ± 0.06 ab	99	110	107
2000	94.66 ± 0.06 a	1.59 ± 0.04 ab	99	106	81
4000	79.83 ± 0.1 b	1.77 ± 0.08 b	96	100	49 *

The data show the mean ± standard deviation for 3 independent plates, with 25 seeds in each one. For each metal, different letters indicate significant differences ($p < 0.05$); IT data marked with * means significant differences with the control.

The initial development of the seedlings was affected by the presence of metals in different ways (Table 1, Figure 1). Copper did not affect the length of the cotyledons, but at 4000 µM it significantly reduced the length of the hypocotyls to 46% of the control, and the length of the radicle was significantly affected from 250 µM, with a reduction of 71% at 250 µM and 28% at 4000 µM (Table 1, Figure 1A). Manganese, at the concentrations tested, did not statistically affect the initial development of *S. vermiculata* seedlings (Table 1, Figure 1B). The presence of nickel significantly reduced the length of the cotyledon at 4000 µM to 85% of the control, and the length of the hypocotyl decreased from 2000 µM to around 70% of the control; the length of the radicle was 69% of the control at 2000 µM and 31% at 4000 µM (Table 1, Figure 1C). Zinc did not affect either the length of the cotyledons or the length of the hypocotyls at any of the concentrations tested; however, this metal induced the growth of the radicle at smaller concentrations (50 µM) reaching 130% of the control, but had a negative effect at 4000 µM, with a significant reduction in radicle length to 49% of the control (Table 1, Figure 1D)

Figure 1 shows the mean and standard deviation. Different letters above the bars indicate significant differences ($p<0.05$): lowercase Latin letters for cotyledons, uppercase Latin letters for hypocotyles, and Greek letters for radicles.

As shown in Figure 2, the metal content inside the seedlings rose significantly with the increase in metal in the germination media, reaching the following maximum mean values: 9186 mg kg^{-1} DW of Cu at 4000 µM; 4373 and 4676 mg kg^{-1} DW of Mn at 2000, and 4000 µM, respectively; 3990 and 3204 mg kg^{-1} DW of Zn at 2000 and 4000 µM, respectively; 7130 mg kg^{-1} DW of Ni at 4000 µM. However, the behavior is different for each metal studied.

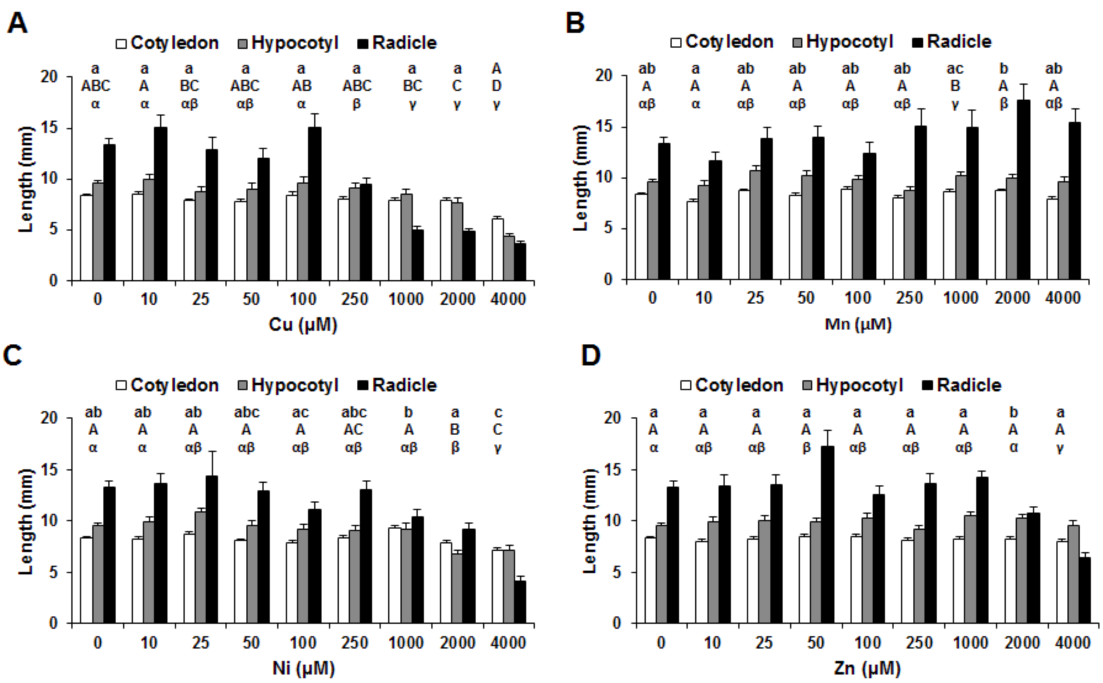

Figure 1. Effects of Cu, Mn, Ni, and Zn on the initial development of the cotyledon, hypocotyl, and radicle of *Salsola vermiculata* seedlings.

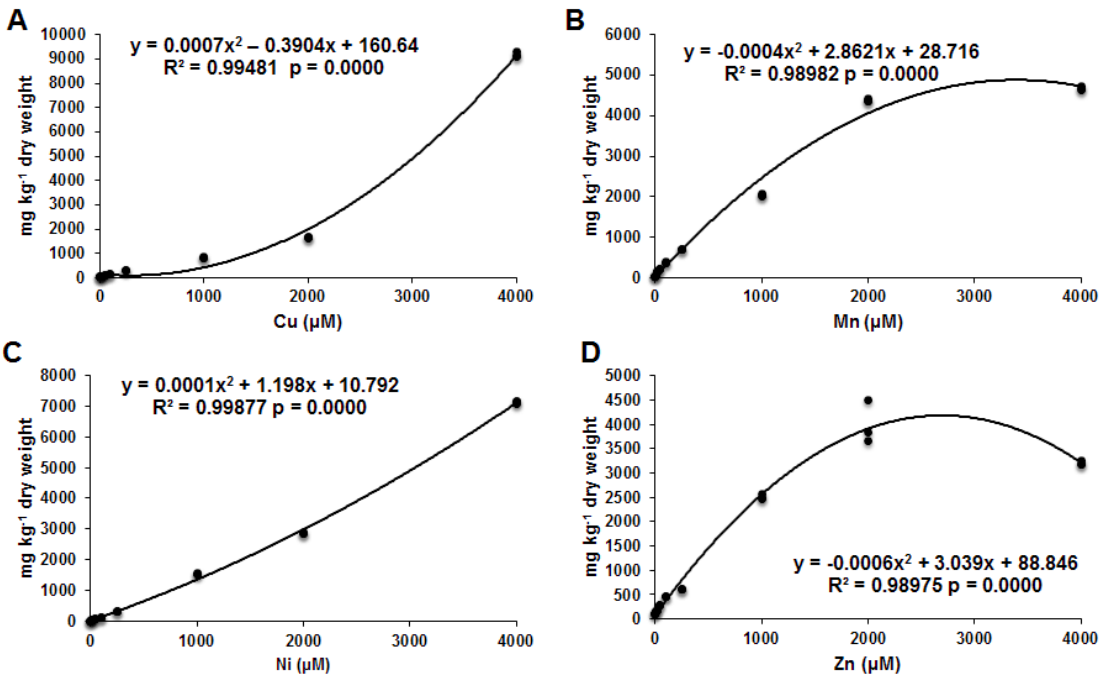

Figure 2. Levels of Cu, Mn, Ni, and Zn (mg kg^{-1} or ppm) accumulated in the seedlings germinated at different concentrations of metals, and quadratic equations for curves with their determination coefficient (R2) and p value.

Accumulation curves can fit significantly ($p < 0.05$) to second-degree polynomials. The equation presents a high positive value for component "a" in the case of copper; therefore, the accumulation increases exponentially with the increase in copper in the medium. However, nickel registers a very low value for component “a”; therefore, the increase in accumulated metal rises arithmetically as its concentration in the culture medium increases. On the other hand, manganese and zinc behave similarly, with a negative “a” component, which means that when a concentration value is reached, the accumulation of metal in the tissue begins to decrease.

2.4. Discussion

I. Copper

In our study, copper reduced the final germination percentage at 4000 µM but had no effect at concentrations of 2000 µM or lower and did not affect the speed of germination (t50). These results are the same for other Chenopodiaceae plants from the Odiel marshes, such as *Atriplex halimus* and *Salicornia ramosissima*, studied by Márquez-García et al. [21].

In addition, copper concentration affected seedling development in hypocotyl growth at 4000 µM and reduced the length of radicles at concentrations up to 250 µM, with significant reductions over 1000 µM, falling below 40% of the control length. *Salsola vermiculata* exhibited a greater tolerance than *Atriplex halimus* and *Salicornia ramosissima*, in which cotyledon and hypocotyl development was affected from 1000 and 2000 µM, respectively. Radicle development was affected in the same way, being reduced from 250 and 1000 µM in both species, respectively [21]. The biggest effect of Cu on the root was due to its accumulation mainly in this organ, with little translocation to the shoots [50,78].

In sensitive plants, Cu can become toxic when it accumulates in plant tissue at levels exceeding 20 mg kg^{-1} dry weight; these data differ according to plant species and growth conditions [79]. Our results showed that copper in *Salsola vermiculata* reached levels of 1664 and 9186 mg kg^{-1} DW, grown in solutions of 2000 and 4000 µM, respectively. However, it presented the first negative effects on the radicle at concentrations of 250 µM, the plants remained unaffected when cultivated in 100 µM solution, accumulating 154 mg kg^{-1} DW. These data match those of Boularbah et al. [70], who found Cu content of 69.5 mg kg^{-1} DW in mining sites in Morocco, with no toxicity symptoms.

The accumulator capacity of *Salsola vermiculata* seems to be higher than that of *Atriplex halimus*, which Mateos-Naranjo et al. [50] studied in the Odiel marshes, where they detected clear phytotoxicity symptoms at tissue concentrations greater than 38 mg kg^{-1} DW.

The maximum concentrations of this metal registered in the Odiel Marshes Natural Park ranged from 500 to 1000 µM. In halophytes in this estuary, Luque et al. [80] found Cu accumulations that ranged from 12.3 mg kg^{-1} DW in Halimione portulacoides to 878 mg kg^{-1} DW in Zostera noltii Hornem., and at the same location Park, Stenner, and Nikless [81] found in the latter species accumulations in tissue of 1350 mg kg^{-1} DW. These levels are far superior to the data collected by other authors for these species in other estuaries around the world [1,2], which clearly demonstrates the high level of contamination in the Odiel Marshes Natural Park.

As described previously, the Cu accumulation curve equation presents a high positive value for component “a”; therefore, the accumulation rises exponentially with the increase in the metal in the medium, which matches Kabata-Pendias and Pendias [79], who established that Cu concentration rises exponentially when concentrations in the medium increase. This also fits with observations by Mateos-Naranjo et al. [50] in *Atriplex halimus* and with the accumulation in Arctium tomentosum Mill. observed by Al Harbawee et al. [77], and in roots, shoots, and leaves of Sesuvium portulacastrum (L.) L. studied by Feng et al. [82]. This exponential accumulation could be linked to damage in the roots, which impacts negatively on the root transport system. This could be explained by the fact that at lower concentrations the relation between the concentration of the metal in the medium and tissue is arithmetic [9,83].

II. Manganese

In our work, manganese did not affect the final germination percentage or the germination dynamics of *Salsola vermiculata*, which coincides with data on *Atriplex halimus* and *Salicornia ramosissima*, other Chenopodiaceae plants from the Odiel marshes presented by Márquez-García et al. [21]. Neither did it affect *Salsola vermiculata* seedling development in concentrations up to 4000 µM. Márquez-García et al. [21] found that manganese had no effect on the cotyledons or hypocotyls of *Atriplex halimus* and *Salicornia ramosissima* in concentrations up to 2000 µM, but there was an increase in radicle length at 10 µM and a reduction in concentrations over 250 µM in *Atriplex halimus,* and over 2000 µM in *Salicornia ramosissima*.

Normal Mn content differs greatly between species (30–500 mg kg^{-1} DW) [84]. The threshold of damage caused by Mn depends on the plant species and cultivars or genotypes within a species [85,86]. In general, plants are negatively affected by Mn concentrations above 500 ppm, although concentrations over 1000 ppm have been described in tolerant species [79].

Salsola vermiculata reached levels of up to 4675 mg kg^{-1} DW, grown in solutions up to 4000 µM, without presenting any negative effects. By contrast, this metal significantly curtailed the growth of Suaeda glauca when accumulation in tissue reached approximately 1000 mg kg^{-1} DW [60], revealing, for the first time, that *Salsola vermiculata* is a better accumulator for this metal.

Mn is present in the Odiel marshes at maximum concentrations of between 50 and 500 µM, occupying third place in metal concentration in plant tissue in these marshes, behind Fe and Zn, and its concentrations range from 25.4 mg kg^{-1} DW in Arthrocnemum macrostachyum plants to 1960 mg kg^{-1} DW in Zostera noltii [80].

Accumulation of Mn in seedlings increased to 4373 mg kg^{-1} DW when exposed to 2000 µM, and maintained this level in higher concentrations, reaching 4676 mg kg^{-1} DW at 4000 µM Mn. This behavior contrasts with that established by Kabata-Pendias and Pendias [79], who determined that Mn concentration in plants is proportional to its presence in soil, and with observations by Zhang et al. [60], who found arithmetic increases in Zn content in Suaeda glauca up to 2812 mg kg^{-1} DW. However, Lidon and Teixeira [87] observed, in Oryza sativa, a stabilization in accumulation when it reached 2000 mg kg^{-1} DW in the culture medium with concentrations that exceeded 8 mg L^{-1}.

III. Nickel

In many plant species, increasing concentrations of Ni inhibit and delay seed germination and seedling growth, generally due to the suppression of amylase and protease activity [88–94]. However, this metal does not affect the final percentage of *Salsola vermiculata* germination, or germination dynamics, as was the case in *Atriplex halimus* and Salicornia ramosissima, other Chenopodiaceae plants from the Odiel marshes studied by Márquez-García et al. [21]. In some species, Ni improves both the rate and percentage of seed germination [22,88], but we did not observe this effect, perhaps due to the high percentage of germination in the control.

On the other hand, some authors state that this metal retards germination in crop plants [95], but this toxic effect was not observed in this study, in *Salsola vermiculata*, *Atriplex halimus*, or *Salicornia ramosissima* [21].

There are many examples of the toxic effects of Ni on seedlings that reduce their growth [93,96]. In the case of *Salsola vermiculata*, nickel reduces cotyledons at 4000 µM, diminishes hypocotyls at 2000 µM, and affects radicles from 2000 µM, causing a drastic reduction at 4000 µM. The threshold concentrations for damage observed in this species are higher than those observed in *Atriplex halimus* and Salicornia ramosissima, studied in the same location, with both species registering damage at 1000, 250, and 100 µM for cotyledons, hypocotyls, and radicle, respectively.

As we have described, the concentration of nickel required for normal growth in most plants is very low; from 0.05 to 0.1 mg kg^{-1} DW to 5 mg kg^{-1} DW, according to Kabata-Pendias and Pendias [79], from 10 mg kg^{-1} DW, in Ain et al. [90], or from 20–30 mg kg^{-1} DW, as described by White and Brown [97]. In most plants studied, Ni in tissue is toxic in concentrations from 10–100 mg kg^{-1} DW [79,91,98].

In our study, *Salsola vermiculata* seedlings reached levels of 7130 mg kg^{-1} DW when grown in 4000 µM of Ni medium, but the highest concentration at which toxic effects on seedlings were not observed was 1000 µM, accumulating in seedlings up to 1537 mg kg^{-1} DW.

In the Odiel estuary, this metal has been found in concentrations below 50 µM, and its accumulation in the plants studied ranged from 13.0 mg kg^{-1} DW in *Salicornia ramosissima* to 45.7 mg kg^{-1} DW in Spartina maritima (Curtis) Fernald [80].

At the concentration levels tested, accumulation of this metal increased arithmetically as concentrations in the medium rose, which is consistent with Lu et al. [9], who established that Ni concentrations in many plants positively correlated to concentrations in the medium. But this is only true until the medium concentration reaches a certain level [79]; this threshold varies in different species, for example, in Lepidium ruderale L. the threshold concentration in the medium was 20 µM, while it reached 30 µM in Capsella bursa-pastoris Moench [99]; it was 100 µM in Arctium tomentosum [77]; in Odontarrhena bracteata (Boiss and Buhse) Spaniel and O. inflata

(Nyár.) D.A. German, the threshold was approximately 150 µM [100]; and in varieties of Brassica juncea (L.) Czern, it was 400 µM [96]. The persistence of Ni intake until the concentration in the medium of 4000 µM could indicate the non-existence of mechanisms involved in the control of Ni intake.

IV. Zinc

Salsola vermiculata germination fell to 79.83% and was delayed at 4000 µM, but no effects were observed at lower concentrations, which coincides with figures for *Atriplex halimus* and *Salicornia ramosissima* reported by Márquez-García et al. [21], who did not observe any reduction at 2000 µM of Zn.

The Zn concentrations tested did not affect cotyledon or hypocotyl size, but at 50 µM the radicles of *Salsola vermiculata* seedlings were, significantly, 30% longer than the control; the effects of hormesis have also been observed in Medicago sativa L. [22]. Nevertheless, at 4000 µM a reduction of almost 50% was observed in the radicles. The study by Márquez-García et al. [21] of *Atriplex halimus* noted a reduction in cotyledons and hypocotyls at 2000 µM, and in radicles from 250 µM. They also described a reduction in the radicles of *Salicornia ramosissima* seedlings from 1000 µM, which demonstrates higher tolerance in *Salsola vermiculata*.

Most plants presented critical toxicity levels for this metal in their tissue from 100–500 mg kg^{-1} DW [79]. In *Salsola vermiculata*, Zn reached maximum accumulations of 3990 mg kg^{-1} DW when cultivated at 2000 µM, showing no seedling damage at this concentration. Our results are consistent with Boularbah et al. [70], who found Zn content of 819 mg kg^{-1} DW in mining areas in Morocco, with no toxicity symptoms.

The maximum concentrations of this metal recorded at the Odiel marshes ranged from 500 µM to 1000 µM, occupying second place in metal concentration in plant tissue in these marshes. In the plants studied, Zn accumulation ranged from 62.9 mg kg^{-1} DW in Arthrocnemum macrostachyum to 2440 mg kg^{-1} DW in Zostera noltii [80,81].

Accumulation of Zn in seedlings increased to 3990 mg kg^{-1} DW of Zn at 2000 µM, and maintained this level in higher concentrations, accumulating to 3204 mg kg^{-1} DW at 4000 µM, showing similar behavior to that observed for Mn. As in the case of Mn, Kabata-Pendias and Pendias [79] determined that Zn concentration in plants is proportional to its presence in the soil, but Al Harbawee et al. [77] observed similar dynamics to those we observed in Arctium tomentosum: that when concentrations in the medium increased to 1000 uM in the plate, they registered an arithmetic increase in accumulation, and the accumulation did not increase at the same rate when the concentration was higher. This was also observed by Pandey [101] in Raphanus sativus L. and Spinacia oleracea L., and by Kozhevnikova et al. [99] in Lepidium ruderale and Capsella bursa-pastoris. Nevertheless, Ivanov et al. [102], observed in Pinus sylvestris L. seedlings, that while Zn accumulation in the radicles increased proportionally to the concentration in the nutrient solution to 300 µM, the Zn content in leaves stabilized at a concentration of 150 µM.

Although plant metal intake behavior depends on the range of concentrations in the medium analyzed [103], some authors [104] have described three patterns for accumulations of heavy metals: as the concentration in the medium increases, (1) accumulator plants show an arithmetic increase in content in tissue to the point where content reaches a maximum level; (2) non-accumulators show a low level of accumulation in tissue until the concentration in the medium reaches a point where the restricting mechanism breaks down and there is unlimited accumulation, resulting in plant death; (3) a linear pattern in which the accumulation in tissue is directly related to the metal concentration in the medium. Observations in our study resemble the first pattern for Mn and Zn, the second pattern for Cu, and the third pattern in the case of Ni.

2.5. Conclusions

Under control group conditions, *Salsola vermiculata* reached germination levels of above 90%, in line with data previously published by Muñoz-Rodríguez et al. [65], with seeds from the same location and under the same conditions. In that study, *S. vermiculata* demonstrated its adaptation to soil salinity, germinating at above 90% in salinities of up to 0.2 M when planted without the calyx, and at over 80% with the calyx; even after exposure to 0.6 M salinities, they germinated in distilled water in a proportion higher than 80%.

Regarding the toxic effects of the metals studied, the roots are the primary target of the metals, and their growth is usually more severely affected than that of the aerial parts, probably since the roots are the first contact point with toxic elements and provide an entrance to the cellular structure inside the plant. This is confirmed by the results in our work, as the roots were affected at lower concentrations in the culture medium than the hypocotyls or cotyledons for all the metals tested.

Our results clearly show how *Salsola vermiculata* can tolerate the presence of metals and successfully germinate. With no toxic effects, its seedlings accumulate Cu up to 299 mg kg^{-1} DW, Mn up to 4675 mg kg^{-1} DW, Ni up to 1537 mg kg^{-1} DW, and Zn up to 2507 mg kg^{-1} DW.

Salsola vermiculata exhibits a higher tolerance to metals than other halophylous Chenopodiaceae species studied, such as *Atriplex halimus* and Salicornia ramosissima, both analyzed at the same location [21]; and higher than Salsola passerine, which is an accumulator for Ni, Cu, Cd, Cr, and Co [9].

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3. Metal effects on germination and seedling development on two closely-related halophyte species inhabiting at different elevations along the intertidal gradient.

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A B S T R A C T

Seed germination and seedling establishment are very sensitive plant stages to metal pollution. Many halophyte species colonizing salt marshes are able to germinate and establish in highly contaminated habitats and low marsh halophyte species seem to show higher tolerance to metals than high marsh species. We analyzed the effects of copper, zinc and nickel in concentrations up to 2000 µM on seed germination and seedling growth in two closely related species of Sarcocornia, S. perennis, a low marsh species, and S. fruticosa, a high marsh species. Germination of both halophytes was not affected by any metal concentration, and their seedling growth, mainly radicle length, was reduced by increasing metal concentrations. Seedlings of *S. perennis* showed higher tolerance to the three metals than those of S. fruticosa. Our results are useful for

designing ecotoxicological bioassays and planning phytoremediation projects in salt marshes.

Keywords: intertidal gradient, Odiel Marshes, metal pollution, radicle, *Sarcocornia fruticosa*, *Sarcocornia perennis*, seedling growth, vegetation zonation.

3.1. Introduction.

Coastal marshes are usually exposed to high loads of contaminants such as metals (Gedan et al., 2009). This pollution, coming from industrial, mining, agricultural and transport activities, accumulates in intertidal sediments in concentrations that often exceed their toxicity threshold values (Sharifuzzaman et al., 2016). In this context, metal pollution is a major environmental problem in many estuaries due to their toxic nature, non biodegradability and accumulative behaviors (Williams et al., 1994). Nevertheless, halophytes adapted to survive in brackish and salty environments frequently show high tolerance to metal pollution (Van Oosten & Maggio, 2015) since tolerances to salinity and metal stress share some common mechanisms such as high levels of antioxidant defenses and vacuolar sequestration (Manousaki & Kalogerakis, 2011).

Seed germination and seedling establishment are the most sensitive stages to metal pollution in the plant life cycle (Munzuroglu & Geckil, 2002; Liu et al., 2005; Ahsan et al., 2007). Metals may provoke concentration-dependent reduction in germination and seedling growth (Kranner & Colville, 2011; Sethy & Ghosh, 2013; Asati et al., 2016), which depends on the ability of the metal to reach embryonic tissues across the seed coats and the effects that the ions cause on the germination metabolism (Ko et al., 2012). Defenses against metals during germination include reduction of metal uptake, chelation and the induction of antioxidant defenses (Kranner & Colville, 2011). Many halophyte species exhibit some of these adaptive mechanisms that let them germinate and survive under high metal concentrations (Thomas et al., 1998; Van Oosten & Maggio, 2015).

Plants colonizing salt marshes in the joint estuary of Odiel and Tinto rivers (Gulf of Cadiz, southwest Iberian Peninsula) are great focus species to study metal pollution effects on halophytes since this is one of the most metal-polluted estuaries around the world (Nelson & Lamothe, 1993; Sainz et al., 2004). The pollution in Odiel Marshes is as a result of industrial activity located on the estuary itself and chiefly of mining activity that develops upstream in the Iberian Pyrite Belt (Pérez-López et al., 2011, Muñoz-Vallés et al., 2017). The high metal concentrations recorded in the Odiel Marshes could have adverse effects on the wildlife as they accumulate in halophyte species (Stenner and Nicless, 1975). Metal accumulation in halophytes in the Odiel Marshes decreases from low to high marshes, being the highest for those species inhabiting the lowest elevations along the intertidal gradient (Luque et al., 1999). This spatial pattern in metal accumulation may be related with a higher bioavailability of several metals under hypoxic and anoxic conditions at lower elevations in the intertidal gradient (O'Reilly Wiese et al., 1997; Reboreda et al., 2007; Wang et al., 2012; Brito et al., 2021). Thus, germination and seedling growth of *Spartina maritima* (Curtis) Fernald, a native low-marsh species, were unaffected by Copper (Cu), Niquel (Ni) and Zinc (Zn) concentrations up to 2000 µM (Infante-Izquierdo et al., 2020). In the same way, final germination percentage and seedling development of *Salicornia ramosissima* J. Woods, an annual species sampled from low elevations in the Odiel Marshes, were not affected by increasing concentrations of Cu, Manganese (Mn) and Zn, but its final germination decreased c. 25% at Ni concentrations higher than 10 µM (Márquez-García et al., 2013). Furthermore, working with middle marsh populations of *S. densiflora* Brongn., an exotic invasive species in the Odiel Marshes, Infante-Izquierdo et al. (2020) found its final germination percentage was not affected by Cu, Ni and Zn concentrations up to 2000 µM, but its seedling growth, mostly radicle development, was reduced at metal concentrations higher than 100-250 µM. From the highest marsh elevations in the Odiel Marshes, germination of *Atriplex halimus* L. was not affected by increasing concentrations of Cu, Mn, Ni or Zn, but its seedling development was reduced at concentrations higher than c. 100 µM (Márquez-García et al., 2013). In view of these previous studies, it seems that low marsh species show higher tolerance to metals during seedling development than halophytes colonizing higher elevations in the intertidal gradient. However, the toxicity of metals in plants varies with plant species, soil characteristics, specific metals and their concentration and chemical forms (Nagajyoti et al., 2010; Asati et al., 2016).

Our focus species in the Odiel Marshes to study germination and seedling development of halophytes inhabiting contrasted elevations along the intertidal gradient were *Sarcocornia perennis* (Mill.) A.J. Scott, a low marsh species (Davy et al., 2006), and *Sarcocornia fruticosa* (L.) A.J. Scott., a high marsh species (Contreras-Cruzado et al., 2017). These are closely-related species that often hybridize and play an important role in the ecological succession (Figueroa et al., 2003). Moreover, they present similar germination responses to salinity characterized by germinating at a wide salinity range but dropping at salt concentrations over 0.3 M NaCl (Muñoz-Rodríguez et al., 2017). In addition, *S. perennis* shows a great potential for phytoremediation of metal polluted salt marshes (Curado et al., 2014). We studied the germination and seedling responses of both *Sarcocornia* species collected from contrasted environments in the Odiel Marshes and exposed to a wide concentration gradient (0-2000 µM) of Cu, Ni and Zn in controlled conditions. We hypothesized that *S. perennis* would be less sensitive to high metal concentrations than *S. fruticosa*, especially during seedling development, since this low marsh species would be exposed to higher bioavailable metal concentrations in its natural habitat. Our results are useful to design ecotoxicological bioassays and to plan phytoremediation projects in salt marshes.

3.2. Materials and methods

I. Plant material sampling

Fruits of *Sarcocornia perennis* and *S. fruticosa* were collected at the Acebuchal area in the Odiel Marshes (37°12'29.71" N, 6°57'32.60" W; Gulf of Cadiz, Southwest Iberian Peninsula) in November 2020. *S. perennis* fruits were collected from a low marsh area and *S. fruticosa* was sampled in an adjacent high marsh area. We collected fresh ripped cymes from more than ten individual plants of each species. Once in the laboratory, fruits were stored in paper bags at 20-25 ºC during a week, when seeds were carefully extracted from the fruits. Plant species were identified following Castroviejo (1990).

II. Soil sampling and analysis

Sediment samples were collected using stainless steel cores of 50 mm diameter and 50 mm height from the same low and high marsh areas where fruits of both *Sarcocornia* species were sampled. Sediment samples were placed in polyethylene bags that were hermetically closed and stored at −20 °C until analysis. For soil electrical conductivity and pH measurements, 20 cc of soil and 20 ml of distilled water were deposited in a falcon tube (1:1), homogenized, and centrifuged at 3000 g for 15 minutes. Electrical conductivity was measured in the supernatant using a conductivimeter (Horiba Laqua, Kyoto, Japan) and the pH using a pHmeter (Crison Basic 20+, Barcelona, Spain) (n = 2).

Sediment samples were pretreated for the quantification of bioavailable metals following Alan & Kara (2019). Samples (n = 3) were dried in an oven at +45 °C for two days and sifted using 100 µm sieve. Once sieved, 40 ml of 20 mM $CaCl_2$ was added to 1 g of sediment and kept stirring overnight at room temperature. Samples were subsequently centrifuged at 3000 g for 15 minutes, recovering the supernatant fraction that was stored at +4 °C. Each supernatant was prepared by adding 5% of nitric acid (analytical reagent grade 65%) and 100 µg l^{-1} of Rh (100 ppb; Sigma-Aldrich, Steinheim, Germany) in the final volume as internal standard. This mix was diluted five-fold with ultrapure water and analyzed with an inductively coupled plasma mass spectrometer (ICP-MS) Thermo XSeries2 (Thermo Scientific, Bremen, Germany) equipped with a MicroMist nebulizer, Ni cones and Cetac ASX-500 autosampler (Agilent, Wilmington, DE, USA). All analyses were performed in triplicate.

III. Germination assays

To prevent fungal contamination, seeds were surface-sterilized in 5% (v/v) sodium hypochlorite for 10 min and then rinsed with distilled water (Infante-Izquierdo et al., 2020). For each species, three replicates of 25 seeds were sown for each metal and tested concentrations on Petri dishes (9 cm diameter) with two layers of autoclaved filter paper adding 5 ml of different treatments solutions: distilled water (control), solutions containing 100, 250, 500, 1000 and 2000 µM Cu, Zn or Ni in their sulphate forms ($CuSO_45H_2O$, $ZnSO_47H_2O$ and $NiSO_46H_2O$). These chemical forms were used because they are the most abundant in Odiel Marshes (Barba-Brioso et al., 2010), and because the toxicity of other forms as chlorides might have inhibitory effects on seed germination (León et al., 2005). These metals and their concentrations were chosen based on the metal concentrations record previously in the sediments of Odiel Marshes (Achterberg et al., 2003; Borrego et al., 2002; Braungardt et al., 2003; Elbaz-Poulichet et al., 2001; Fernández-Caliani et al., 1997; Galán et al., 2003; González-Pérez et al., 2008). Once sown, Petri dishes were sealed with adhesive tape (Parafilm™) to avoid desiccation. Germination assays were carried out under controlled-environmental conditions at +20–25 °C and a 12 h/12 h photoperiod. Radiation was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 µmol m^{-2} s^{-1}. Seeds were exposed to treatments for 30 days, and germination was recorded every 3-4 days (Keiffer & Ungar, 1997). A seed was recorded as germinated when the radicle emerged. The percentage of the 25 seeds that germinated and the number of days necessary to reach 50% of the final germination (T_{50}) was calculated for each Petri dish (Muñoz-Rodríguez et al., 2017). Seedling are highly sensitive to the germination environment, therefore their growth is a useful indicator of environmental stresses such as high concentrations of metals (Mabrouk et al., 2019). For this reason, we measured, under a magnified glass, the lenght of cotyledons, hypocotyl and radicle for 5 seedlings per Petri dish 30 days after their germination.

IV. Statistical analyses

Statistical analyses were carried out using STATISTICA 8.0 (StatSoft Inc., USA), applying a significance level (α) of 0.05. Deviations from the arithmetic mean were calculated as standard error (SE). Normality and homogeneity of variance of data series were tested using the Kolmogorov–Smirnov and the Levene test, respectively. Sedimentary variables were compared between zones using Student t-test or Man-Whitney U-test. To allow interspecific comparisons, we used the relative size of each seedling organ that was calculated dividing the data obtained in each species and organ series by the mean calculated in control conditions. Cotyledon relative size was compared between species, metals, their concentrations and their interactions using general linear models (GLM). Since normality or homogeneity of variance was not achieved, final germination percentage, T_{50}, and hypocotyl and radicle relative size were analyzed using generalized linear models (GLZ) with Chi-square (χ^2) de Wald (Ng and Cribbie, 2017). The effect of each metal on seedling traits of each species was analyzed using one-way ANOVA and Tukey's honest significant difference (HSD) as post hoc test, or nonparametric Kruskal-Wallis and Mann-Whitney U as post hoc test.

3.3. Results.

I. Sedimentary environment

Low salt marshes colonized by *S. perennis* presented c. 20% lower electrical conductivity and higher total Ni (+55%) and Zn (+72%) concentration than *S. fruticosa* high marshes (Table 1).

	Conductivity	pH	Cu	Ni	Zn
S. perennis low marsh	28.8 ± 0.1^a	5.7 ± 0.3^a	194.7 ± 4.4^a	11.5 ± 0.2^a	157.7 ± 32.9^a
S. fruticosa high marsh	36.5 ± 0.3^b	6.2 ± 0.1^a	201.6 ± 6.2^a	5.2 ± 0.2^b	44.8 ± 1.8^b

Table 1. Electrical conductivity (mS cm^{-1}), pH and total content of metals (μM) for salt marsh sediments colonized by *Sarcocornia perennis*, a low marsh species, and *Sarcocornia fruticosa*, a high marsh species, in the Odiel Marshes (Southwest Iberian Peninsula). Values are mean ± SE (n=2-3). Different letters indicate significant differences between species habitats (Student t-test or Man-Whitney U-test, $p<0.05$).

II. Effects of metals on germination

Final germination percentage ranged from 41.4 ± 11.0% to 65.1 ± 5.0% for *S. perennis*, and from 72.2 ± 3.7% to 95.8 ± 2.4% for *S. fruticosa* (Table 2). Final germination percentage did not changed significantly between species, metals, concentrations or their interactions (Table S1). T_{50} was lower for *S. perennis* (8.6 ± 0.4 days) than for *S. fruticosa* (4.9 ± 0.2 days) (Table 2), without showing differences among metals and their concentrations (Table S1).

		S. perennis		*S. fruticosa*	
	Concentration (µM)	Germination (%)	T_{50} (days)	Germination (%)	T_{50} (days)
Control	0	49.1 ± 6.4	4.5 ± 0.2	94.0 ± 3.8	6.4 ± 0.4
Cu	100	58.2 ± 1.9	5.3 ± 0.9	90.4 ± 1.5	6.8 ± 0.6
	250	46.7 ± 4.4	4.1 ± 0.2	81.6 ± 1.7	1.4 ± 2.0
	500	52.3 ± 2.5	4.3 ± 0.2	75.8 ± 0.8	8.1 ± 0.7
	1000	61.6 ± 0.4	5.9 ± 0.8	80.1 ± 3.6	1.2 ± 1.5
	2000	56.4 ± 1.8	4.9 ± 0.8	72.2 ± 3.7	5.2 ± 0.9
Ni	100	65.1 ± 5.0	4.6 ± 0.8	79.6 ± 4.5	6.5 ± 0.3
	250	41.4 ± 11.0	5.5 ± 1.3	78.1 ± 1.1	9.1 ± 0.9
	500	53.4 ± 13.5	6.6 ± 1.4	69.7 ± 7.2	10.0 ± 1.9
	1000	57.6 ± 3.1	4.8 ± 0.9	84.9 ± 1.6	7.6 ± 1.5
	2000	49.9 ± 5.9	4.4 ± 0.2	76.9 ± 3.8	10.6 ± 1.1
Zn	100	64.0 ± 5.1	3.9 ± 0.1	95.8 ± 2.4	9.7 ± 1.2
	250	47.4 ± 4.2	4.5 ± 0.1	80.7 ± 5.1	9.7 ± 1.5
	500	55.6 ± 6.3	4.9 ± 0.6	83.2 ± 5.1	13.3 ± 1.3
	1000	49.2 ± 4.0	5.1 ± 0.7	89.1 ± 3.7	7.7 ± 0.8
	2000	50. 7 ± 2.9	5.4 ± 0.8	82.4 ± 1.6	9.2 ± 0.4

Table 2. Final germination percentage and the number of days necessary to reach 50% of the final germination (T50) for *Sarcocornia perennis* and *S. fruticosa* at different concentrations of Copper (Cu), Niquel (Ni) and Zinc (Zn). Values are mean ± SE (n=3).

III. Effects of metals on seedlings

The relative size of cotyledons, hypocotyl and radicle changed between species, metal concentrations, and the interaction between species and metal concentrations. Additionally, the relative size of cotyledons and radicle also changed among the tested metals, and cotyledon relative size was also affected by the interactions between species and metals, and metals and their concentrations (Table S2).

Increasing Cu concentration did not affect cotyledon and hypocotyl size of *S. perennis*, but its radicle was c. 80% shorter at concentrations higher than 250 µM Cu than at control conditions (Fig. 1A). Cotyledons, hypocotyl and radicle length of *S. fruticosa* decreased gradually and significantly at concentrations higher than 100 µM Cu than under control conditions (Figure 1B) (Table S3).

In *S. perennis*, cotyledons were 22% shorter at 2000 µM Ni than under control conditions. The longest hypocotyls were recorded at 250 µM Ni, decreasing c. 40% at concentrations higher than 500 µM Ni. Radicle length decreased more than 80% at concentrations higher than 250 µM Ni compare to control conditions (Figure 1C). In *S. fruticosa*, cotyledon length decreased gradually at higher Ni concentrations, whereas hypocotyl and radicle length decreased more than 57 and 93%, respectively, at concentrations higher than 100 µM Ni than at control conditions (Figure 1D) (Table S3).

Increasing Zn concentrations did not affect cotyledon and hypocotyl size in *S. perennis*, but its radicle was 66% shorter at 2000 µM Zn than at control conditions (Figure 1E). In *S. fruticosa*, cotyledons were c. 35% shorter at concentrations higher than 500 µM Zn than at control conditions, whereas hypocotyl and radicle growth reduction was recorded at concentrations higher than 100 µM Zn (Figure 1F) (Table S3).

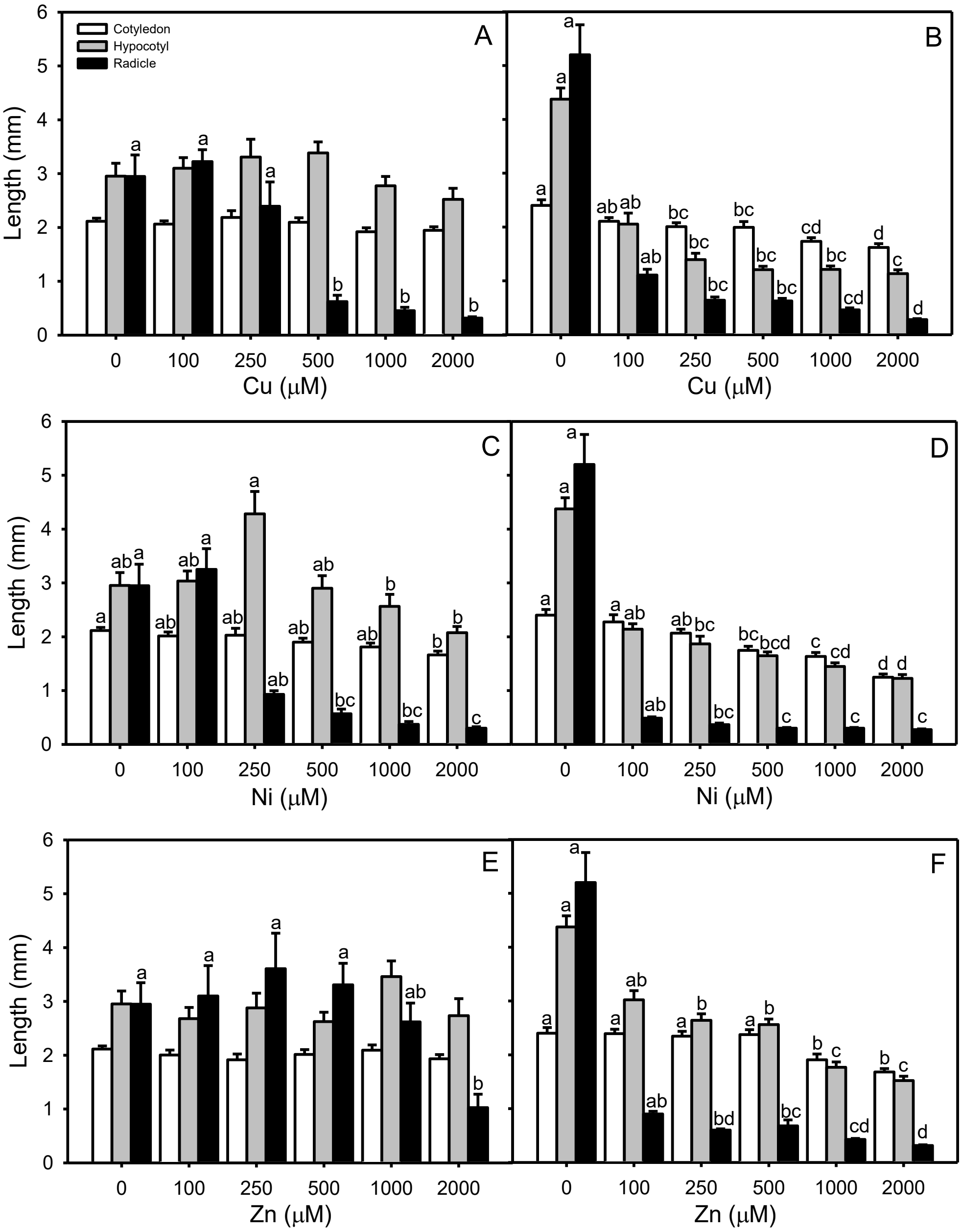

Figure 1. Size (mm) of cotyledons (white bars), hypocotyl (grey bars) and radicle (black bars) for *Sarcocornia perennis* (A, C, E) and *S. fruticosa* (B, D, F) seedlings under different (A, B) Copper (Cu), (C, D) Niquel (Ni) and (E, F) Zinc (Zn) concentrations. Different letters indicate significant differences between treatments for a given organ (Tukey HSD test or Mann-Whitney test). Values are mean ± SE (n=3).

3.4. Discussion.

Our results show that the germination of both studied halophyte species was not affected by concentrations up to 2000 µM Cu, Ni or Zn. Nevertheless, their seedling growth was reduced by increasing metal concentrations. According to our hypothesis, seedlings of *S. perennis*, a low marsh species, showed higher tolerance to the three tested metals than those of *S. fruticosa*, a high marsh halophyte.

It is generally assumed that high metal concentrations inhibit germination (Kranner & Colville, 2011) and that halophytes show higher tolerance to metals than glycophytes (Thomas et al., 1998; Van Oosten & Maggio, 2015). Mrozek and Funicelli (1982) recorded none inhibitory effects of Zn on seed germination in the low marsh halophyte *Spartina alterniflora* Loisel., and recent studies have recorded a gradual reduction in the germination percentage of different halophytes from non-inundated areas under increasing metal concentrations (Jiang et al. 2020; Zhang et al., 2020; Yao et al., 2021). Nevertheless, our results and previous studies in the joint Estuary of Odiel and Tinto rivers, one of the most metal polluted estuaries in the world, recorded none effects on the germination of halophytes from all along the intertidal gradient under metal loads up to 2000 µM (Mateos-Naranjo et al., 2011; Márquez-García et al., 2013; Infante-Izquierdo et al., 2020) (Fig. 2). In fact, the germination of the invasive halophyte *S. densiflora* is reduced in the Odiel-Tinto Estuary only when very high metal loads are combined with very acidic sediments (pH < 4.5) (Curado et al., 2010).

Regarding early seedling growth, detrimental effects of metals on both studied halophytes were more evident in their radicle than in cotyledons and hypocotyl. In this sense, Cu, Ni and Zn mainly accumulate in root tissue in many species (Sheldon and Menzies, 2005; Varhammar et al., 2019; Yusuf et al., 2011). Moreover, *S. perennis* was more tolerant to increasing metal concentrations than *S. fruticosa*. This result corroborates our hypothesis and may reflect that *S. perennis* was exposed to higher total concentrations of Ni and Zn in low marshes than *S. fruticosa* in high marshes. Moreover, sediment redox potentials in low marshes colonized by *S. perennis* (c. +100 mV) are lower than those recorded in high marshes colonized by *S. fruticosa* (between +70-270 mV) (Gallego-Tévar et al., 2018; Castillo et al., 2021), which would increase metal bioavailability at lower elevations under hypoxic conditions (Alhdad et al., 2015; Wang et al., 2012; Brito et al., 2021). In this context, *S. perennis* exhibited similar to higher tolerance to Cu, Ni and Zn than low marsh *Salicornia ramosissima* and lower tolerance than the primary colonizer *Spartina maritima* (Márquez-García et al., 2013; Infante Izquierdo et al., 2020). The sensitivity levels to Cu, Ni and Zn recorded for these three low marsh halophytes follow their position in the tidal frame and the accumulation of metals in their tissues. Thus, *Spartina maritima* presents the lowest sensitivity to metals, colonizes the lowest elevations and shows the highest metal accumulation, whereas *S. perennis* presents the highest sensitivity to metals, occupies the highest elevations and accumulates the least metals (Luque et al., 1999; Gallego-Tévar et al., 2018; Infante Izquierdo et al., 2020). Looking at halophytes from high salt marshes, our results show that *S. fruticosa* seedlings present lower metal tolerance than those of the low marsh species and similar levels to those of middle-high marsh species *Spartina densiflora* and *Atriplex halimus* (Márquez-García et al., 2013; Infante- Izquierdo et al., 2020) (Fig. 2). In this sense, previous studies showed that metal exposure led to marked reductions in seedling growth in different high marsh and salt desert halophytes (Thomas et al., 1998; Zhang et al., 2020; Duarte et al., 2021; Yao et al., 2021). According to these data, the tolerance to metals of seedlings of halophytes colonizing tidal marshes increase at lower elevation in the intertidal gradient.

Germination	No effects on final germination percentage and T_{50}[1]	No effects on final germination percentage and T_{50}	No effects on final germination percentage and T_{50}[2]	No effects on final germination percentage and T_{50}[1]	No effects on final germination percentage and T_{50}	No effects on final germination percentage. Lower T_{50} when exposed to Cu[2]
Radicle	Reduced length at > 100 µM Cu, Zn and > 50 µM Ni[1]	Reduced length at > 100 µM Cu, Ni, Zn	Reduced length at > 100 µM Cu, Ni and > 500 µM Zn[2]	Reduced length at > 250 µM Cu, Zn and > 50 µM Ni[1]	Reduced length at > 250 µM Cu, Ni and > 1000 µM Zn	No effects[2]

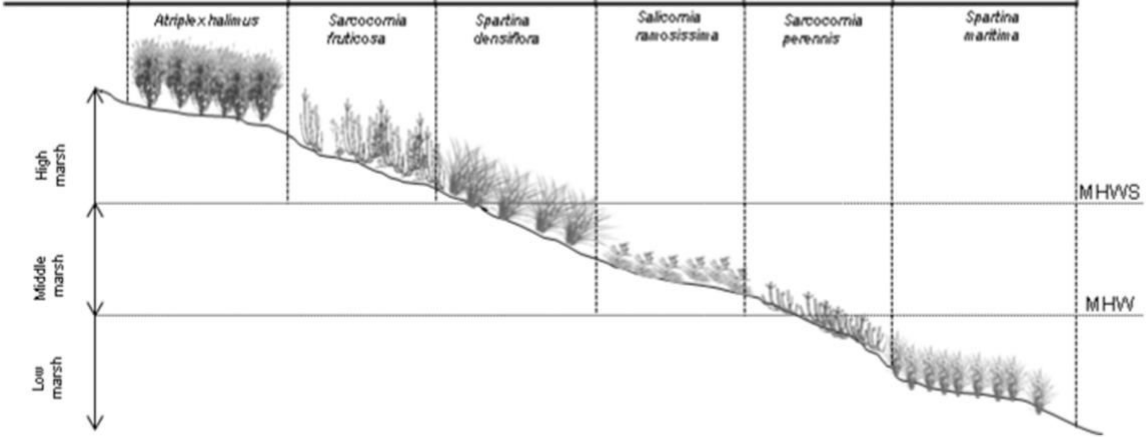

Figure 2. Main effects of metals on germination and radicle growth for six halophyte species distributed along the intertidal gradient in Odiel Marshes (Southwest Iberian Peninsula). Metals: Cu, copper; Ni, niquel; Zn, zinc. Tidal levels: MHWS, mean high water spring; MHW, mean high water. T50, number of days necessary to reach 50% of the final germination. References: 1, Márquez-García et al., 2013; 2, Infante-Izquierdo et al., 2020.

In view of our results, the recorded concentrations in solution in the Odiel Marshes for Cu (0.07-745 µM) and Zn (61 to 4220 µM) (Achterberg et al., 2003; Borrego et al., 2002; Braungardt et al., 2003; Elbaz-Poulichet et al., 2001; Fernández-Caliani et al., 1997; Galán et al., 2003; González-Pérez et al., 2008) may reduce the early development of both studied *Sarcocornia* species, which may affect their establishment mainly by diminishing their radicle growth.

3.5. Conclusions.

Based on our results, the growth of *S. fruticosa* seedling is a good candidate for ecotoxicological bioassays in salt marshes, providing a good set of metal pollution morphological biomarkers that are easy to record such as the radicle length. Additionally, our results allow identifying metal toxicity thresholds to sow *S. perennis* and *S. fruticosa* in phytoremediation projects in salt marshes.

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4. The Bioconcentration and the Translocation of Heavy Metals in Recently Consumed Salicornia ramosissima J.Woods in Highly Contaminated Estuary Marshes and Its Food Risk.

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Abstract: Salicornia species are halophyte plants that are an important source for food, pharmacy, and bioenergy. They can be consumed as a leafy vegetable, but they can accumulate heavy metals that carry a health risk when knowledge of how each species behaves in different types of soil is lacking. This present work aimed to determine to what extent *S. ramosissima* can be cultivated as food in estuaries contaminated by heavy metals and to what extent it can be used in phytoremediation works, by studying its behavior in populations that grow naturally in contaminated soils. We analyzed accumulation and translocation in different parts of the plant for 14 heavy metals and calculated the Health Risk Index value associated with their consumption as a leafy vegetable. The results obtained mean that the *S. ramosissima* plants that grow in most of the soils of this estuary are unfit for human consumption in some of the populations studied. In conclusion, *Salicornia ramosissima* J.Woods can accumulate Cd, As, and Pb among other metals—in its leaves so its consumption should be limited to plants that grow in soils free of these metals.

Keywords: safety food; Health Risk Index; salt marshes; Chenopodiaceae; heavy metals

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4.1. Introduction.

Heavy metals are enriched in the environment by human activities such as mining, industrial and traffic emissions, untreated sewage effluents, fertilizer, pesticides, etc., reaching the soil and the sediment where they become bound [1–3], generating problems by their contamination of the atmosphere, water and soil that have become a global concern [4–7]. For this reason, soil contamination by heavy metals has been regulated by various organizations and public administrations, which have established maximum permitted limits for metal concentrations in order to protect the soil and the environment, normally depending on the soil's pH value. This is the case in the European Union [8] and more specifically it is the situation defined by the Spanish government [9], which both set limits for Cd, Cr, Cu, Hg, Ni, Pb, and Zn and, more locally in our case, the government of the autonomous region of Andalusia, which fixed levels for As, Cd, Co, Cr, Cu, Ni, Pb, Tl, and Zn [10].

When heavy metals accumulate to a certain degree of concentration in soils, they can easily enter the human body by plant enrichment via the food chain, which acts as the major pathway for human exposure to heavy metals [5,7,11,12]. Unsurprisingly, food safety is fast becoming a major concern worldwide, hence the growing demand for research on the risks associated with the consumption of food contaminated by heavy metals [13–16].

The capacity of plants to absorb heavy metals from soil depends on the bioavailability of the metal in the soil and on the plant species. As vegetables are an important component of the human diet, many studies have assessed their levels of heavy metal contamination in different situations and the risk to health by their intake—specifically leafy vegetables—which potentially accumulate more heavy metals than other fruit and vegetable crops [4,13,14,17–19].

Hazards due to heavy metal intake in the human body have led many countries and agencies, such as the Food and Agriculture Organization of the United Nations, the World Health Organization [20], and the European Union [21,22] to establish tolerance levels for food and animal feed based on investigations of their effect according to their degree of toxicity or to establish rates of periodic intake of which the most widely used are PTWI (permitted tolerable weekly intake) (mg kg^{-1} body weight $week^{-1}$)—whose levels were established by the FAO and WHO [20,23]—and TUIL (Tolerable Upper Intake Level) (mg day^{-1}), whose levels are used by different agencies such as the Panel on Micronutrients of the US Institute of Medicine [24], the European Food Safety Authority [25–27], the Spanish Agency for Food Safety and Nutrition [28], and the WHO [29].

Various parameters could be used to quantify these hazards but the most common is the Health Risk Index (HRI), which is based on the Estimated Daily Intake (EDI) (mg kg^{-1} body weight day^{-1}) and on the dose reference (RfD) (mg kg^{-1} body weight day^{-1}); this parameter generally uses the values established by the United States Environmental Protection Agency [30–32], widely used for vegetable consumption [33–37].

Similar to EDI is the Chronic Daily Intake (CDI) (mg kg^{-1} body weight day^{-1}) index, which also considers the years of exposure to one food and the mean days of exposure in a year, comparing this value with the RfD value yields the Target Hazard Quotient (THQ), which has been used by several authors [15,16,19,34].

Meanwhile, degradation of agricultural land by salinization continues worldwide [38,39] due to a decrease in fresh water and groundwater; this has stimulated interest in the use of halophytes, which have more salt resistance than conventional agricultural crops [40–45] because they are naturally adapted to salt resistance, which can also extend tolerance to other toxic elements [46–49].

In Europe, halophytes play an increasingly important role in human consumption patterns, and the European Union is mulling a decision to establish maximum levels for heavy metals for such foods; monitoring has already begun of Salicornia europaea L. (an aggregate species to which *S. ramosissima* belongs) to enable an accurate estimate of exposure to be made [50] since halophytes and seaweeds could contain high concentrations of heavy metals such as arsenic in comparison to terrestrial plants [51].

As in the rest of the glassworts Salicornia species, *S. ramosissima* is an annual halophyte species in which water is stored in the succulent leaves that are opposite and fused, forming a ring around the stems [52,53]. It is a pioneer species that colonizes European and North African salt marshes, occurring in a range of salt marsh habitats [54], including those with hypersalinity conditions, due to which its seeds are capable of germinating at high salinity levels [55,56] and are an important source of food [16,42,57,58], pharmaceuticals [42,43,59–61], and bioenergy [41,43]. Its cultivation also has ecological applications, thanks to its ability to survive and to reproduce in saline environments [62], as a biofilter to recycle the water and nutrients contained in the effluents of marine aquaculture, and as a phytoremediator of saline soils and soils contaminated with heavy metals [43,63–67].

In the case of S. ramosissima, it has been proposed for phytoremediation due to its ability to accumulate Cd in its roots, but this accumulation recedes with increasing salinity, especially at high concentrations [68,69]; this species is considered as a phytoaccumulator at root level for As, Cd, Cu, Ni, Pb, and Zn and that plant growth promoting inoculation could enhance this phytoaccumulation due to its increase in plant biomass.

Salicornia species primarily inhabit saline coastal habitats, such as estuarine salt marshes [54], where heavy metals have been added to the environment by polluted water through various anthropogenic activities—such as manufacturing, mining, and agricultural industries [70]—as is the case of the estuary of the Tinto and the Odiel rivers in Huelva (SW Spain), which is one of the systems most heavily polluted by heavy metals in the world [71–73].

The aim of this present study was to determine to what extent *S. ramosissima* can be farmed for food in estuaries polluted by heavy metals and to what extent it can be used in phytoremediation work by studying its behavior in populations growing naturally in the heavy metal-contaminated soils of the Odiel river estuary. To do this, we analyzed heavy metal accumulation and translocation in different parts of the plant for 14 heavy metals: Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Tl, U, V, and Zn, mostly selected for their abundance in the area and/or for their toxic potential when eaten.

The heavy metal concentrations observed in leaves for the 14 heavy metals in all populations were compared to the limit of tolerable content in food for these metals established by the FAO/WHO [20], the European Union [21,22], and China's Food and Drug Administration [74] to verify its edibility and its commercialization in accordance with these criteria and then we calculated the Health Risk Index value associated with their consumption as a leafy vegetable.

The following hypotheses were tested: (1) Heavy metal accumulation would be site specific and depend on soil concentrations; (2) Heavy metal intake by roots could be affected by soil pH and soil conductivity; (3) Heavy metal accumulation would vary in the different habitats that this species inhabits; and (4) Heavy metal accumulation in edible parts of the plant can compromise human consumption due to its toxicity.

4.2. Materials and Methods.

I. Study Area and Species Studied.

The study was carried out in the Odiel Marshes Natural Park (Huelva; SW Spain), located in the estuary of Huelva, formed by the convergence of the rivers Tinto and Odiel, protected by law in Andalusia as a Natural Park, and internationally recognized as a Biosphere Reserve by UNESCO. The major habitats in the park are tidal marshes, canals, saltworks, and islands covered by sclerophyllous shrubland vegetation and pine woodlands [75]. Tidal salt marshes show a clear community zonation pattern, based on tidal influence and elevation [76], which are sometimes separate in different habitats [75,77,78]. In the Odiel Marshes, *S. ramosissima* inhabits four types of habitats: low marsh, medium marsh, saltpan, and saltwork.

A total of 14 populations of *Salicornia ramosissima* were sampled in May 2019 throughout the Natural Park (Figure 1), including populations collected in the four habitats mentioned (Table 1).

Table 1. Coordinates of the sample points, correspondent habitat, and mean and standard error of soil pH and soil conductivity (mS cm^{-1}).

Sampling Point	Longitude	Latitude	Habitat	Soil pH	Soil Conductivity
1	37°16′18.75″ N	6°58′59.81″ W	Saltpan	6.14 ± 0.01	38.05 ± 0.25
2	37°15′40.85″ N	6°58′34.57″ W	Saltwork	5.66 ± 0.06	24.95 ± 0.15
3	37°15′39.13″ N	6°58′37.06″ W	Saltwork	5.92 ± 0.01	32.45 ± 0.05
4	37°15′12.12″ N	6°57′59.79″ W	Low marsh	6.20 ± 0.00	31.60 ± 0.20
5	37°14′31.80″ N	6°57′56.65″ W	Saltpan	5.92 ± 0.00	32.45 ± 0.05
6	37°14′31.54″ N	6°57′54.44″ W	Saltpan	5.03 ± 0.01	23.30 ± 0.00
7	37°13′32.80″ N	6°57′52.52″ W	Medium marsh	5.11 ± 0.02	34.40 ± 0.20

Table 1. *Cont.*

Sampling Point	Longitude	Latitude	Habitat	Soil pH	Soil Conductivity
8	37°13′17.47″ N	6°57′41.09″ W	Saltpan	6.33 ± 0.02	15.60 ± 0.00
9	37°13′16.31″ N	6°57′43.75″ W	Medium marsh	6.48 ± 0.00	23.70 ± 0.40
10	37°13′04.99″ N	6°57′48.85″ W	Medium marsh	5.73 ± 0.01	36.05 ± 0.05
11	37°12′29.71″ N	6°57′32.60″ W	Low marsh	5.69 ± 0.01	36.50 ± 0.30
12	37°12′26.06″ N	6°57′33.75″ W	Saltpan	5.55 ± 0.25	28.75 ± 0.15
13	37°12′20.09″ N	6°57′04.41″ W	Medium marsh	5.96 ± 0.03	21.25 ± 0.15
14	37°11′02.84″ N	6°56′29.89″ W	Medium marsh	6.21 ± 0.01	32.00 ± 0.10

Figure 1. Location of the sample points in the Odiel Marshes Natural Park. Numbers in white circle indicate sampling points.

II. Soils Sampling and Analysis

In each sampling population, a soil sample and a sample of plant material were collected. Soil samples were collected from the *S. ramosissima* population using stainless steel cores of 50 mm height and diameter. These samples were hermetically sealed in polyethylene bags and stored at 20 C for analysis in the laboratory [6].

The day before analysis, the soil samples were thawed to room temperature. A sample of 20 cc from each soil sample was placed in a Falcon tube with 20 cc of distilled water (1:1), then homogenized by vigorous vortex shaking for two minutes, and centrifuged at 3000 g for 15 min. The supernatant was transferred to a glass tube for the rest of analysis. Electrical conductivity in the supernatant was measured by a conductivity meter (Horiba Laqua, Kyoto, Japan), and the pH was measured by a pHmeter (Crison Basic 20+, Barcelona, Spain). The pH and the conductivity analyses were performed in duplicate, and two measures of pH and conductivity were taken for each soil sample [77].

For the quantification of bioavailable metals, the samples were pretreated using the Alan and Kara protocol namely, the thawed soil samples were deposited in Petri dishes and oven-dried at 45 C for two days. A sufficient amount of soil sample was sifted using a 100 m sieve. Once sifted, 1 g of each soil sample was weighed and 40 mL of 20 mM CaCl2 added, then constantly stirred overnight at room temperature. The following day, the soil samples were centrifuged at 3000 g for 15 min to recover the supernatant fraction containing the bioavailable metals and stored at 4 C until further quantification by ICP-MS [79].

Each supernatant was five-fold diluted with 5%HNO3 (trace metal grade 65%), containing 100 ug/L of Rh as internal standard, and analyzed in an inductively coupled plasma mass spectrometer, (ICP-MS) Thermo XSeries2 (Thermo Scientific, Bremen, Germany), equipped with a MicroMist nebulizer, Ni cones, and Cetac ASX-500 autosampler (Agilent, Wilmington, DE, USA). All analyses were performed in triplicate, and three measures of each heavy metal were taken for each soil sample. The validation of the methodology was carried out using the standard reference material NIST 1646a (estuarine sediment).

The heavy metals recorded were: Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Tl, U, V, and Zn. This list includes the 10 most abundant metals in solutions and in sediments found in the Odiel Marshes Natural Park: Al, As, Cd, Co, Cu, Fe, Mn, Ni, Pb, and Zn [80–89]. The list also includes three of the heavy metals most commonly associated with poisoning in humans—lead (Pb), arsenic (As), and cadmium (Cd)—which cause significant health problems associated with neurotoxic and carcinogenic actions, even when exposed to low concentrations [90], and other metals required by living organisms in traces, such as chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), and zinc (Zn), which at excessive levels can be harmful to humans, causing degenerative diseases of the central nervous system, the cardiovascular and gastrointestinal systems, lungs, kidneys, liver, endocrine glands, and bones [91]. To complete the study, we also included V, Tl, and U.

III. Plants Sampling and Analysis.

At each of the 14 populations, 20 or more complete plants were carefully selected and transported in paper bags to the laboratory and stored at 20–25 C for analysis the following day. Plant material was processed using a modified protocol from Alzahrani et al. [92] namely, plants from each sampling point were dissected in three parts: roots; basal parts of the stem with dried leaves, referred to as stems; and the upper parts of the stems and branches with fresh and turgent leaves around, referred to as leaves. The different parts were carefully washed with ultrapure water, thoroughly dried in a forced convection oven at 60 C for 48 h and pulverized with a mortar and pestle (the mortars were previously washed by immersion in pure nitric acid for half an hour, rinsed with deionized water and dried in an oven at 60 C), and the powder stored in hermetically sealed polypropylene tubes at 4 C for analysis. A total of 50 mg of a powdered sample were mixed with 640 L HNO3 and 160 L of H2O2 in polytetrafluoroethylene vessels and incubated for 10 min. Mineralization, using a CEM Matthews microwave oven (CEM Corporation, Matthews, NC, USA, model MARS) was carried out at 800 W at room temperature, ramped up to 180ºC for 10 min, and maintained for 20 min at that temperature. Then, the solutions were prepared with up to 5 mL of ultrapure water, and the metals were analyzed with an inductively coupled plasma mass spectrometer (ICP-MS), as described in the soil analysis. All analyses were performed in triplicate, and three measures of each heavy metal in each plant part were taken for each population. The validation of the methodology was carried out using the standard reference material NIST 1573a.

IV. Translocation and Bioconcentration Factors.

The heavy metal concentrations recorded were used to estimate the translocation and the bioconcentration factors. The translocation factors (TF) were calculated by dividing the heavy metal concentrations in the different parts of the plants: stems/roots and leaves/stems; the bioconcentration factors (BCF) were calculated by dividing the heavy metal concentrations in the different parts of the plants by soil concentration [7].

V. Assessment of Food Risk to Human Health.

First, we compared the heavy metal concentrations in leaves to the limit of tolerable content in food for these metals established by the FAO/WHO [20], the European Union [21,22], and China's Food and Drug Administration [74].

For each heavy metal included in this study we also calculated the Estimated Daily Intake (EDI) (mg kg^{-1} body weight day^{-1}): EDI = (C_{metal} x Cf_{actor} x D_{food} intake)/B_w according to the concentration of metal in food (mg kg^{-1} dry weight) (C_{metal}); the conversion factor (C_{factor}) that converts the fresh vegetable weight into dry weight; the daily intake of the respective food (kg wet weight day^{-1}) (D_{food} intake); and the body weight (B_w).

For the C_{factor} in leafy vegetables, we used the value of 0.2 [93]; for the daily intake of leafy vegetables, we used the mean population data in Spain, 70.4 g, which is considerably higher than in most European countries and the United States, due to influence of the Mediterranean diet [94]; and for body weight, we used the European average of 70.8 kg [95].

Then, we calculated the Health Risk Index (HRI):

HRI = EDI/RfD, applying the United States Environmental Protection Agency values for most of the metals studied [30–32].

If the HRI is <1, then adverse health effects are not expected, but there could be adverse health effects if the HRI is >1. However, these health risk parameters could add uncertainty and limitation to the risk assessment as metals in food are not totally absorbed by humans when ingested, so metal concentrations may overestimate the actual health risk value [15].

VI. Statistical Analyses.

Statistical analyses were carried out using STATISTICA 8.0 (StatSoft Inc., Minneapolis, MN, USA) with results considered significant when p =< 0.05. The normality and the homogeneity of variance in the data series were tested using the Kolmogorov–Smirnov and the Levene tests, respectively.

In each population, the mean and standard error was calculated for soil pH and conductivity (n = 2) and for the content of the 14 heavy metals studied in soil, roots, stems and leaves (n = 3). The means of these parameters were compared among the populations using the non-parametric Kruskal–Wallis test, as none of the data series had normal distribution and homogeneity of variance. To examine the strength of association between the population means of the different parameters, we used Pearson's correlation coefficient, when the series of means had normal distribution, or otherwise Spearman's correlation coefficient, applying the Bonferroni correction to each series of analyses.

In each population, the TF root/stem, TF stem/leaf, BCF root, BCF stem, and BCF leaf were determined, and the mean and standard error for each metal was calculated. For habitat analysis, the populations were grouped in the four habitats considered:

low marsh (2 populations), medium marsh (5 populations), saltpan (5 populations) and saltwork (2 populations) (Table 1). In each habitat, the mean and standard error was calculated for soil pH and conductivity (n = 4 for low marsh and saltwork, and n = 10 for medium marsh and saltpan) and for content of the 14 heavy metals studied in soil, roots, stems and leaves (n = 6 for low marsh and saltwork, and n = 15 for medium marsh and saltpan). The means of these parameters were compared among habitats using the ANOVA test and Tukey as a post-hoc test, when the data series had normal distribution and homogeneity of variance, or otherwise, the Kruskal–Wallis test and Mann–Whitney as a post-hoc test.

In each habitat the TF root/stem, TF stem/leaf, BCF root, BCF stem, and BCF leaf were determined, and the mean and standard error for each metal was calculated and compared among habitats using the non-parametric Kruskal–Wallis test as none of the data series had normal distribution and homogeneity of variance.

4.3. Results.

I. pH and Soil Conductivity Found in Populations and Habitats

The mean soil pH in the different populations studied ranged from 5.03 to 6.48 (5.85 +- 0.11) (Table 1), with significant differences among populations (Kruskal–Wallis test

H(12.28) = 26.47, p = 0.0147). At habitat level, the means ranged from 5.79 in saltpans and saltworks to 5.95 in low and medium marshes (Figure 2), with no significant differences among habitats (ANOVA test F(4.28) = 0.279, p = 0.8399).

The mean soil conductivity in the populations ranged from 15.60 to 38.05 mS cm^{-1} (29.36+-1.77) (Table 1), with significant differences among populations (Kruskal–Wallis test H(12.28) = 26.80, p = 0.0133). At habitat level, the means ranged from 27.63 in saltpans to 34.05 in low marshes (Figure 2), with no significant differences among habitats (ANOVA test F(4.28) = 0.929, p = 0.4419).

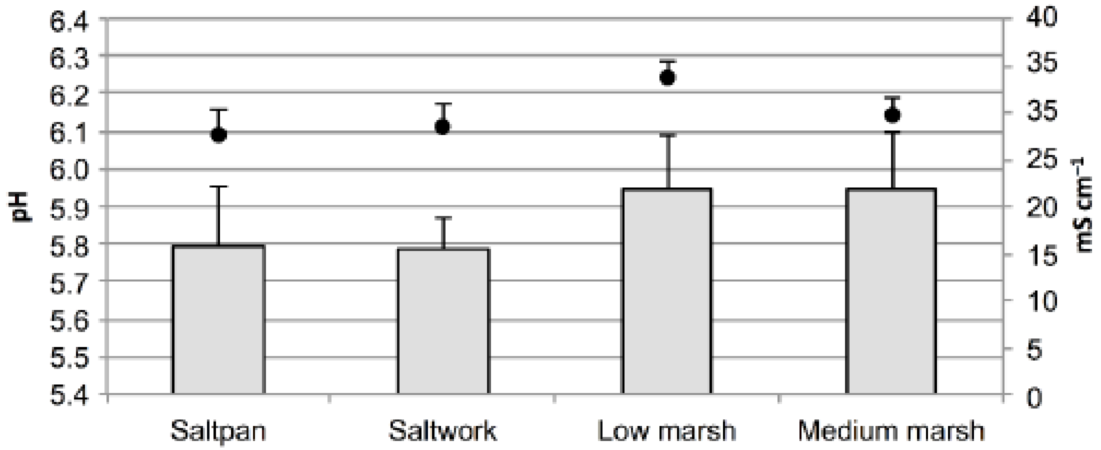

Figure 2. Mean and standard error for soil pH (grey bars) and conductivity (black point) for populations grouped by different habitats.

II. Heavy Metal Concentrations in Soil

The supplementary material table shows the heavy metal concentrations for each population in soil, roots, stems, and leaves. The order of metals by mean concentration for all populations in the soils was: Zn > Mn > Cu > Fe > Al > As > Ni > Co > Pb > V > Cr > Cd > Tl > U. For all metals, there were significant differences among soil concentrations in different populations. Significantly greater concentrations of Al, Fe, and U were found in population 1; Cr in population 3; Mn, Ni, and Cu in population 7; V in population 11; and As, Cd, Co, Pb, Tl, and Zn in population 14. Significantly lower concentrations of Cr were observed in population 2; As, Co, Cu, Tl, U, and Zn in population 3; V in population 6; Al, Fe, and Ni in population 9; and Mn and Cd in population 11.

There were no significant differences among soil concentrations in the different habitats studied for Al, Cr, Mn, Fe, and Pb (Table 2). Soil available concentrations of V and Cu were significantly greater in low-marsh soils; those of Co, Ni, Zn, As, Cd, and Tl were significantly greater in soils of the medium marshes; and those of U were significantly greater in saltpan soils. Soil available concentrations of Ni and Tl were significantly lower in low-marsh soils; those of As were lower in saltpan soils; and those of V, Co, Cu, Zn, Cd, and U were lower in saltwork soils.

Table 2. Mean and standard error of metal content (mg kg^{-1}) in soil and in the different parts of the plants for each habitat and results of the statistical test for comparing means among populations. Different letters after the means indicate significant differences among means for habitats.

Metal	Material	Low Marsh	Medium Marsh	Saltpan	Saltwork	Kruskal Wallis Test	
Al	SOIL	102.96 ± 27.32	74.83 ± 7.20	147.22 ± 47.14	49.98 ± 2.80	$H_{(3, N=42)} = 2.78$	$p = 0.4273$
	ROOT	795.79 ± 40.70	1468.69 ± 319.61	1994.83 ± 178.26	1677.93 ± 474.13	$H_{(3, N=42)} = 6.01$	$p = 0.1109$
	STEM	165.21 a ± 48.65	178.08 a ± 35.05	574.17 b ± 92.46	133.67 a ± 10.26	$H_{(3, N=42)} = 21.29$	$p = 0.0001$
	LEAF	26.24 a ± 1.19	46.57 a ± 12.94	101.24 b ± 15.28	36.67 ab ± 2.16	$H_{(3, N=42)} = 16.64$	$p = 0.0008$
V	SOIL	5.43 a ± 1.96	2.05 a ± 0.17	0.85 b ± 0.11	0.62 b ± 0.03	$H_{(3, N=42)} = 25.57$	$p = 0.0000$
	ROOT	1.82 a ± 0.15	29.19 b ± 17.38	3.73 b ± 0.48	2.63 ab ± 0.67	$H_{(3, N=42)} = 8.17$	$p = 0.0425$
	STEM	0.54 ± 0.09	0.78 ± 0.09	1.52 ± 0.39	0.49 ± 0.01	$H_{(3, N=42)} = 8.85$	$p = 0.0314$
	LEAF	0.06 a ± 0.01	0.17 b ± 0.03	0.16 b ± 0.03	0.07 ab ± 0.00	$H_{(3, N=42)} = 15.08$	$p = 0.0018$
Cr	SOIL	1.67 ± 0.15	1.68 ± 0.09	1.70 ± 0.13	1.98 ± 0.52	$H_{(3, N=42)} = 0.91$	$p = 0.8228$
	ROOT	1.02 ± 0.04	8.85 ± 4.99	1.88 ± 0.13	1.46 ± 0.36	$H_{(3, N=42)} = 6.92$	$p = 0.0744$
	STEM	0.71 ± 0.07	0.53 ± 0.03	0.86 ± 0.13	0.57 ± 0.01	$H_{(3, N=42)} = 5.84$	$p = 0.1198$
	LEAF	0.09 a ± 0.01	0.17 b ± 0.02	0.17 b ± 0.02	0.08 a ± 0.00	$H_{(3, N=42)} = 20.71$	$p = 0.0001$
Mn	SOIL	410.51 ± 180.09	1190.85 ± 336.31	799.61 ± 240.70	203.83 ± 67.45	$H_{(3, N=42)} = 2.53$	$p = 0.4704$
	ROOT	42.54 ± 8.27	93.23 ± 27.75	88.53 ± 16.86	53.45 ± 16.39	$H_{(3, N=42)} = 2.11$	$p = 0.5498$
	STEM	24.01 ab ± 5.75	68.42 a ± 15.99	44.65 ab ± 9.37	13.70 b ± 1.90	$H_{(3, N=42)} = 10.96$	$p = 0.0120$
	LEAF	5.90 a ± 0.17	43.92 b ± 10.74	28.92 b ± 7.14	18.66 ab ± 3.52	$H_{(3, N=42)} = 13.19$	$p = 0.0043$
Fe	SOIL	119.55 ± 31.78	118.98 ± 23.01	144.75 ± 41.20	29.55 ± 1.85	$H_{(3, N=42)} = 4.68$	$p = 0.1971$
	ROOT	989.57 ± 152.21	1305.59 ± 228.56	2167.75 ± 277.07	1197.09 ± 360.23	$H_{(3, N=42)} = 8.52$	$p = 0.0365$
	STEM	177.32 a ± 10.97	203.62 a ± 27.24	504.88 b ± 98.36	152.74 a ± 12.89	$H_{(3, N=42)} = 21.68$	$p = 0.0001$
	LEAF	39.57 ± 7.95	66.28 ± 17.17	58.82 ± 10.55	27.93 ± 1.15	$H_{(3, N=42)} = 3.81$	$p = 0.2832$
Co	SOIL	4.93 ab ± 1.92	14.21 a ± 2.94	9.01 ab ± 1.41	2.44 b ± 0.94	$H_{(3, N=42)} = 10.10$	$p = 0.0177$
	ROOT	1.87 ± 0.04	5.01 ± 1.84	3.31 ± 0.46	1.96 ± 0.74	$H_{(3, N=42)} = 2.76$	$p = 0.4300$
	STEM	0.63 ab ± 0.05	1.06 a ± 0.14	1.24 a ± 0.13	0.34 b ± 0.05	$H_{(3, N=42)} = 16.10$	$p = 0.0011$
	LEAF	0.10 ± 0.01	0.38 ± 0.13	0.24 ± 0.04	0.09 ± 0.01	$H_{(3, N=42)} = 6.81$	$p = 0.0782$
Ni	SOIL	4.91 a ± 0.15	12.93 b ± 2.70	10.34 b ± 1.13	6.41 ab ± 0.33	$H_{(3, N=42)} = 14.00$	$p = 0.0029$
	ROOT	1.78 ± 0.15	6.35 ± 2.70	4.62 ± 0.79	3.42 ± 0.49	$H_{(3, N=42)} = 6.99$	$p = 0.0723$
	STEM	2.06 ± 0.74	1.05 ± 0.15	1.94 ± 0.62	2.59 ± 0.99	$H_{(3, N=42)} = 2.92$	$p = 0.4035$
	LEAF	0.28 ab ± 0.05	0.45 a ± 0.04	0.24 b ± 0.02	0.26 ab ± 0.04	$H_{(3, N=42)} = 16.98$	$p = 0.0007$
Cu	SOIL	246.83 a ± 20.72	188.17 a ± 31.15	172.81 a ± 22.11	13.23 b ± 3.86	$H_{(3, N=42)} = 16.60$	$p = 0.0009$
	ROOT	43.09 ab ± 2.74	71.31 a ± 9.56	70.61 a ± 3.77	24.91 b ± 7.02	$H_{(3, N=42)} = 15.89$	$p = 0.0012$
	STEM	22.91 ab ± 3.72	30.58 ab ± 1.22	34.74 a ± 2.28	18.70 b ± 4.29	$H_{(3, N=42)} = 10.67$	$p = 0.0137$
	LEAF	3.57 a ± 0.02	8.76 b ± 1.21	8.91 b ± 0.93	6.96 ab ± 0.82	$H_{(3, N=42)} = 16.01$	$p = 0.0011$
Zn	SOIL	166.23 ab ± 54.54	1706.96 a ± 500.86	647.84 a ± 194.64	88.14 b ± 22.28	$H_{(3, N=42)} = 13.18$	$p = 0.0046$
	ROOT	50.00 ab ± 0.69	67.58 a ± 6.95	59.25 a ± 1.78	39.09 b ± 4.42	$H_{(3, N=42)} = 11.94$	$p = 0.0076$
	STEM	47.12 ab ± 1.32	57.82 a ± 2.98	49.62 a ± 2.49	34.38 b ± 0.44	$H_{(3, N=42)} = 19.78$	$p = 0.0002$
	LEAF	16.11 a ± 0.78	27.86 b ± 2.69	26.88 b ± 2.82	17.76 ab ± 0.77	$H_{(3, N=42)} = 14.56$	$p = 0.0022$
As	SOIL	8.64 ab ± 1.07	24.28 a ± 4.31	6.23 b ± 0.43	7.74 ab ± 2.68	$H_{(3, N=42)} = 22.75$	$p = 0.0000$
	ROOT	7.79 ± 1.55	13.84 ± 4.21	9.71 ± 1.28	7.78 ± 2.80	$H_{(3, N=42)} = 1.19$	$p = 0.7562$
	STEM	1.29 ± 0.04	1.49 ± 0.15	2.26 ± 0.35	1.28 ± 0.27	$H_{(3, N=42)} = 6.07$	$p = 0.1084$
	LEAF	0.22 ± 0.03	0.42 ± 0.12	0.36 ± 0.05	0.24 ± 0.06	$H_{(3, N=42)} = 2.49$	$p = 0.4776$
Cd	SOIL	0.49 ab ± 0.09	1.53 ab ± 0.57	1.05 a ± 0.09	0.39 b ± 0.02	$H_{(3, N=42)} = 13.58$	$p = 0.0035$
	ROOT	0.14 ab ± 0.01	3.69 a ± 2.36	0.20 ab ± 0.03	0.12 b ± 0.00	$H_{(3, N=42)} = 14.82$	$p = 0.0020$
	STEM	0.19 ± 0.05	0.14 ± 0.01	0.13 ± 0.01	0.12 ± 0.02	$H_{(3, N=42)} = 2.33$	$p = 0.5062$
	LEAF	0.03 ± 0.00	0.10 ± 0.01	0.08 ± 0.02	0.06 ± 0.01	$H_{(3, N=42)} = 6.93$	$p = 0.0740$
Tl	SOIL	0.19 a ± 0.01	0.44 ab ± 0.09	0.37 b ± 0.04	0.21 ab ± 0.02	$H_{(3, N=42)} = 12.83$	$p = 0.0050$
	ROOT	0.18 ± 0.03	0.49 ± 0.06	0.98 ± 0.28	0.42 ± 0.16	$H_{(3, N=42)} = 5.31$	$p = 0.1502$
	STEM	0.12 ± 0.02	0.29 ± 0.06	0.65 ± 0.18	0.34 ± 0.11	$H_{(3, N=42)} = 4.39$	$p = 0.2221$
	LEAF	0.09 ± 0.01	0.22 ± 0.04	0.70 ± 0.20	0.19 ± 0.06	$H_{(3, N=42)} = 5.82$	$p = 0.1205$
Pb	SOIL	4.74 ± 0.38	4.71 ± 0.53	3.55 ± 0.24	4.52 ± 1.37	$H_{(3, N=42)} = 3.99$	$p = 0.2625$
	ROOT	4.57 ± 0.22	5.62 ± 0.94	5.03 ± 0.56	4.66 ± 1.66	$H_{(3, N=42)} = 0.75$	$p = 0.8604$
	STEM	1.52 ± 0.30	1.96 ± 0.25	2.36 ± 0.36	1.38 ± 0.04	$H_{(3, N=42)} = 2.45$	$p = 0.4843$
	LEAF	0.31 ± 0.03	0.57 ± 0.08	0.43 ± 0.05	0.55 ± 0.03	$H_{(3, N=42)} = 7.37$	$p = 0.0611$
U	SOIL	0.27 ab ± 0.05	0.23 ab ± 0.01	0.28 a ± 0.03	0.16b ± 0.01	$H_{(3, N=42)} = 10.69$	$p = 0.0135$
	ROOT	0.25 ± 0.01	0.38 ± 0.10	0.50 ± 0.08	0.43 ± 0.16	$H_{(3, N=42)} = 6.86$	$p = 0.0764$
	STEM	0.08 ab ± 0.01	0.13 ab ± 0.03	0.16 a ± 0.01	0.07 b ± 0.02	$H_{(3, N=42)} = 11.95$	$p = 0.0075$
	LEAF	0.01a ± 0.00	0.01 ab ± 0.00	0.02 b ± 0.00	0.01 ab ± 0.00	$H_{(3, N=42)} = 11.79$	$p = 0.0081$

III. Heavy Metal Concentrations in Plants

At root level (Table S1 in Supplementary Material), the order of metals by mean concentration for all populations was: Al >Fe>Mn>Cu>Zn>V>As>Pb>Ni>Cr>Co>Cd>Tl>U. For all metals, there were significant differences among root concentrations in different populations. Significantly greater concentrations of Al and Fe were found in population 1; Tl in population 8; V, Cr, Mn, Co, Ni, Cu, As and Cd in population 10; and Zn, Pb, and U in population 13. Significantly lower concentrations of V, Cr, Fe, Co, Cu, Zn, As, Tl, and Pb were observed in population 3; Cd in population 5; Ni in population 9; and Al, Mn, and U in population 14. At habitat level, for Al, Cr, Mn, Fe, Co, Ni, As, Tl, Pb, and U, there were no significant differences among root concentrations in the different habitats studied (Table 2). Concentrations of V, Cu, Zn, and Cd were significantly greater in the roots of medium-marsh populations. Concentrations of V were significantly lower in the roots of low-marsh populations, and those of Cu, Zn, and Cd were lower in the roots of saltwork plants.

At stem level (Table S1 in Supplementary Material), the order of metals by mean concentration for all populations was: Al > Fe > Zn > Mn > Cu > Pb > Ni > As > V > Co > Cr > Tl > Cd > U. For all metals, there were significant differences among stem concentrations in different populations. Significantly greater concentrations of Ni were found in population 2; Cd in population 4; Tl in population 8; Al, V, Cr, Fe, Co, Cu, As, and Pb in population 12; and Zn and U in population 13. Significantly lower concentrations of V, Mn, and Pb were observed in population 1; Cd in population 2; Cu and Zn in population 3; Tl in population 10; Ni in population 11; and Al, Cr, Fe, Co, Zn, and U in population 14. For V, Cr, Ni, As, Cd, Tl, and Pb, there were no significant differences among stem concentrations in the different habitats studied (Table 2). Concentrations of Mn, Co, and Zn were significantly greater in stems of medium-marsh populations; and those of Al, Fe, Cu, and U were significantly greater in plants from saltpans. Concentrations of Al, Mn, Fe, Co, Cu, Zn, and U were lower in the stems of saltwork plants.

At leaf level (Table S1 in Supplementary Material), the order of metals by mean concentration for all populations was: Al > Fe > Mn > Zn > Cu > Pb > Tl > As > Ni > Co > Cr > V > Cd > U. For all metals, there were significant differences among leaf concentrations in different populations. Significantly greater concentrations of U were found in population 1; V, Mn, Fe, Co, Ni, and Cu in population 4; Al, Cr, Zn, and Cd in population 7; Tl in population 10; and Pb in population 11. Significantly lower concentrations of V, Cu, and Zn were observed in population 3; Cd and Pb in population 6; Fe in population 8; Al, Co, and As in population 11; Mn in population 12; Ni in population 13; and Cr, Tl, and U in population 14. For Fe, Co, As, Cd, Tl, and Pb, there were no significant differences among leaf concentrations in the different habitats studied (Table 2). Concentrations of V, Cr, Mn, Ni, Cu, and Zn were significantly greater in leaves of medium-marsh populations; and those of Al and U were significantly greater in plants from saltpans. Concentrations of Al, V, Mn, Zn, and U were significantly lower in leaves of low-marsh populations; those of Ni were lower in leaves of saltpan plants; and those of Cr were lower in the leaves of saltwork plants.

There was no significant correlation in any of the metals studied between soil pH or soil conductivity and metal concentration in the roots. Neither was there any significant correlation in any of the metals between soil concentrations and concentrations in roots, stems, or leaves, except in the case of concentration in soil and leaf for Fe (Spearman correlation coefficient $r = 0.736$; $p = 0.0027$). Among correlations between concentration in the different parts of the plant, the only significant correlations appeared between metal concentration in the root and the stem for Tl (Spearman correlation coefficient $r = 0.851$; $p = 0.0001$) and for U (Spearman correlation coefficient $r = 0.885$; $p = 0.0000$).

3.4. Translocation and Bioconcentration Factors

Figure 3 shows values, means, and standard error values for BCF and for TF for all the metals studied in the populations. Maximum BCF means in the roots were observed for Al (21.6) and Fe (28.3), with values that exceeded 1 in all populations. V, Tl, Pb, and U showed BCF means in the roots greater than 1, respectively, in 12, 9, 9, and 10 populations. For the rest of the metals, BCF means in the roots were lower than 1, but Cr surpassed this limit in 5 populations: Mn in 3; Co in 2; Cu in 4; Zn in 1; and As in 6. The means were lower than 1 in all the populations for Ni and Cd.

For the stems (Figure 3), BCF means were greater than 1 for Al, which showed a mean value of 4.8 and values above 1 in 12 populations; Fe had a mean value of 6.8, and values greater than 1 in 11 populations; and Tl presented a mean value of 1.2, with values greater than 1 in 6 populations. The rest of the metals presented means lower than 1, but V concentrations exceeded this limit in 4 populations; likewise, Cr in 1 population; Mn in 2; Cu in 2; Pb in 1; and U in 1.

For the leaves (Figure 3), BCF means were lower than 1 for all the metals studied, except for Al, which showed a mean value of 1 and values greater than 1 in 5 populations; Fe had a mean value of 1.2, with values greater than 1 in 4 populations; and Tl presented a mean value of 1, with values greater than 1 in 6 populations. In the rest of the metals, only 1 population exceeded a value of 1 for Cu.

For all the metals studied, mean TF factors between root and stem were lower than 1 (Figure 3). However, 2 populations surpassed this level for Mn; 1 for Co; 3 for Ni; 2 for Cu; 5 for Zn; 2 for Cd; 1 for Tl; and 1 for Pb. For TF between stem and leaf (Figure 3), except Mn which showed a mean value of 1.1 and surpassed the limit of 1 in 6 populations, the rest of the means for the metals were lower than 1, with this level only surpassed in 1 population for Al; 6 for Mn; 1 for Fe; 2 for Zn; 3 for Cd; 3 for Tl; and 1 for U.

The mean values for BCF and TF in the different habitats showed no significant differences among them, except in the case of BCF in stems for As (Kruskal–Wallis test: $H(3.14) = 8.49$, $p = 0.0369$) that reached its significant greatest value in saltpans (Figure 4).

3.5. Assessment of Food Risk to Human Health

Table 3 presents the values for the limit for heavy metal content in food, PTWI, TUIL, R_fD, and the minimum and maximum values calculated for EDI and HRI based on the minimum and maximum concentrations observed in the different populations for each heavy metal studied.

For Cd, no population reached the limit established by the FAO/WHO [20] and ChFDA [74] for leafy vegetables, but in 3 of the 14 populations studied the Cd concentrations in leaves surpassed the limit for leafy vegetables established by EUCR 2021/1323 [21]. For Pb, no population reached the limit established by the FAO/WHO [20] for food in general, but in 11 of the 14 populations Pb concentrations in leaves exceeded the limit for leafy vegetables set by EUCR 2021/1317 [22] and ChFDA [74]. In the case of Cr, no population reached the limit established by ChFDA [74] for vegetables, while for As, 3 of the 14 populations exceeded this limit.

For HRI, all the values calculated were lower than 1 except for Tl in which HRI ranged from 1.2 to 37.2 in the populations studied.

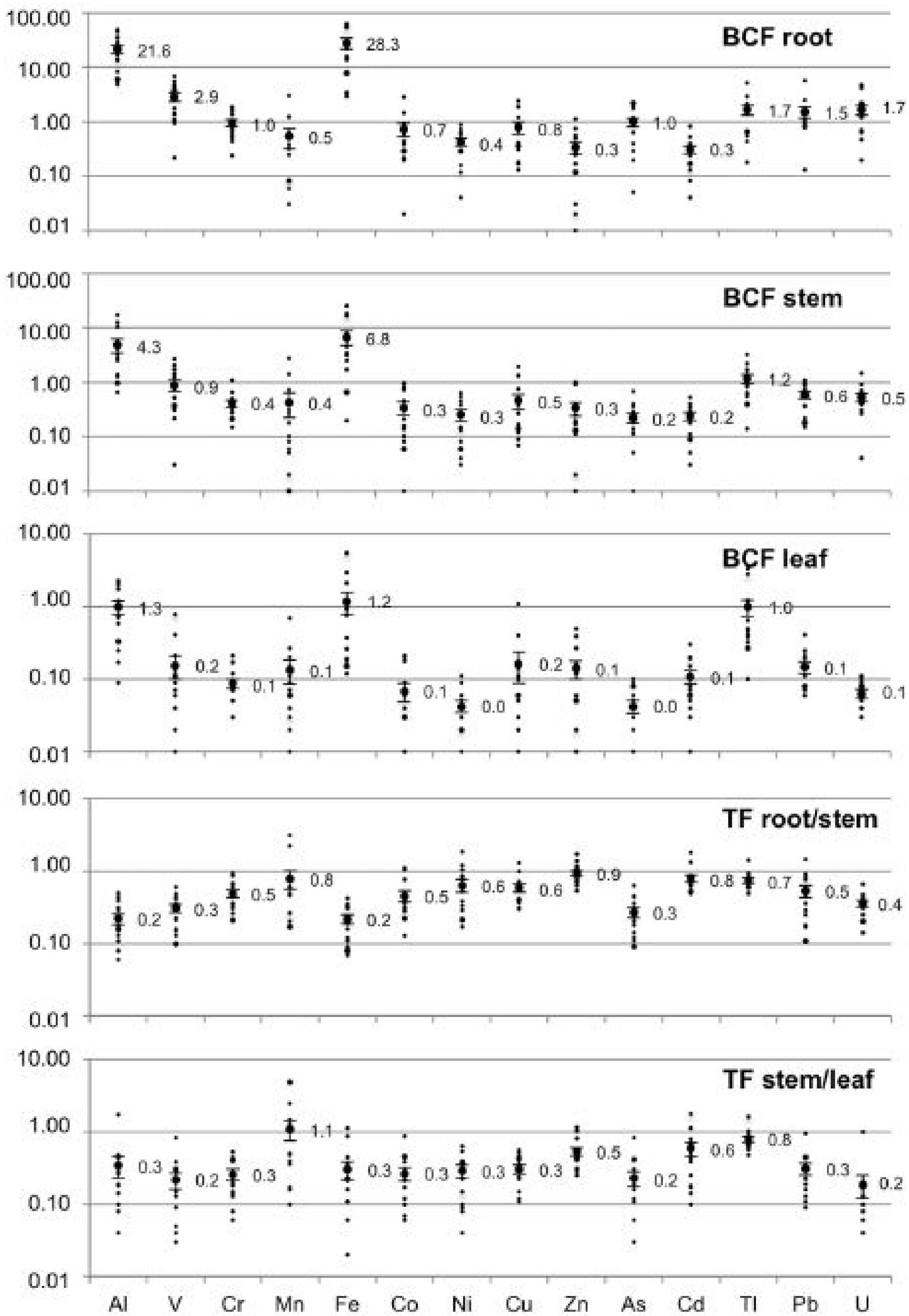

Figure 3. Values for the different populations, mean and standard error for bioaccumulation factors (BCF) in roots, stems, and leaves, and translocation factors (TF) from roots to stems and from stems to leaves, represented in logarithmic y axis.

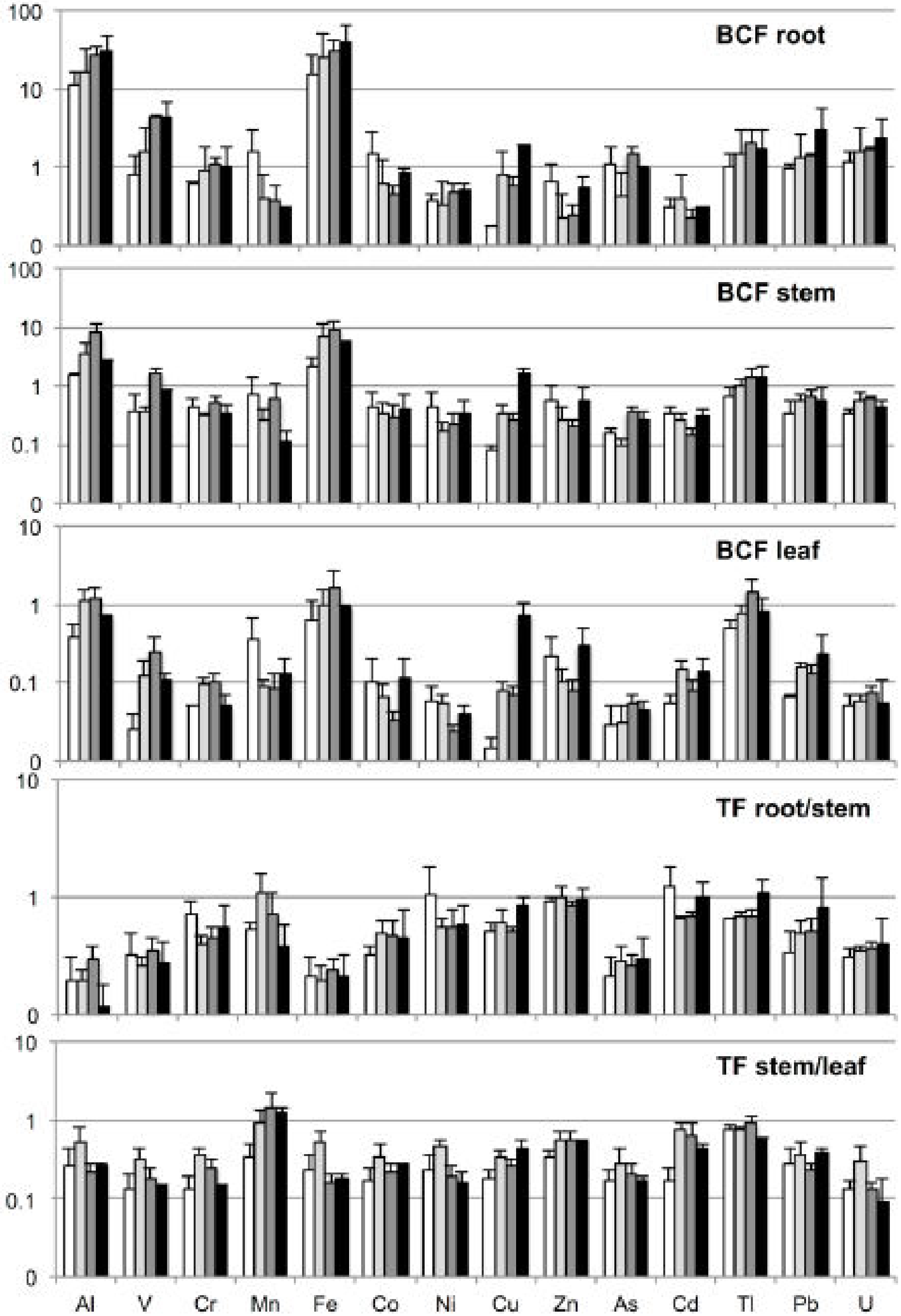

Figure 4. Mean and standard error for bioaccumulation factors in roots, stems, and leaves, and translocation factors from roots to stems and from stems to leaves in the habitats studied: low marsh (white bars); medium marsh (light grey bars); saltpan (dark grey bars); and saltwork (black bars).

Table 3. Limit content in food, PTWI (Permited Tolerable Weekly Intake), TUIL (Tolerable Upper Intake Level), RfD (Oral Reference Dose), and minimum and maximum values calculated for EDI (Estimated Daily Intake) and HRI (Health Risk Index) based on the minimum and maximum concentrations observed in the different populations for each heavy metal studied.

Metal	Limit Content in Food	PTWI	TUIL	R_fD	EDI Min.	EDI Max.	HRI Min.	HRI Max.
Al	-	2 (1)	-	1(14)	0.001352	0.034180	0.001352	0.034180
V	-	-	1.8 (2,3)	0.005(14)	0.000008	0.000082	0.001600	0.016400
Cr	0.5 for vegetables (15)	-	-	0.003(14)	0.000014	0.000064	0.004640	0.021213
Mn	-	-	11 (2)	0.14(14)	0.001104	0.022785	0.007884	0.162747
Fe	-	0.8 (4)	45 (2)	0.7(14)	0.003697	0.038215	0.005281	0.054593
Co	-	-	-	0.0003(14)	0.000012	0.000274	0.040000	0.913333
Ni	-	-	1 (2); 0.15–0.90 (5)	0.02(14)	0.000028	0.000139	0.001392	0.006960
Cu	-	-	10 (2); 2–3 (4); 5 (6)	0.04(14)	0.000708	0.003248	0.017699	0.081189
Zn	-	1(4)	40 (2); 25 (6)	0.3(14)	0.002917	0.009319	0.009725	0.031064
As	0.5 for vegetables (15)	0.015 (7)	0.003–0.008 (8)	0.0003(12)	0.000014	0.000263	0.046403	0.875028
Cd	0.2 (7); 0.1 (9); 0.2 (15) for leaf vegetables	0.025 (4); 0.0025 (10)	-	0.0001(14)	0.000002	0.000046	0.019890	0.457400
Tl	-	-	-	0.00001(14)	0.000012	0.000372	1.200000	37.200000
Pb	2 for food (7); 0.3 (11); 0.3 (15) for leaf vegetables	0.025 (16)	-	0.004(13)	0.000039	0.000181	0.008452	0.045243
U	-	-	-	0.0002(14)	0.000000	0.000008	0.000000	0.040000

Limit content in food (mg kg^{-1} dry weight), PTWI (mg kg^{-1} body weight $week^{-1}$), TUIL (mg day^{-1}), RfD (mg kg^{-1} body weight day^{-1}). Numbers between brackets: (1) [23]; (2) [24]; (3) [25]; (4) [29]; (5) [28]; (6) [26]; (7) [20]; (8) [27]; (9) [21]; (10) [96]; (11) [22]; (12) [32]; (13) [15]; (14) [31]; (15) [74]; and (16) [97].

4.4. Discussion.

I. pH and Soil Conductivity

It has been described that soil conductivity and pH affect metal availability [1,98–100]. According to the United States Department of Agriculture Soil Survey Division and the United States Division of Soil Survey [102], we can classify the soils in our study as strongly acidic (populations 6 and 7), moderately acidic (populations 2, 3, 5, 10, 11, 12, and 13), and slightly acidic (populations 1, 4, 8, 9, and 14), which fits with the calculations made by Bermejo et al. [87] in the Odiel estuary, who determined that water acidity decreases as a consequence of the tidal action of sea salt water in the estuary. In terms of salinity, all the populations' soils were strongly saline [103].

At habitat level, this study's values for pH and conductivity for low- and mediummarsh populations matched those obtained by Contreras-Cruzado et al. [77] in other tidal marshes of the Gulf of Cádiz (SW Iberian Peninsula), who also found no significant differences between the means of these habitats for both parameters. Likewise, our data for pH and for conductivity for saltpan populations coincide with those obtained by Polo-Ávila et al. [78] in the Odiel estuary.

II. Heavy Metal Concentrations Found in Soil

The differing concentrations of metals observed in the sampled populations of *S. ramosissima* may be due to the variations of heavy metal concentrations in soils due to their spatial location, which affects their level of exposure to fluvial or marine influence, their location with respect to industrial effluents, and their sediment context [82,104]; it could also be due to soil characteristics such as pH, cation exchange capacity, clay content, and organic matter content [1,13,98,99].

The available Cu, Ni, Zn, As, Cd, and Mn concentrations determined in the soils in our study were within the ranges described by other authors who had previously studied their available content in sediments in the Odiel and Tinto estuary [82,83], however, our concentrations were lower for As and Cu than those registered by Mesa-Marín et al. [69] in a location of the Odiel marshes; these authors observed a more than two-fold increase in these marshes in the concentration of Pb and As compared to data compiled in the last decade due to anthropogenic influence, as this increase has not been observed in other salt-marshes of the province with lower human activity [69,101]. Our results are of the same order of magnitude but somewhat less than those of other authors who studied their total concentrations in sediments in the same area, for example, the available Cr (1.26–2.70 mg kg^{-1}) and Fe (11.58–431.49 mg kg^{-1}) observed in concentrations were lower than the total concentrations registered previously in sediments, 8–5003 mg kg^{-1} and 9168–88,429 mg kg^{-1}, respectively [73,86–89].

In the case of Pb, the available concentrations we observed (1.45–8.00 mg kg^{-1}) were much less than the available concentrations registered by Borrego et al. [82] (58–2060 mg kg^{-1}) and by Mesa-Marín [69] (1139.85 mg kg^{-1}), who classified the soils of the Odiel estuary as heavily polluted and less than the concentrations in total sediments observed by other authors (19–1660 mg kg^{-1}) [73,86–89].

According to levels for soil contamination established by EUCD 1986/278 [8], SGRD 1310/1990 [9], and Andalusia's Ministry of Environment [10], for soils with a pH under 7, 11 populations surpassed the limit of 50 mg kg^{-1} for Cu; 9 populations surpassed the European and the Spanish limit of 150 mg kg^{-1} for Zn (8 surpassed the Andalusian limit of 200 mg kg^{-1}); 4 populations surpassed the limit of 1 mg kg^{-1} for Cd (1 surpassed the Andalusian limit of 2 mg kg^{-1}); 3 populations surpassed the Andalusian limit of 20 mg kg^{-1} for As; 2 populations surpassed the Andalusian limit of 20 mg kg^{-1} for Co; 1

population surpassed the European and the Spanish limit of 30 mg kg^{-1}, but none exceeded the Andalusian limit of 40 mg kg^{-1} for Ni; 1 population surpassed the Andalusian limit of 1 mg kg^{-1} for Ti; and none of the populations surpassed the European, Spanish, and Andalusian limit of 50 mg kg^{-1} for Pb; nor the Spanish and Andalusian limit of 100 mg kg-1 for Cr.

The greatest soil concentrations of 9 of the 14 heavy metals studied were reached at population or habitat level in the middle-marsh populations. This habitat showed the highest levels for soil organic matter content and soil conductivity, and it had the most acidic soils among the marsh habitats [77], factors that favored metal availability and could explain the greater concentrations in this habitat [1,98,99]. Low pH affects cationic metal availability since the hydrogen ion has a higher affinity for negative charges on the colloids, releasing metals [1,100]; some authors have suggested that the availability of heavy metals increases with a rise in salinity, caused by greater ionic strength that raises the level of concentration of the metals released [48,105,106]. This habitat also presented the highest levels for soil water content, due to twice-daily flooding by tides and evaporation, which increases the concentration of the solute dissolved thus improving precipitation, mainly during summer drought [77].

The soil in saltpan population 1 reached the highest concentrations in 3 of the 14 metals studied, namely Al, Fe, and U, and its concentrations of Al and Fe were much greater than those of the rest of the populations. This could be due to the historical location of this sampling point, next to an old ship-loading dock where minerals arrived by train from the mines of Tharsis (Huelva) [107].

The soils of the saltwork populations showed the lowest concentrations in 9 of the 14 heavy metals studied, and none surpassed the contamination levels established by European, Spanish, and Andalusian regulations. This is undoubtedly due to the fact that these habitats are not connected to the Odiel river, as they are inundated with seawater from open ocean via the Punta Umbría estuary (Figure 1) that flows through shallow ponds, where it evaporates and deposits sodium chloride in crystallizing ponds [82].

As commented before, pH and salinity affect heavymetal availability in soil [1,48,101,106]. However, in none of the metals studied were there significant correlations between soil pH or soil conductivity and the available metal concentrations in the soils. Therefore, it is logical to think that the different concentrations observed in the populations were due to the heterogeneity of the estuary in terms of total content in the soil, as indicated in other studies of the area [86–89].

III. Heavy Metal Concentrations in Plants

The ranges of concentrations of As, Cr, Fe, Mn, and Ni in the different parts of *S. ramosissima* found in the populations studied include the means obtained by Luque et al. [108] for the whole plant in the same species and in the same estuary, but the means for concentrations that these authors registered for Cu (279 mg kg^{-1}), Pb (51.6 mg kg^{-1}), and Zn (348 mg kg^{-1}) exceeded the range observed in our study.

The data obtained in this study for Mn, Fe, Ni, Cu, Zn, As, and Pb matched those of Sánchez-Gavilán et al. [109] for whole plants of *S. patula* Duval-Jouve in a location near the Odiel Marshes Natural Park as well as the data obtained by Mesa-Marín et al. [69] for Cd and Ni. However, these authors recorded higher values than ours for As, Cu, Pb, and Zn.

At population or habitat level, as was the case with soil metal concentrations, the middle marsh showed the highest root concentrations for 11 of the 14 heavy metals studied, and the saltworks showed the lowest root concentrations for 10 heavy metals. However, at stem level, the saltworks showed the highest stem concentrations for 10 heavy metals and the lowest concentrations for 7. At leaf level, the highest concentrations were reached in the medium marsh for 8 metals, and the lowest concentrations in leaves were reached in the low marsh for 7 metals.

We found no significant correlations in any of the metals studied between available soil concentration and concentrations in roots, as Sánchez-Gavilán et al. [109] observed for Fe concentrations in whole plants and total soil content in Salicornia patula, and as Otte et al. [105] observed for Cd, Cu, and Zinc in different parts of plants of Spartina anglica C.E. Hubb. and Aster tripolium L., when using fresh soil samples, but they found some significant correlations when they used dry soil samples. In Salicornia spp., Williams et al. [110] found that metal concentrations within sediments were not reflected in the plants, except for Zn; and Smillie [64] found significant correlations with both the roots and the aerial portion of the plants of Salicornia spp. with sediment Cu and Zn concentrations, although he found no significant relationships with either Mn or Fe.

This is not unusual as Greger [1] observed when establishing that the phytoavailability of metals for a plant species need not correlate linearly with the bioavailable metal concentration in the soil or the total metal concentration, and so *S. ramossisima* was not included in the indicator group of plants, as defined by this author, in which tissue concentration reflected external metal concentration. We also found no significant correlation in any of the metals studied between soil pH or soil conductivity and metal concentrations in roots.

Many reasons could explain this lack of correlation regarding the absorption of heavy metals, including metal species, soil properties, and salinity [3,111–113]. Other reasons could be the exclusion of metal ion uptake at root level [114], saturation in root tissues at high levels of soil concentration [1,7], transpiration rates [115], the presence of root exudates that can improve the solubility of heavy metal ions and thus enhance metal accumulation in plants, such as Pb in Salicornia europaea [116], or the interactions among metals for absorption [4]. Furthermore, Smillie [64] and Khalilzadeh et al. [117] found in Salicornia spp. and *S. europaea*, respectively, that concentrations of Mn, Zn, Cu, Cd, Pb, and Ni in roots and aerial parts changed significantly in the plant development stage. Additionally, in dense populations, as in the case of *S. ramosissima* [78], the plants compete in the uptake of metals and uptake efficiency diminishes [100]; it is possible that there were differences in the metal uptake mechanism among populations of the same species due to the genotypic differences among them [1,14].

IV. Translocation and Bioconcentration Factors

Plants with a high Bioconcentration Factor (BCF) indicate efficient metal transport from soil to root, and they are called hyperaccumulators. Plants with a high Translocation Factor (TF) show efficient metal translocation from root to stem [7,118–120]. Both factors are important in the scientific evaluation of risks that chemicals may pose to humans and the environment, and they are a current focus of regulatory initiatives [121].

For Cr, Mn, Co, Ni, Cu, Zn, As, and Cd, the mean concentrations in roots were lower than the concentrations available in the soil, so their BCF was less than 1 in most of their populations, therefore, *S. ramosissima* could be considered a root excluder [114,122]. For V, Tl, Pb, and U, the BCF values ranged between 1 and 3, with most of their populations having values greater than 1. These results coincide with those observed by Fuente et al. [123] and Sánchez-Gavilán et al. [109] in S. patula. They compared the concentrations in whole plants with the concentrations of total metals in the soil of Mn, Ni, Cu, Zn, and As, and they found a BCF of less than 1 in a population near the Odiel Marshes Natural Park and in a population of the Tinto River estuary and with those obtained for Zn, Cu, Pb, and Ni by Khalilzadeh et al. [117] in the roots and the stems of S. europaea plants.

Our results are also similar to those for the perennial shrub *Arthrocnemum subterminale* (Parish) Standl. (known as Salicornia subterminalis Parish) for Zn, Cu, Cd, Pb, and for As by Sánchez-Martínez et al. [124], who observed BCF values in the roots of two populations that were lower than 1, except in one population for Cu (4.25) and Zn (1.03). In the case of Cd, Pedro et al. [68] and Pérez-Romero et al. [125] proposed *S. ramosissima* for the phytoremediation of contaminated soil due to its capacity to accumulate Cd in the roots, with a BCF above 1, but the former authors observed this accumulation in a non-saline medium and the latter observed it in a growth medium of 171 mM NaCl, which is in the lower range for soil salinity observed in this paper.

Maximum BCF means in the roots were observed in Al (21.6) and Fe (28.3), exceeding 1 in all populations. It seems that *S. ramosissima* could accumulate these metals in the root in concentrations higher than those in the soil, which indicates that there must be mechanisms at work that enhance their uptake. This could be related to pH and the redox potential in the rhizosphere, especially in the case of Al and Fe [126], as plants that live in environments with low redox potential, as in salt marsh plants, are able to transport the oxygen produced in photosynthesis to the roots and release it to the rhizosphere; this increase in the redox potential releases the metals from sulfides and the plant can take them up [1,127]. Additionally, some plants in the presence of low bioavailable Fe are able to synthesize and to release phytosiderophores, macromolecules that can release Fe and other metals from colloids and transport the complex to the root tissue [128].

BCF in stems and leaves was less than 1 for V, Cr, Mn, Co, Ni, Cu, Zn, As, Cd, Pb, and U. These results coincide with those obtained by Sánchez-Martínez et al. [124] in the perennial shrub *Arthrocnemum subterminale* in the presence of Zn, Cu, Cd, Pb, and As, based on the results of Liu et al. [129] for Cd, Pb, Cr, and AS in 23 plant species under natural conditions in China. Our results also fit with those of Pal et al. [36] for Cd, Pb, Cu, Zn, Cr, and Ni in six plant species irrigated with domestic wastewater in India and with those of Chabchoubi et al. [16] for Zn, Cu, Pb, Cd, Cr, and Ni in three plants, including the perennial scrub *Sarcocornia fruticosa* (L.) A.J. Scott (known as *Salicornia arabica* L.), which grows in phosphogypsum-contaminated fields in Tunisia.

For Al, Fe, and Tl, the mean BCF values in stems and leaves were close to 1, exceeding 1 for the stem values in most of the populations, while for the leaves the majority of the populations had values lower than 1. These results are compatible with those of Sánchez-Gavilán et al. [109] for *S. patula* and with those of Fuente et al. [123] who found in this species values that exceeded 1 for Mn, Fe, Ni, Cu, Zn, As, and Pb in whole plants in a population located in the Tinto River estuary; they also observed similar results for populations of *Arthrocnemum macrostachyum* (Moric.) K. Koch, *Sarcocornia perennis* (Mill.) AJ Scott and *Halimione portulacoides* Aellen, other halophytic species of the Chenopodiaceae family.

So, we can assume that *S. ramosissima* is not an accumulator plant in terms of the description by Nikalje and Suprasanna [114]. Additionally, as expressed by other authors, hyperaccumulator plants may accumulate more than 100 mg kg^{-1} for Cd and As; 1000 mg kg^{-1} for Co, Cu, Cr, Ni, and Pb; and 10,000 mg kg^{-1} for Zn and Mn [130 133]. These levels were not reached by *S. ramosissima* in any of the populations studied.

As expected in view of the BCF results, TF values were lower than 1 or close to 1 for all the metals studied in root-stem and stem-leaf transport, which indicates a trend to reduce metal concentration from roots to stems and from stems to leaves, a common behavior, as during their transportation through the plant, metals become bound largely to the cell walls [1]. Additionally, some metals are retained at root level, as in Pb and Ni [16]. What is remarkable is the low TF values from root to stem for Al and Fe, with their concentrations reducing drastically from root to stem, as observed by Kabata-Pendias [134].

Our results coincide with those by Pedro et al. [68] who observed in *S. ramosissima* that Cd accumulation in the roots exceeded that of the aerial portions in all of the Cd concentrations tested. Our findings also match those by Mesa-Marín et al. [69] for As, Cd, Cu, Ni, Pb, and Zn, and the TF between roots and leaves observed by Sánchez-Martínez et al. [124] in the perennial shrub *Arthrocnemum subterminale* (Parish) Standl (known as *Salicornia subterminalis* Parish), [16]. In contrast, it has been observed that in *Sarcocornia fruticosa* the concentrations of Cu and Zn in leaves were higher compared to roots and stems, with Cu concentrations in leaves 15 times higher than in roots, and 24 times higher than in stems, and 1.8 times higher than in roots and 2.3 times higher than in stems for Zn, while our study found the same decreasing order of concentrations from the underground part to the aerial part for these same metals. The difference in this different pattern could be explained by the perennial nature of Sarcocornia species [57], so that the time of exposure to accumulation is greater than in the annual *Salicornia ramosissima* and, as in other leafy plants, it could accumulate heavy metals with increasing exposure time [135].

A major concentration in leaves rather than in roots has also been observed by other authors in some leafy vegetables. Intawongse and Dean [136] analyzed heavy metal concentrations in lettuce, spinach, radish, and carrot, and they observed that the accumulation of Cd, Zn, and Mn was higher in the leafy portion than in the root portion of the plants. This greater accumulation of heavy metals in the leaves of leafy vegetables could be due to the plant's higher transpiration rate in order to maintain growth and the moisture content of the plant [115].

The means values of BCF and TF in the different habitats showed no significant differences among them, except in the case of BCF in stems for As, which reached its significant greatest value in saltpans.

V. Assessment of Food Risk to Human Health.

The results obtained make it impossible for European consumers to contemplate consumption of the *S. ramosissima* plants that grow in most of the soils in this estuary because in 3 of the 14 populations studied the Cd concentrations in the leaf exceeded the limit of heavy metal content for leafy vegetables, 11 populations exceeded the Pb limit in the leaf, and some populations exceeded the limit for As [21,22,74]. If we compare our results to the limits set by the FAO/WHO [20] for foods in general, the limit established for any of the metals was not exceeded.

Sánchez-Gavilán et al. [109] stated that concentrations of Cu in plants of *S. patula* from different areas of the Iberian Peninsula surpassed the intake levels of 10 mg kg^{-1} established by USIM [24] on the basis that these concentrations in plants reached values greater than 10 mg kg^{-1} dry weight. However, this intake level established by USIM [24] is the daily upper limit of Cu with no observed adverse effect in adults and so is a daily TUIL (Tolerable Upper Intake Level), and it depends on the daily intake of fresh vegetables consumed each day and on the conversion factor that converts the fresh vegetable weight into dry weight, not only on the Cu concentration on dry weight. In the same way, these authors stated that Pb concentrations in *S. patula* plants also surpassed the intake levels of 0.01 mg kg^{-1}, which they attributed to EFSA [97], according to which reference this value given as a limit for food is a PTWI (Permitted Tolerable Weekly Intake) of 25 g kg^{-1} b.w., which these authors stated was no longer appropriate.

Our results also show that human consumption is not recommended based on the HRI values obtained for Tl, which was present in all populations at a level above 1, ranging from 1.2 to 37.2.

Cd was present in soil and in plant parts in low concentrations, and S. ramosissima showed BCF and TF values of less than 1. However, this metal had been classified as carcinogenic in humans by the International Agency for Research on Cancer, with numerous toxic effects, the main outcome being kidney dysfunction [28], hence the limits established by EUCR 2021/1323 [21] for its content in leafy vegetables even though the Tolerable Weekly Intake (TWI) estimated by EUCR 2014/193 [96] (0.0025 mg kg^{-1} body weight) is very near to the mean weekly intake of Cd in Spain (0.00203 mg kg^{-1} body weight) [28]. Nevertheless, HRI results for Cd showed no potential risk by intake of this plant as food, and this discrepancy between the established limit in food content and the risk calculated by its ingestion has also been observed in *Sarcocornia ruticose* (=*Salicornia arabica*), which grows in phosphogypsum-contaminated fields in Tunisia, where Chabchoubi et al. [16] studied concentrations of Zn, Cu, Pb, Cd, Cr and Ni in soils and plants, and they found that Cd concentrations in leaves were higher than the FAO/WHO [20] threshold, but no risk was observed to human health due to its intake as food, according to the Target Hazard Quotient (THQ) values they calculated.

The Pb concentrations observed in soil were relatively low with respect to the other heavy metals studied and with respect to the concentrations observed by other authors, as commented above; none of the populations studied surpassed the Pb soil contamination levels established by European [8], Spanish [9], and Andalusian [10] regulators. However, it showed a mean BCF root of 1.5, which means a certain capacity of these populations to increase their Pd content compared to soil content, which is in accordance with observations by Kaviani et al. [66], who stated that *Salicornia iranica* is able to accumulate Pb, and it can be used for phytoremediation in polluted soils.

Pb has been associated with a decrease in intelligence quotient in children and with an increase in blood pressure in adults [29], and it is listed as a probable carcinogen in humans [28]. Our results showed that, in most of the populations studied, Pb concentrations in the leaf surpassed the limit for content of this metal in leafy vegetables established by EUCR 2021/1317 [22], although the HRI was lower than 1 in all of them. Similar contradictory results were found by Rahmdel et al. [38], who studied Pb, Cd, Cu, Zn, Ni, and Co contamination in 100 samples of leafy vegetables cultivated in different agricultural sites in Iran and found that, according to the HRI, the consumption of these leafy vegetables was less than the established safety limit, with the Pb level observed exceeding the permissible limit set by the FAO/WHO [20] in 44% of samples taken.

Tl concentrations in soil were low, making it the second lowest heavy metal in terms of concentration, but it had a BCF leaf value above 1 in 9 of the populations studied, making it the third highest metal in BCF leaf value, behind Al and Fe; thus, its concentration in leaves was greater than for other metals that were more abundant in the soil. Tl represents a rare cause of heavy metal poisoning. Because thallium is not a common environmental or workplace contaminant and it is not readily available to the public, any thallium-poisoned patient should be considered a victim of a criminal act until proven otherwise [137]. For this reason, there is little information on this metal, and its low R_fD value (0.00001 mg kg^{-1} body weight day^{-1}) as estimated by USEPA [31] is an unreliable estimate, perhaps spanning an order of magnitude, with the previous RfD values for its soluble salts ranging from 0.00008 to 0.00009 mg kg^{-1} body weight day^{-1}, according to a document from 2009 included in the database by USEPA [32].

Al and Fe were the heavy metals with the highest levels of concentration in *S. ramosissima* leaves, despite not being the most abundant metals in the soil; they showed the highest BCF values in roots, stems, and leaves.

Fe is an essential element in human nutrition and, as with Al, there is little indication that it is acutely toxic to humans when orally ingested, despite the widespread occurrence of this element in foods. It has been hypothesized that aluminum exposure is a risk factor for the development or acceleration of onset Alzheimer disease in humans [29]. Both metals have high established values for PTWI, TUIL, and R_fD, so, as expected, both showed low HRI.

4.5. Conclusions.

In the specific case of the marshes of the estuary of the Tinto and the Odiel rivers in Huelva—one of the systems most contaminated by heavy metals in the world—the results obtained discourage the consumption of *S. ramosissima* obtained in most studied locations since it exceeds the limits established in the regulations regarding concentrations of Cd, Pb, and Tl in leaves.

S. ramosissima is an accumulator of Al and Fe, chiefly in its roots and stems, so it could be used in soils contaminated by these metals. With respect to the rest of the studied heavy metals, it is a root excluder of As, Cd, Co, Cr, Cu, Mn, Ni, and Zn, and its BCF values in leaves are lower than 1 for Pb, U, and V. Following these results, we can set down that this species can be cultivated and/or harvested from most of the environments that do not reach the extreme levels of contamination that occur in the Huelva estuary and especially in salt marsh habitats. Regarding Tl, for which this species has average BCF levels of 1, food safety problems could arise, although it is not a common environmental or workplace contaminant nor is it readily available to the public. Based on what was observed, we would recommend carrying out specific studies on the content of Al and Tl in the soils where this species will be cultivated.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/d14060452/s1, Table S1: Heavy metal concentrations for each population in soil, roots, stems and leaves.

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