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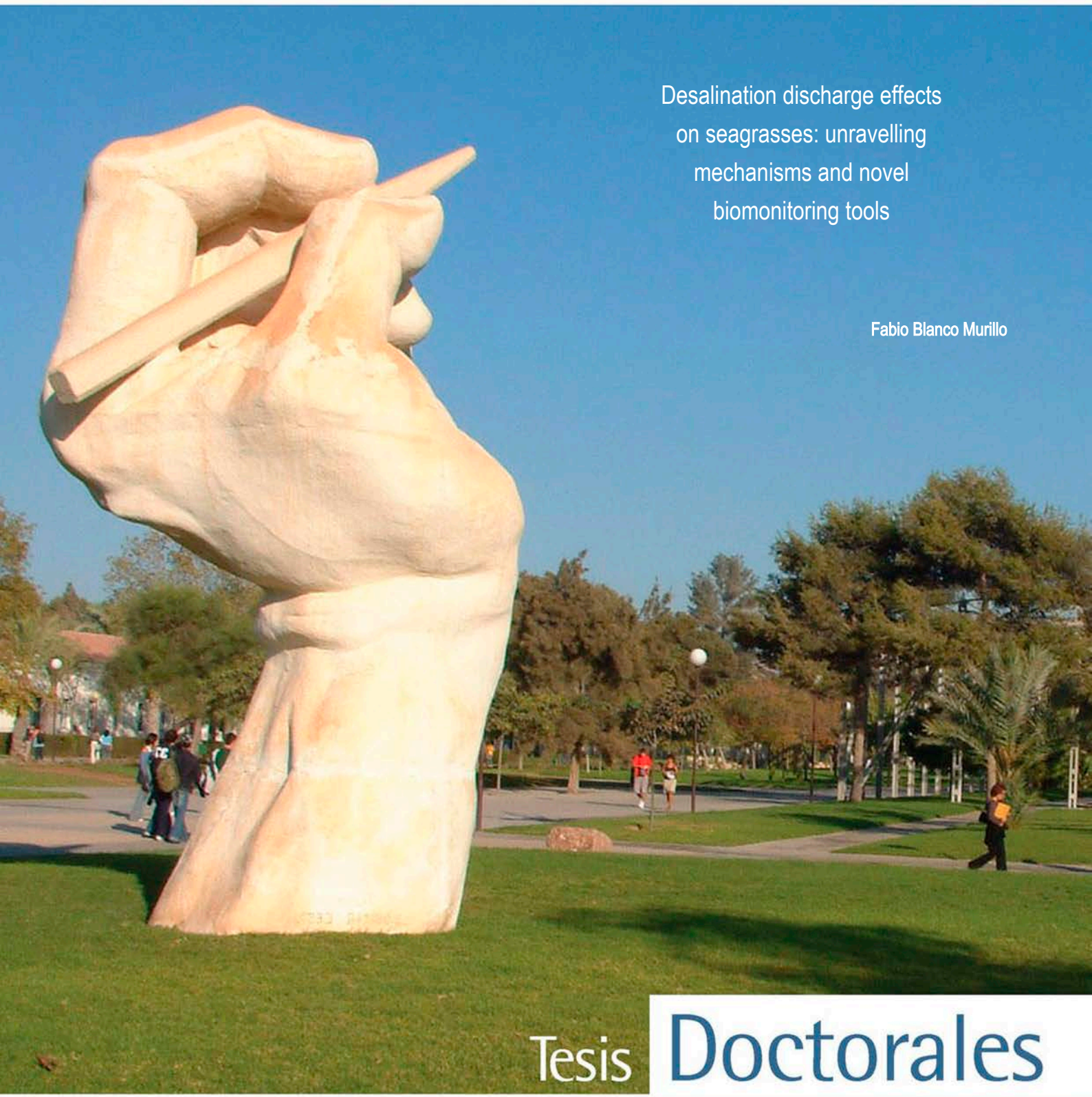
Desalination discharge effects on seagrasses: unravelling mechanisms and novel biomonitoring tools

Fabio Blanco Murillo



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Desalination discharge effects on seagrasses: unravelling mechanisms and novel biomonitoring tools

Fabio Blanco Murillo

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Dr. Jose Luis Sánchez Lizaso y Dr. Claudio Alejandro Sáez Avaria

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"The more clearly we can focus our attention on the wonders and realities of the universe about us, the less taste we shall have for destruction"

Rachel Carson

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1. Resumen de los resultados obtenidos

1.1 Contexto y antecedentes

Angiospermas marinas en el Antropoceno

Las angiospermas marinas son plantas espermatofitas que se adaptaron completamente a la vida en el medio marino, hace aproximadamente 90 millones de años (Periodo Cretácico) (Les et al., 1997) y actualmente pueden encontrarse en aguas someras (0-50 m) de regiones templadas y tropicales de todo el mundo (Kuo & den Hartog, 2007). Este grupo polifilético está formado por 4 familias (Posidoniaceae, Cymodoceaceae, Hydrocharitaceae, and Zosteraceae) que evolucionaron paralelamente desde un ancestro común; una planta acuática del orden Alismatales. Para adaptarse al medio marino, las angiospermas marinas desarrollaron una serie de características para lidiar con sedimentos anóxicos, la baja disponibilidad de CO_2 y, particularmente, tolerar la salinidad del agua de mar (Hemminga & Duarte, 2000b; Touchette, 2007).

Una vez adaptadas al medio marino, estas plantas han logrado formar uno de los ecosistemas marinos más biodiversos y productivos del planeta (Larkum et al., 2007). Las praderas de angiospermas marinas generan una amplia variedad de servicios ecosistémicos esenciales para los ambientes costeros. Como organismos fotosintéticos, estas praderas son importantes productores primarios que suponen la base de multitud de ecosistemas marinos y además contribuyen a la producción de oxígeno en aguas costeras. Debido a su estructura y a la generación de una rizosfera bajo el sedimento, el crecimiento de las praderas marinas es responsable de un importante porcentaje del carbono marino secuestrado, convirtiéndose así en un sumidero de carbono y un agente mitigador de la emisión de gases de efecto invernadero (Fourqurean et al., 2012; Macreadie et al., 2021). Además, la compleja estructura de las praderas marinas

aumenta la heterogeneidad ambiental y las convierte en refugio, hábitat y zona de cría de multitud de especies animales, así como sustrato para otros organismos fotosintéticos (Hemminga & Duarte, 2000b). La praderas al crecer generan una barrera física llegando a formar estructuras de arrecife en algunas especies y que reducen la energía del oleaje que llega al borde costero e influyen en las dinámicas sedimentarias (Manca et al., 2012). Con respecto a los beneficios directos para el ser humano, estas praderas son esenciales para el mantenimiento de especies de interés comercial (Unsworth et al., 2019), actúan como filtros para ciertos contaminantes (de los Santos et al., 2021) y aportan un capital natural que está directamente relacionado con sectores económicos como el turismo (Vassallo et al., 2013). Sin embargo, a pesar de la relevancia de las praderas marinas tanto para las comunidades marinas como para la economía costera, éstas han sufrido una importante regresión a causa de la actividad humana (Orth et al., 2006; Short et al., 2011).

En general, estas plantas son relativamente sensibles a cambios ambientales, destacando su vulnerabilidad ante el desarrollo humano en zonas costeras. De hecho, es la actividad humana el principal factor condicionando la salud y el desarrollo de las praderas de angiospermas marinas (Duarte & Hemminga, 2000), al igual que lo es de numerosos procesos climáticos, geológicos y biológicos en la época geológica actual, conocida como Antropoceno (Lewis & Maslin, 2015). Las construcciones costeras, contaminación marina, fondeo de embarcaciones son algunos de los estresores que, junto a ciertos eventos naturales, han llevado a las angiospermas marinas a un declive en diversos lugares del mundo (Waycott et al., 2009). En algunas ocasiones estos procesos de regresión han sido generalizados (Orth et al., 2006), mientras que en otros casos se han producido de forma local (Guillén et al., 2013). En algunos casos, la causa de estas pérdidas ha sido un impacto específico como el fondeo de embarcaciones (Ceccherelli et al., 2007) o la regeneración de playas (González-Correa et al.,

2008). Sin embargo, en la mayoría de los casos hay múltiples estresores coexistiendo en el borde costero que han provocado la regresión de las praderas de angiospermas marinas (Turschwell et al., 2021; Vieira et al., 2020). Es por ello que el análisis a escala local de los estresores que afectan a una pradera de angiospermas marinas y la discriminación de impactos son necesarias para una correcta protección y conservación de estos valiosos ecosistemas.

La desalación y su impacto en las comunidades marinas

La actividad humana en la costa está asociada a numerosas alteraciones de las distintas variables ambientales (concentración de nutrientes, turbidez, salinidad, etc.), que pueden afectar negativamente a las comunidades marinas, como las praderas de angiospermas marinas (Duarte & Hemminga, 2000). En el contexto actual de cambio climático, la escasez hídrica está siendo uno de los problemas más urgentes a los que deben enfrentarse las poblaciones de zonas costeras, especialmente en climas áridos y semiáridos (Kummu et al., 2016). Es por ello que en las últimas décadas se ha desarrollado ampliamente la industria de la desalación de agua de mar que, principalmente por medio de ósmosis inversa, permite obtener agua dulce a partir de agua salada (Eke et al., 2020). La industria desaladora ha experimentado un desarrollo exponencial en las últimas décadas, especialmente zonas áridas y semiáridas como el golfo Arábigo, el Mediterráneo occidental o la cosa australiana. En la actualidad esta industria está comenzando incrementar su desarrollo en otras zonas del mundo como es el caso de la costa de Chile, donde se espera un incremento del 100% de las plantas operativas actuales durante la próxima década, para uso industrial, urbano y agrícola (Herrera-León et al., 2019). El creciente desarrollo de esta industria ha desencadenado la atención científica por los impactos ambientales que pueda tener en su entorno. De entre los impactos ambientales derivados de la desalación el más relevante para el medio marino es el vertido de las aguas de rechazo en las aguas costeras. Como resultado del proceso de desalación el agua

con exceso de sales resultante, denominada salmuera, es vertida de nuevo al mar (Darre & Toor, 2018; Jones et al., 2019). Este efluente, al tener una alta salinidad, suele tener mayor densidad que el agua de mar y, por tanto, generalmente se desplaza por el fondo, afectando a las comunidades bentónicas. La salmuera está principalmente compuesta por agua de mar concentrada (hasta el doble de su salinidad natural), además de concentraciones variables de distintos compuestos químicos añadidos durante el proceso (antiincrustantes, biocidas, …). Esta composición puede producir un efecto de estrés osmótico y toxicidad que puede causar estrés fisiológico e incluso el desplazamiento de las comunidades afectadas (Fernández-Torquemada, Sánchez-Lizaso, et al., 2005; Lattemann & Höpner, 2008).

En el caso de las angiospermas marinas, los aumentos de salinidad sobre ciertos umbrales han mostrado generar diversas respuestas adversas, desde reducciones en sus tasas fotosintéticas (Marín-Guirao, et al., 2013), hasta declives en el crecimiento y de su supervivencia (Fernández-Torquemada & Sánchez-Lizaso, 2005). Sin embargo, a pesar de que la vulnerabilidad de ciertas especies de angiospermas marinas a la hipersalinidad ha sido estudiada previamente, la mayoría de los estudios sobre la tolerancia de plantas marinas a la hipersalinidad han sido aproximaciones en laboratorio que se han centrado en fenómenos morfológicos (crecimiento, necrosis, supervivencia) o fisiológicos (fotosíntesis, respiración) en periodos de tiempo de semanas o meses (Sandoval-Gil et al., 2023). Los incrementos de salinidad producen un estrés osmótico en las células vegetales por el exceso extracelular de iones, generando procesos de deshidratación o de toxicidad de ciertos iones como el sodio (Na^+) que puede desplazar otros elementos esenciales para el metabolismo como el potasio (K^+). Por ello, las angiospermas marinas deben reaccionar desencadenando mecanismos de exclusión de iones que puedan causar toxicidad, así como una asimilación eficiente de aquellos más escasos. Además, la salinidad puede alterar

el correcto funcionamiento de las cadenas de transporte de electrones en mitocondrias y tilacoides, generando especies reactivas de oxígeno (ROS). Estos compuestos, al encontrarse en exceso, pueden comenzar a oxidar componentes celulares como enzimas, membranas celulares o ácidos nucleicos (Arora et al., 2002; Bartosz, 1997). Para combatir el desbalance en las ROS, las células vegetales cuentan con mecanismos enzimáticos y no enzimáticos con función antioxidante para eliminar el exceso de estos compuestos. Sin embargo, si la respuesta antioxidante no es lo suficientemente eficiente el daño oxidativo, éste puede desembocar en la degradación de estructuras intracelulares y, eventualmente la muerte de tejidos (necrosis) y así comprometer la fisiología y supervivencia de la planta. Aunque estos procesos de regulación osmótica y control del estrés oxidativo han sido ampliamente descritos en plantas terrestres, hay escasa información sobre su rol en las angiospermas marinas y, al tratarse de procesos de activación muy temprana (horas, días) pueden permitir la detección de estrés en sus primeras fases y pueden tener el potencial de prevenir daños más graves sobre las angiospermas marinas y, por ende, en las comunidades asociadas a éstas.

Efecto de la desalación sobre angiospermas marinas: Posidonia oceanica y Zostera chilensis

Dada la relevancia ecológica de las angiospermas marinas y su sensibilidad a perturbaciones ambientales, el estudio de sus rangos de tolerancia es esencial para determinar su vulnerabilidad a los vertidos procedentes de la desalación. Como se ha descrito anteriormente la mayoría de estudios relativos al efecto de la hipersalinidad en angiospermas marinas se ha centrado en su fisiología, revelando la sensibilidad de estos organismos ante este tipo de perturbación ambiental (Sandoval-Gil et al., 2023). Sin embargo, la mayoría de los estudios publicados hasta la fecha se han realizado en unas pocas especies y con

incrementos poco realistas de salinidad, considerando las condiciones reales de un vertido de salmuera.

Los rangos de tolerancia a los cambios de salinidad son variables en función de la especie, y es por ello por lo que en esta tesis se han elegido como modelos de estudio dos angiospermas marinas de linajes evolutivos diferentes que se encuentran en regiones donde la desalación ha tenido o se prevé tener una importante expansión, como son el Mediterráneo occidental y la costa de norte de Chile. Por un lado, *Posidonia oceanica* (L.) Delile es una especie endémica del Mar Mediterráneo y que además es la angiosperma marina con mayor distribución y relevancia ecológica en su área de distribución. Su tolerancia a distintos estresores ambientales y, especialmente la hipersalinidad, ha sido ampliamente estudiada por su alta sensibilidad e importancia (Gobert et al., 2007; Ruiz et al., 2012; Sandoval-Gil et al., 2022). En el caso del Mediterráneo occidental, la desalación ha tenido un amplio desarrollo en las últimas décadas y *P. oceanica* ha sido incluso utilizada como especie bioindicadora en planes de vigilancia ambiental (PVA) de vertidos de salmuera (Sánchez-Lizaso et al., 2008).

Por otro lado, *Zostera* (o *Heterozostera*) *chilensis* (J. Kuo) S.W.L. Jacobs & D.H. Les es una especie endémica y única representante de las angiospermas marinas en el Pacífico Sudamericano. En la actualidad hay escasa información sobre esta especie y su tolerancia a estresores ambientales es aún desconocida. Su distribución se encuentra limitada a 3 bahías de centro y norte de Chile, dónde se espera el mayor desarrollo de plantas desaladoras en los próximos años (Herrera-León et al., 2019). Dada su escasa distribución y su situación como especie vulnerable según la UICN, es esencial conocer su tolerancia a los incrementos de salinidad derivados de plantas desaladoras para proteger y preservar esta especie icónica.

Ambas especies se encuentran distanciadas geográfica y evolutivamente, pero dado que las angiospermas marinas han sufrido una evolución convergente, se cree que sus mecanismos de tolerancia podrían ser similares frente a los incrementos de salinidad. Además, ambas especies son endémicas y forman ecosistemas esenciales en sus respectivas áreas de distribución

1.2 Objetivos principales de la tesis

Dada la relevancia de las angiospermas marinas tanto para el medio natural como para el ser humano, especialmente considerando su potencial rol en la mitigación del cambio climático, es esencial desarrollar medidas de gestión y protección que permitan frenar su regresión y facilitar su recuperación. Para ello es imprescindible conocer la escala a largo de plazo de los procesos de regresión sufridos por estos organismos y desarrollar herramientas de monitoreo que detecten de forma temprana de estrés y permita así la toma de medias para evitar su degradación. Es en este contexto en el que se enmarca la investigación de esta tesis doctoral. Con el objetivo de profundizar en el impacto de la desalación sobre las praderas de angiospermas marinas se estudiaron las respuestas a gran escala espacial y temporal para evaluar las tendencias y los procesos de regresión, así como indicadores de estrés fisiológico, para después aplicar herramientas bioquímicas y moleculares específicas para el impacto de desalación. De esta forma se complementarían las respuestas a corto plazo (días) en parámetros de los más bajos niveles de organización (metabolismo, expresión de genes), con el largo plazo (décadas) usando descriptores poblacionales (densidad de haces, cobertura de la pradera).

Como **objetivo general** de la tesis se plantea profundizar en el conocimiento sobre la influencia de los vertidos de salmuera sobre plantas marinas en la búsqueda de biomarcadores más eficaces que pueda usarse en futuras tareas de seguimiento y como indicadores de alerta temprana.

Para ello se plantean los siguientes **objetivos específicos**:

1- Establecer la tendencia poblacional de las praderas a largo plazo en el litoral de Alicante y la magnitud de regresión de una pradera marina en el largo plazo bajo condiciones de perturbación ambiental

2- Determinar las respuestas fisiológicas y metabólicas de las angiospermas marinas (*Z. chilensis* y *P. oceanica*) a los incrementos de salinidad tanto con sales artificiales como salmuera procedente de plantas desaladoras

3- Comprobar la respuesta de biomarcadores específicos en plantas marinas bajo la influencia de un vertido de salmuera en el corto plazo y su potencial aplicabilidad como herramientas de seguimiento específicas

4- Determinar el rol de los meristemos apicales en la tolerancia a la salinidad, así como el potencial uso de estos órganos en tareas de monitoreo

Esta tesis se desarrolló en cotutela entre la Universidad de Alicante (España) y la Universidad de Playa Ancha (Chile), realizando parte de los experimentos en cada una de las universidades y permitiendo el estudio de las dos especies objetivo (*P. oceanica* y *Z. chilensis*) en sus respectivos ambientes. Además, para el desarrollo de los objetivos 3-5 se realizó una colaboración con el grupo de Ecología de Angiospermas Marinas del Centro Oceanográfico de Murcia (IEO, CSIC).

1.3 Resultados obtenidos y discusión

Evolución espacio-temporal de las praderas de Posidonia oceanica a lo largo de la provincia de Alicante (SE España)

Debido a impactos de origen antrópico, se ha postulado que las praderas de *P. oceanica* se encuentran en un proceso general de regresión en las últimas décadas. Sin embargo, aún es controvertido si este proceso de declive es un

fenómeno global o si es causado por perturbaciones ambientales a escala local. Con el objetivo de evaluar los cambios sufridos en el límite superior de las praderas de *P. oceanica*, en este estudio, se analizaron los datos de monitoreo de 20 años de 14 localidades a lo largo de 200 km de costa en la provincia de Alicante (SE España). Los datos pertenecen al proyecto POSIMED que tiene como objetivo realizar un seguimiento anual de praderas marinas para determinar su estado ecológico y evolución temporal. El porcentaje de cobertura de *P. oceanica* viva y de mata muerta, así como la densidad de haces fueron analizados para determinar la tendencia global durante los 20 años de datos a dos profundidades 3-5 metros y 7-9 metros en cada localidad. Tanto la cobertura como la densidad de haces mostraron una tendencia general positiva en la mayoría de los puntos de muestreo. Sin embargo, el porcentaje de mata muerta también mostró un ligero incremento a lo largo del tiempo y 6 localidades mostraron una disminución de la cobertura de *P. oceanica* viva. Esto indica que, si bien hay una situación general de estabilidad en estos descriptores, algunas localidades están sufriendo procesos de regresión. La densidad de haces disminuyó con la profundidad y la cobertura de rocas, mientras que mostró una relación poco clara con la mata muerta, indicando que puede haber diversas causas que puedan aumentar la presencia de plantas muertas. Estos resultados apoyan la idea de que son perturbaciones locales las que producen el declive de las poblaciones de angiospermas marinas en el Mediterráneo, resaltando la necesidad de desarrollar herramientas de gestión enfocadas en los estresores que afectan a estos ecosistemas a escala local. Programas de seguimiento a gran escala y largo plazo permiten evaluar el estado y evolución de ecosistemas de crecimiento lento como son las praderas de *P. oceanica* y son necesarios para determinar correctamente la escala de los impactos ambientales sobre ellas.

Regresión a largo plazo de una pradera de Posidonia oceanica sometida a múltiples impactos

El desarrollo costero tiene un innegable impacto sobre las comunidades marinas, en detrimento de las comunidades más sensibles. En el mar Mediterráneo, el ecosistema costero clímax más abundante con las praderas de *P. oceanica*, las cuales se ven amenazadas por diversas actividades humanas en el borde costero. La ciudad de Alicante es una ciudad costera en el sudeste español en la que pueden encontrarse diversas actividades nocivas con el medio marino, típicas de las ciudades costeras: construcción de un puerto, vertido de aguas residuales, industrias metalúrgicas y dos plantas desaladoras.

Para determinar el efecto conjunto de estos impactos coexistiendo en la misma área, se realizó un análisis de la extensión de estas praderas a largo plazo usando cartografías bionómicas y mapas elaborados con sónar de barrido lateral y buceo autónomo (1984 – 2014). Además, se analizaron datos de descriptores poblacionales (porcentaje de cobertura de *P. oceanica*, porcentaje de cobertura de mata muerta y densidad de haces) en 14 puntos a lo largo de la bahía en las dos últimas décadas (2003, 2007, 2014 y 2021). Los resultados mostraron una pérdida total de pradera de 619 hectáreas, es decir, el 25 % del total de la superficie estudiada. La cobertura de *P. oceanica,* así como la densidad de haces sufrieron una disminución durante los últimos 20 años, especialmente en el límite superior de la pradera. La mata muerta de Posidonia mostró, como consecuencia, un incremento durante el mismo periodo de tiempo, siguiendo una tendencia inversa a los dos descriptores anteriores. En el área de estudio han coincidido diversos estresores durante las últimas décadas (ampliación del puerto de Alicante, vertido de aguas residuales, instalación de una planta desaladora, etc.), contribuyendo al declive de la pradera de *P. oceanica*. La actual tendencia negativa en los descriptores poblacionales utilizados resalta la necesidad de desarrollar herramientas de gestión a escala local que atiendan a

los impactos ambientales actualmente presentes y actúen sobre ellos con el fin de detener la degradación de estos vitales ecosistemas y promover su conservación.

Vulnerabilidad de Zostera chilensis, el último relicto de angiospermas marinas en al Pacífico Sudamericano, ante el desarrollo de la industria desaladora en Chile

Las angiospermas marinas están consideradas entre los ecosistemas costeros más valiosos y amenazados del planeta. Estas plantas tienen una limitada distribución en el Pacífico Sudamericano, donde *Z. chilensis* es la única especie presente en la actualidad. Debido a la escasez hídrica, la industria de la desalación ha crecido exponencialmente en las últimas décadas en las costas del centro y norte de Chile y existe escasa información en cuanto al efecto de los vertidos hipersalino de esta industria sobre las comunidades bentónicas. En este trabajo se analizaron las respuestas ecofisiológicas y celulares de *Z. chilensis* ante incrementos de salinidad extrapolables a vertidos de salmuera en la costa chilena. Se llevaron a cabo experimentos de mesocosmos durante 10 días donde las plantas fueron expuestas a 3 tratamientos de salinidad: 34 psu (control), 37 psu y 40 psu. Se midió el desempeño fotosintético, la acumulación de H_2O_2 y el contenido de ascorbato (reducido y oxidado), además de la expresión relativa de genes relacionados con la regulación osmótica y el estrés oxidativo. Estos parámetros se midieron los días 1, 3, 6y 10 del experimento. *Z. chilensis* mostró una reducción de descriptores fotosintéticos como la tasa de transporte de electrones (ETR_{max}) y la irradiancia de saturación (Ek_{ETR}) en los tratamientos de hipersalinidad, mientras que el enfriamiento no fotoquímico (NPQ_{max}) presentó un incremento inicial (hasta el día 3) y una posterior reducción a 40 psu. Los niveles de H_2O_2 incrementaron en condiciones de hipersalinidad, mientras que el ascorbato y dehidroascorbato sólo aumentaron a 37 psu, aunque se redujeron a lo largo del periodo experimental. Las plantas a 37 y 40 psu además estimularon la expresión de genes relacionados con el transporte de iones y la síntesis de

osmolitos orgánicos, pero los genes que se expresaron más en relación con el aumento de salinidad fueron aquellos relacionados con el metabolismo de especies reactivas de oxígeno. En este estudio *Z. chilensis* mostró la capacidad de responder ante incrementos de salinidad simulada equivalentes a las producidas por un vertido de salmuera de una planta desaladora en el corto plazo. Sin embargo, el consumo de energía de estas respuestas podría potencialmente producir efectos más graves a largo plazo y comprometer la supervivencia de la plata y, considerando su limitada distribución, se desaconseja la instalación de puntos de descarga de salmuera cerca de praderas de esta especie endémica.

Efecto de los vertidos de salmuera más allá de la hipersalinidad: descubriendo indicadores específicos de estrés y respuestas en la angiosperma marina Posidonia oceanica.

Considerando el potencial efecto negativo de los vertidos de salmuera procedentes de plantas desaladoras sobre organismos marinos, es necesario conocer las respuestas específicas que este tipo de contaminación causa en especies sensibles. En este contexto, la angiosperma marina como *P. oceanica*, endémica del mar Mediterráneo, es una especie que ha mostrado una alta sensibilidad a los cambios de salinidad y que, además, posee un alto valor ecológico. La mayoría de estudios ecotoxicológicos relativos al efecto de la salmuera sobre angiospermas marinas se han llevado a cabo usando sales artificiales, a pesar de que los vertidos de salmuera pueden, además, contener ciertos aditivos añadidos durante el proceso de desalación que podrían incrementar los efectos perjudiciales de esta salmuera además del incremento de salinidad. Para determinar el potencial efecto de salmuera real en *P. oceanica* se realizó un experimento de mesocosmos durante 10 días simulando incrementos de salinidad con sales artificiales y con salmuera procedente de una planta desaladora (a 43 psu, 6 psu por encima de la salinidad natural de 37 psu). Descriptores morfométricos (crecimiento y necrosis), fotoquímicos

(Fluorometría de la clorofila a del fotosistema II), metabólicos como el peróxido de hidrógeno (H_2O_2), sustancias reactivas del ácido tiobarbitúrico (TBARS) y ascorbato/dehidroascorbato (ASC/DHA), y moleculares (expresión de genes de tolerancia al estrés osmótico y oxidativo) se analizaron en cada tratamiento. Aunque hubo un crecimiento positivo en todos los tratamientos, aquellas plantas sometidas a incrementos de salinidad tuvieron in incremento en longitud y biomasa foliar menor que los controles. Los parámetros fotoquímicos no mostraron cambios asociados a la hipersalinidad, excepto para el enfriamiento no fotoquímico (NPQ_{max}), que fue menor en plantas expuestas a salmuera. La peroxidación lipídica (TBARS) y la expresión de genes relacionados con estrés oxidativo (GR, MnSOD y FeSOD) o exclusión de iones (SOS3 y AKT 2/3) se vieron incrementados en ambos tratamientos de hipersalinidad. Por otro lado, la ratio ASC/DHA fue menor y la expresión de SOS1, CAT y STR1 fue superior en las plantas bajo influencia de salmuera. Este estudio reveló que, a pesar de que se detectaron diferencias en ciertas respuestas entre sales artificiales y salmuera, la mayoría de las respuestas revelaron un comportamiento similar, indicando que el efecto más relevante de la salmuera es la hipersalinidad, aunque los efectos de ésta se puedan ver agravados por aditivos o ciertos componentes de los efluentes de la desalación.

Aplicación de nuevos indicadores y herramientas de monitoreo en Posidonia oceanica expuesta a vertidos de salmuera

En el contexto de cambio climático y de aumento de población a escala mundial, la escasez hídrica está aumentando y la industria de la desalación está creciendo como principal fuente de agua alternativa en las zonas costeras. Tras haber comprobado el estrés por hipersalinidad en *Z. chilensis* y de probar diversos descriptores fisiológicos y metabólicos en *P. oceanica* bajo efecto de salmuera en condiciones de mesocosmos, se propone aplicar éstos últimos descriptores en condiciones naturales. Los experimentos de laboratorio revelaron que los

descriptores metabólicos y la expresión de genes respondieron activamente a la hipersalinidad causada por la salmuera. Por ello, plantas de *P. oceanica* se trasplantaron cerca del punto de vertido de una planta desaladora en Alicante (España). Las plantas, procedentes de una pradera en condiciones inalteradas, se ubicaron en 3 puntos: un control (~37 psu), un lugar de influencia moderada (IB, ~39.5 psu) y otro de alta influencia del vertido de salmuera (HB, ~42 psu). Se analizaron parámetros relativos al metabolismo de especies reactivas de oxígeno (ROS) como H_2O_2, TBARS y ASC/DHA, y respuestas de regulación de genes relativos al estrés osmótico y oxidativo. Las muestras de hojas se tomaron en los días 1, 3 y 6 tras el tras el trasplante.

H_2O_2 y TBARS aumentaron con el aumento de exposición al vertido de salmuera y ASC disminuyó en las plantas en HB, indicando producción de ROS, daño oxidativo y consumo de antioxidantes a mayor influencia hipersalina. Los genes relativos a la osmorregulación (*SOS1*, *SOS3*, *AKT2/3*, *STRK1*) y a la respuesta antioxidante (*GR*) se vieron sobreexpresados en las plantas en HB, indicando una respuesta correlacionada con el aumento de salinidad. El hecho de que parte de las respuestas se vieran incrementadas con respecto a los experimentos de laboratorio indica que el estrés causando por los vertidos de salmuera puede verse incrementado en condiciones naturales, debido a la interacción con estresores tanto naturales (hidrodinamismo, herbivoría) como antrópicos (incrementos de nutrientes). Estos parámetros se proponen como eficientes biomarcadores de alerta temprana que reaccionan a la influencia de salmuera sobre *P. oceanica* en el corto plazo y podrían incorporarse como herramientas de seguimiento de plantas desaladoras en la costa mediterránea.

El rol de los meristemos apicales en la tolerancia a la hipersalinidad en Posidonia oceanica: uso como órgano de biomonitoreo

Las angiospermas marinas son pantas que han experimentado una serie de adaptaciones evolutivas para poder desarrollarse en el medio marino completamente sumergidas, siendo la adaptación a la salinidad una de las más complejas a escala metabólica y fisiológica. Además, las angiospermas marinas son sensibles a los cambios de salinidad, que pueden ser dañinos para su desarrollo y supervivencia, convirtiéndose en un factor determinante en su biología y distribución. Dado que la distribución de las angiospermas marinas comprende gran parte de las costas templadas del planeta, donde el crecimiento poblacional y los cambios de regímenes de precipitación están incrementando la escasez de agua dulce. En este contexto, el desarrollo de la desalación es un potencial estresor para las praderas de plantas marinas, al producir incrementos locales de salinidad que podrían generar estrés en estos organismos. Tras haber determinado en los capítulos previos el estrés generado por los vertidos de salmuera y las respuestas metabólicas detectadas en las hojas, se propuso estudiar el mismo efecto en los meristemos apicales. Este órgano contiene células pluripotenciales de las cuales depende directamente el crecimiento foliar y estudios previos han postulado este órgano como un indicador más directo de la salud de la planta que el tejido foliar. Para determinar la respuesta y el rol de este órgano ante la exposición a salmuera en *P. oceanica*, se tomó tejido de los experimentos de los dos capítulos anteriores en el último día de muestreo; tras 10 días en el mesocosmos comparando salmuera y sales artificiales (43 psu) y 6 días en el terreno comparando exposición moderada (~39 psu) y alta (~42 psu) a un vertido de salmuera. Se realizaron los mismos análisis a escala metabólica (H_2O_2, TBARS y ASC/DHA) y molecular (expresión de genes), para comparar la respuesta de ambos órganos frente a distintas exposiciones a hipersalinidad.

Aunque los niveles de H_2O_2 y TBARS fueron superiores en las hojas en ambos experimentos, se vieron incrementados en relación a la salinidad en los meristemos, mostrando una respuesta más sensible a los cambios en este parámetro ambiental. La ratio ASC/DHA mostró comportamientos inversos en los experimentos, pero siempre mostró una reducción a mayor salinidad, independientemente del órgano. De forma general, la expresión relativa de genes en los meristemos fue mayor en comparación a las hojas, mostrando una respuesta metabólica más activa en este tejido ante condiciones de hipersalinidad, especialmente la provocada por salmuera. Estos resultados revelan el rol esencial que los meristemos pueden cumplir en relación a la tolerancia de *P. oceanica* a las variaciones de salinidad y, por lo tanto, de su adaptación evolutiva al medio marino. Además, el mayor grado de respuesta de este órgano lo hace un objetivo ideal para el uso de biomarcadores como herramientas de seguimiento ambiental específicas para el impacto de la desalación en angiospermas marinas.

1.4 Conclusiones generales

- El límite superior de las praderas de *P. oceanica* en la costa de Alicante se ha mostrado estable según descriptores poblacionales a gran escala en los últimos 20 años.
- La pradera de *P. oceanica* de la bahía de Alicante sometida a múltiples impactos han sufrido una importante regresión en sus poblaciones.
- Los procesos de regresión de praderas de *P. oceanica* se producen principalmente a escala local, debido a perturbaciones específicas y a sus potenciales sinergias.

- *Z. chilensis* mostró una reducción de su capacidad fotosintética ante condiciones de hipersalinidad, una mayor producción de especies reactivas de oxígeno (H_2O_2) y un mayor consumo de antioxidantes (ASC).
- La hipersalinidad generó una respuesta transcriptómica activa en *Z. chilensis* recurriendo a mecanismos de síntesis de osmolitos, exclusión de iones y consumo de ROS, sin embargo, el estrés osmótico podría comprometer la fisiología de esta especie endémica en el largo plazo.
- Los efectos de la salmuera sobre *P. oceanica* a escala morfométrica y metabólica son principalmente debidos al incremento de salinidad.
- Ciertas respuestas, como el NPQmax, el consumo de ASC, o la regulación de genes como STRK1 y CAT, se vieron incrementadas en ejemplares de *P. oceanica* expuestas a salmuera, en comparación con la misma salinidad alcanzada usando sales artificiales.
- Los biomarcadores metabólicos y moleculares probados en *P. oceanica* han mostrado ser eficientes y específicos al responder activamente a la exposición a salmuera en condiciones naturales, como también para determinar el grado de contribución de este estresor donde existen múltiples presiones ambientales.
- Se propone el uso de descriptores moleculares como indicadores de alerta temprana de plantas de *P. oceanica* bajo el efecto de vertidos de salmuera con el fin de prevenir daños fisiológico y potencial regresión de las praderas formadas por esta especie.
- La producción de ROS y la peroxidación lipídica aumentó más en correlación a la exposición a salmuera en meristemos en comparación con hojas de *P. oceanica*.
- Los meristemos presentaron una mayor regulación de genes tanto de osmorregulación como de estrés oxidativo en comparación con las

hojas, indicando una mayor sensibilidad, así como un rol esencial en la tolerancia de *P. oceanica* a la hipersalinidad.

- Los meristemos tienen el potencial de ser analizados como órganos sensibles para descriptores metabólicos como indicadores de alerta temprana.
- En futuros estudios relativos al efecto de los vertidos de salmuera sobre angiospermas marinas se recomienda: El uso de descriptores de distintos niveles de organización (con énfasis en transcriptomas completos con RNA-seq), el uso de salmuera real de plantas desaladoras, la simulación de condiciones naturales y el estudio de los meristemos apicales como órgano de biomonitoreo.

2. Introduction

2.1 Seagrasses in the Anthropocene

Seagrasses, or marine angiosperms, are an unique group of monocotyledonous plants from the order Alismatales which completely adapted to marine submerged life (Hemminga & Duarte, 2000b; Larkum & den Hartog, 1989). This a polyphyletic group, as the marine colonization occurred in 3 different occasions along seagrass evolutionary history during the Cretacic period (Les et al., 1997). This group is now considered to be formed by 4 families (Posidoniaceae, Cymodoceaceae, Hydrocharitaceae, and Zosteraceae) which have undergone a parallel evolution, adapting to the marine environment through similar morphological, physiological and genetic modifications (Larkum & den Hartog, 1989; Papenbrock, 2012; Wissler et al., 2011).

Seagrasses evolved from freshwater aquatic plants, which had already to develop adaptations to submerged life such as lower light intensity, anoxic sediments, or low available CO_2. In this regard, aquatic plants developed morphological and physiological adaptations to cope with these conditions such as photosynthetically active epidermis, gas transport system from leaves to roots under and the use of HCO_3^- as carbon source. Moreover, the marine environment demanded the development of specific mechanisms to deal with higher salinities to keep water homeostasis, such as negative water potential, low cytosol:vacuole volume ratio or shoot apical meristems protected by sheaths (Fig. 2.1) (Touchette, 2007; Tyerman, 1982).

Seagrasses form extensive meadows along infralittoral bottoms in tropical and temperate regions across the planet (Kuo & den Hartog, 2007) and provide a variety of ecosystem services. As photosynthetic organisms, seagrasses capture inorganic carbon in its tissues and bury part of it in the sediment, thus becoming

a carbon sink. In fact, seagrasses are considered, together with mangroves and saltmarshes the main coastal carbon fixers (Duarte et al., 2013). This "blue carbon" stored by seagrasses comprised the 18% of all stored carbon by the ocean, despite meadows only account for the 0.1% of ocean surface (UNEP, 2020). But seagrasses do not only contribute as an essential node in marine carbon biogeochemistry but also to the cycling and transformation of nutrients (Papadimitriou et al., 2006), taking part in the nitrogen cycle as well. These role in biogeochemical cycles also allow seagrasses to increase marine communities resilience to ocean acidification by (Unsworth et al., 2012), although this effect might be limited to a certain extent (Koweek et al., 2018).

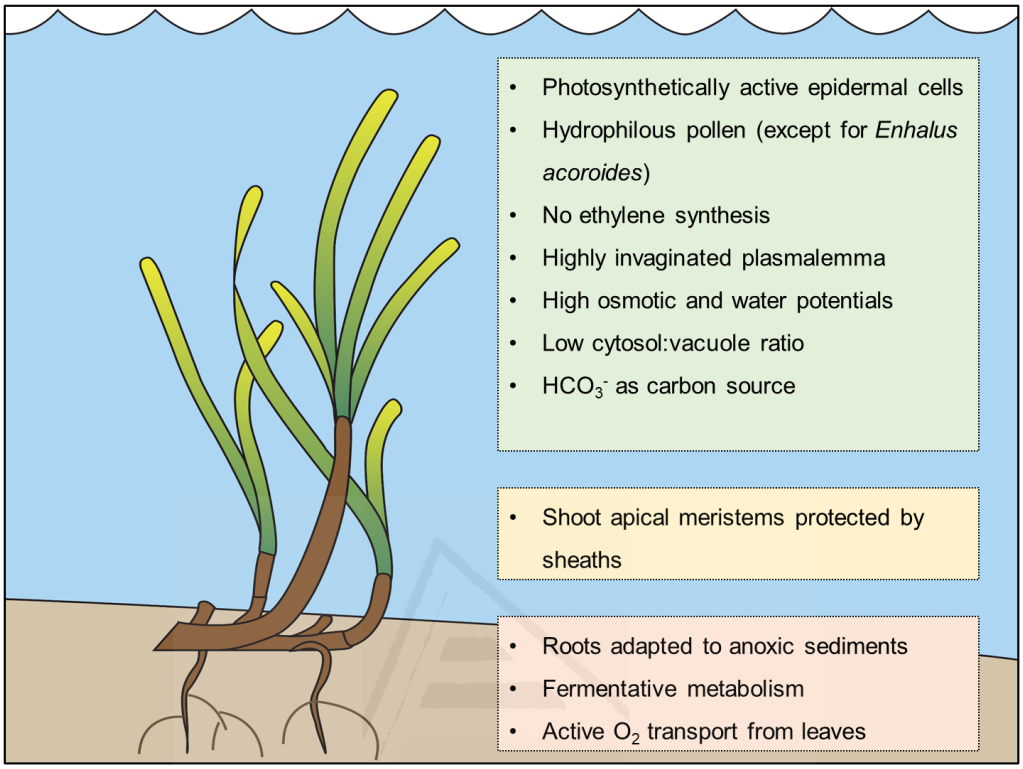

Figure 2.1: Scheme showing the most relevant adaptations of seagrasses to the subaquatic marine environment.

The meadow ecosystems formed by seagrasses are bioconstructions of high spatial complexity which increase bottom heterogeneity, thus creating a variety of microhabitats which turn this meadows into marine biodiversity hotspots (UNEP, 2020). Seagrass meadows work as feeding, shelter and nursery for hundreds of species, many of them of commercial value (Unsworth et al., 2019). Also, sessile animals, macroalgae and microorganisms use seagrass roots, rhizomes and leaves as substratum (Dalla Via et al., 1998), turning seagrass meadows into a diverse and complex holobiont (Ugarelli et al., 2017).

Some seagrasses can form reef-like structures which have a direct effect on hydrodynamics. These barriers protect coasts by reducing wave energy and

erosion (Manca et al., 2012). Furthermore, both seagrass rhizosphere and canopy have an effect in sediment retention and stabilization (Gacia & Duarte, 2001; Short & Short, 1984). In fact, this "filter effect" has also shown to retain other particulate elements present in seawater, such as microplastics (de los Santos et al., 2021). Apart from particulate materials, seagrasses also capture and store dissolved nutrients, pollutants, and other elements, like metals (Marín-Guirao et al., 2005; Sandoval-Gil et al., 2016), highlighting their role in marine pollution mitigation.

Consequently, these ecosystem services also benefit local communities culturally and economically by supporting fisheries production (Nordlund et al., 2018; Unsworth et al., 2019), promoting tourism and coastal recreational activities by maintaining beach structure and clear water (Ondiviela et al., 2014; Short & Short, 1984), maintaining water quality by buffering the effect of human-derived pollution sources (Sandoval-Gil et al., 2016), and preventing the potential negative consequences of climate change due to its mitigation role (de los Santos et al., 2020; Duarte et al., 2013).

Table 2.1: Summary of ecosystem services provided by seagrasses.

Ecological level	Service	References
Species	Primary production	(Hemminga & Duarte, 2000b; Udy & Dennison, 1997)
Species	Nutrient cycling	(Papadimitriou et al., 2006; Sandoval-Gil et al., 2016)
Species	Oxygen production	(Ramesh et al., 2018)
Species	Carbon sequestration	(Mateo et al., 2006;

		Pergent-Martini et al., 2021)
Species	Water acidification buffer	(Unsworth et al., 2012)
Species	Water purification	(Short & Short, 1984)
Species	Pollutant sequestration	(Serrano et al., 2020)
Habitat	Spawning, nursery and living area for various species	(Unsworth et al., 2019)
Habitat	Epifauna and epiflora assemblages	(Dalla Via et al., 1998; Romero, 1988)
Bioconstruction	Coastal protection	(Manca et al., 2012; Ondiviela et al., 2014)
Bioconstruction	Sediment dynamics	(Gacia & Duarte, 2001)
Others	Protection against invasive species	(Ceccherelli & Pinna, 2014)
Others	Microplastic sequestration	(de los Santos et al., 2021)
Others	Pathogen protection	(Lamb et al., 2017)
Others	Bioactive compounds	(Papenbrock, 2012)

Seagrasses have even shown to protect other organisms, including humans, from pathogens (Lamb et al., 2017), and some of seagrass secondary metabolites have antibacterial and antifungal properties (Papenbrock, 2012) with the potential to become useful biotechnology tools.

However, and despite the essential role of seagrasses both to the natural and the human environment, they are threatened by human development. In fact, human activity is the main driver conditioning seagrass meadows health and

development (Duarte & Hemminga, 2000), as it is for several other climatic, geologic and biological processes in the present geological epoch, known as Anthropocene (Lewis & Maslin, 2015). Coastal zones (i.e. 100 km from the shoreline) are the world's most populated areas, with a higher human presence in tropical and temperate regions (Barragán & de Andrés, 2015). As a consequence, there are several human activities coexisting in these areas which can be harmful for the marine environment like, for example, coastal development, sewage loads, plastic pollution, recreational and professional sailing, ... (Crain et al., 2009). This set of environmentally harmful activities specially affects sensitive organisms such as seagrasses and their associated communities.

Seagrasses have suffered a relevant decline along most of the world's coasts in the last few decades, both at the local (Green et al., 2021; Kendrick et al., 2002; Marbà et al., 2014) and global scales (Dunic et al., 2021; Orth et al., 2006; Short et al., 2011; Waycott et al., 2009). In some cases, seagrass meadows regression has been caused by one specific impact like anchoring (Ceccherelli et al., 2007) or beach replenishment (González-Correa et al., 2008). However, in most cases, coastal areas are subject of several stressors coexisting in space and their additive effects might be causing the regression of these valuable ecosystems (Mancinelli & Vizzini, 2015; Turschwell et al., 2021; Vieira et al., 2020). Therefore, it is of great importance to understand the cumulative or synergistic effects this might have on marine communities and develop biomonitoring and management tools which consider impacts in a wider perspective (McMahon et al., 2022).

Given the well-known importance and vulnerability of seagrasses, its study and understanding becomes essential for an effective conservation. Also, as the restoration initiatives remain in development and are limited to a certain extent

(Boudouresque et al., 2021; Ferretto et al., 2021; Pansini et al., 2022), the prevention of seagrass meadows lost becomes the main tool for its conservation. This prevention cannot be done exclusively by using population of even phenologic descriptors (Ceccherelli et al., 2018; Pergent-Martini et al., 2005), supporting necessity for early-warning indicators at individual level (Macreadie, Schliep, et al., 2014) which could allow stress detection and mitigation measures taking before irreversible decline takes place.

Research focused on the environmental impact of human activities on seagrass-based ecosystems should adapt to a multi-scale approach. From the use of molecular tools as early indicators of intracellular stress to the determination of ecosystem functioning and services, all the different complementary approximations would help to provide a better understanding of the seagrass vulnerability and tolerance to their changing environment in the Anthropocene.

2.2 Hypersalinity as environmental stressor

As mentioned above, salinity was one of the major evolutive pressures for seagrasses in order to colonize the marine environment. Salinity changes and fluctuations have even proven to determine seagrass species distributions and viability (Lirman & Cropper, 2003). Adaptations to the sea in seagrasses are surprisingly similar considering their lineages diverged before colonizing the marine environment. This suggest that morphological, physiological and metabolic requirements to thrive under seawater has led to parallel evolution (Wissler et al., 2011), making this polyphyletic group fairly homogeneous regarding marine adaptation. For example, there are common responses to hypersalinity such as the maintenance of negative water potentials to avoid dehydration, growth reduction, or organic osmolyte synthesis (*e.g.,* proline) (Sandoval-Gil et al., 2023). However, salinity tolerance is variable between seagrass species (Berns, 2003; Lirman & Cropper, 2003) and among different

populations of the same species due to local adaptations and the physiological plasticity of each species (Meinesz et al., 2009; Tomasello et al., 2009; Vermaat et al., 2000). These differences are related with the different environments in which seagrasses can live, from estuaries (hyposaline) to coastal lagoons (hypersaline). However, besides osmolyte contents and some transport proteins, little is known about the metabolic processes undergoing in seagrass cells to cope with salinity stress.

The Marine Biology research group form the University of Alicante began its investigation on seagrass and hypersalinity interaction with Fernández-Torquemada & Sánchez-Lizaso (2005), revealing an increment in shoot mortality and reduced growth under hypersaline conditions in *P. oceanica* (25-57 psu, 15 days). After that, different experimental approaches have shown how hypersalinity also affected seagrass survival (Fernández-Torquemada & Sánchez-Lizaso, 2011, 2013), ion content (Garrote-Moreno et al., 2014, 2016), plan-water relations (Garrote-Moreno et al., 2015), osmolyte content (Marín-Guirao et al., 2013; Sandoval-Gil et al., 2012b) and photochemistry and respiration rates (Marín-Guirao et al., 2011; Sandoval-Gil et al., 2012a). On the other hand, the research group from the Laboratory of Aquatic Environmental Research (LACER, HUB AMBIENTAL UPLA), studied physiological and metabolic responses of macroalgae to hypersaline stress (Muñoz et al., 2020, 2023a, 2023b; Rodríguez-Rojas et al., 2020). Due to the lack of metabolic and molecular indicators tested in seagrasses against hypersalinity and following the previous research record, this thesis pretends to combine the experience of both research groups and deepen in the osmoregulatory and oxidative metabolism responses of seagrasses under hypersaline stress.

Osmoregulation responses and physiology

In a nutshell, living in a saline environment require plant cells to control water content and ion uptake, exclusion or compartment (Munns & Tester, 2008). Like other halophytes, marine angiosperms had to evolve a set of adaptations to thrive in seawater by controlling water and osmotic potentials and keeping the more negative than the surrounding water (Touchette, 2007). This allows the pass of water to the cells, while keeping most ions (mainly Na^+ and Cl^-) out of the plant cells.

Na^+ increment is possibly the most relevant stressor regarding the effects of hypersalinity. This monovalent cation can substitute K^+ or Ca^{2+} in essential metabolic processes, causing a metabolic impairment (Wu, 2018). In this sense, Na^+ influx and efflux control are essential mechanisms regarding salinity tolerance coupled with other ion (K^+, Ca^{2+}, Li^+) uptake through specific transport proteins (Carpaneto et al., 2004). Normal cell functioning requires certain Na^+ levels from which seagrasses take advantage. Due to the negative membrane potential and the Na^+ influx force, some transport proteins and ion channels have evolved to be Na-dependent (Rubio et al., 2005, 2011), while at the same time there are efficient efflux mechanisms to control Na^+ intracellular levels (Rubio et al., 2011), keeping them even lower than other marine halophytes (Touchette, 2007). This fact could be explained by the strategy followed by seagrasses to cope with osmotic stress, which have focused their ion-tolerance in Na^+ extrusion rather than osmolyte synthesis in the short term (Van Diggelen et al., 1987). This selective ion flux might be essential for marine plants metabolic and physiological performance. For example, *P. oceanica* has inward-rectifying K^+ channels (KIR), and outward-rectifying K^+ channels (KOR) which are highly impermeable to other cations, including Na^+ (Carpaneto et al., 2004); and *Zostera marina* also proved to have a high ion selection (Fernández et al., 1999), although

this selectivity is lower in seagrasses adapted to estuarine environments (Garrill et al., 1994).

Previous works regarding the effect of hypersalinity on seagrasses have been mostly focused on physiological responses such as respiration rates and photochemistry and organic osmolyte production (free amino acids, carbohydrate content) (Fig. 2.3). As reviewed by Sandoval-Gil et al. (2023), responses are variable depending on the species, the salinity range and the measured parameters. In general terms, photochemistry is not usually affected by salinity increments in the short term, but a decline in photosynthetic quantum yields and gross photosynthesis has been detected at longer exposures or elevated salinity increments (Cambridge et al., 2017; Marín-Guirao et al., 2013). On the other hand, respiration rates tend to increase against hypersalinity, causing carbon balance impairment and a subsequent growth reduction.

These studies have shown the deleterious effects of hypersalinity mainly in plant morphometry and physiology (growth, ion content, photochemistry) while the higher (meadow structure) (Gacia et al., 2007; Ruiz et al., 2009) and lower (metabolism, gene expression) (Booth et al., 2022; Capó et al., 2020) levels of biological organization have been less studied (Fig. 2.3).

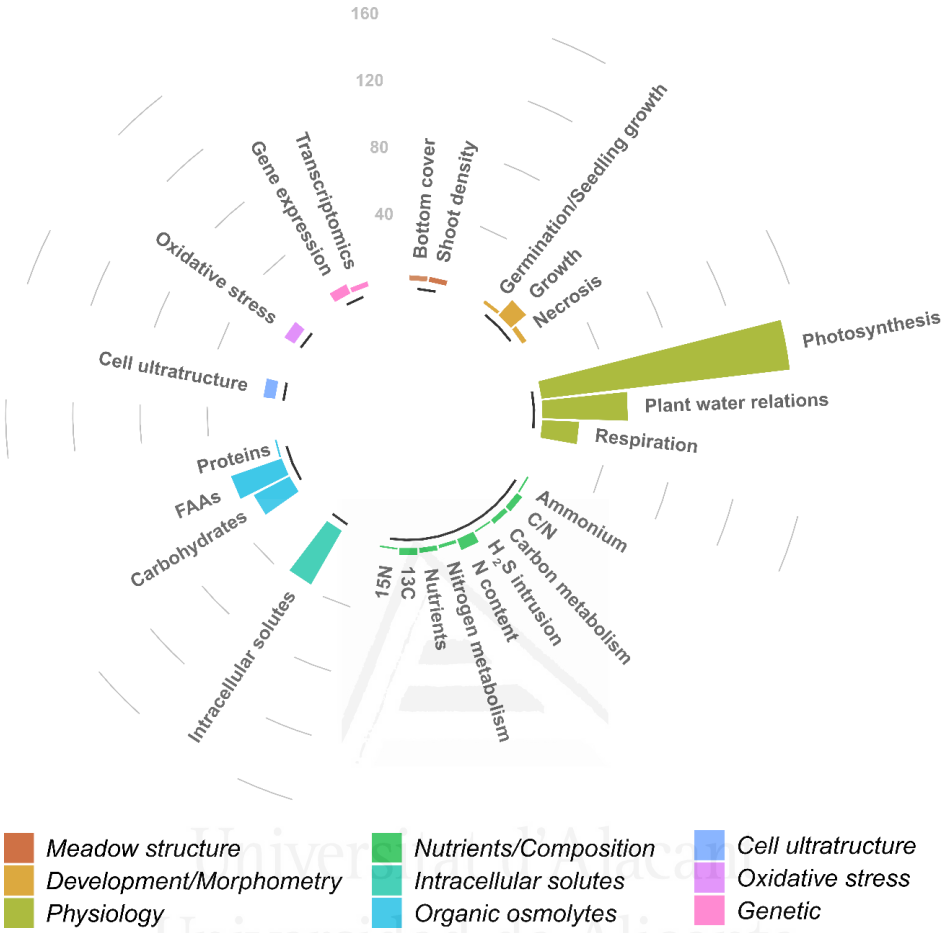

Figure 2.2: Number of publications about seagrass response to hypersalinity. Revisited from Sandoval-Gil et al. (2023).

Reactive oxygen species metabolism

Few studies have focused on the molecular and metabolic responses against salinity increments in seagrasses. Apart from osmoregulatory processes, salinity excess can also cause the formation of reactive oxygen species (ROS). Ion anomalous increments can affect the potential of electron transport chains causing malfunctions and favouring electrons jump out of the system (Bose et al., 2014). These free electron will probably join the most common gas in living plant cells, oxygen (O_2), forming superoxide ion (O_2^-) (Bartosz, 1997). Then, ROS can be

found in the form of hydrogen peroxide (H_2O_2) or hydroxide ion (OH^-). Although ROS have biological functions such as secondary messengers in cellular metabolism, an anomalous increment is the main cause of cellular oxidative damage (Bartosz, 1997). They can oxide and be harmful for different biomolecules and cause deleterious processes such as lipid peroxidation, protein carbonylation and DNA degradation (Arora et al., 2002).

To cope with oxidative stress, living cells have developed a set of mechanisms to scavenge ROS, the reactive oxygen species metabolism (ROM). These series of enzymatic and non-enzymatic responses have the function of maintaining ROS levels in balance and avoid oxidative damage (Foyer & Noctor, 2011). Enzymes like superoxide dismutase (SOD) and catalase (CAT) catalyse reactions to reduce ROS like superoxide and hydrogen peroxide, respectively. Antioxidants like ascorbate and glutathione also buffer the oxidative effect (Arora et al., 2002), conferring cells resistance to ROS increments within their tolerance threshold.

ROS formation as a consequence of hypersalinity has been shown in macroalgae (Luo & Liu, 2011; Muñoz et al., 2020) and seagrasses (Trevathan et al., 2011), but few studies have assessed the tolerance against hypersalinity-derived oxidative stress (Sandoval-Gil et al., 2023). Recently, (Capó et al., 2020) detected ROS production, oxidative damage and antioxidant response in a *P. oceanica* meadow under a desalination brine discharge in Spain. These findings support the hypothesis of ROM taking part of the complex set of metabolic responses of seagrass cells to cope with stress source, acting together with ion homeostasis mechanisms to maintain normal cell functioning (Fig. 2.4).

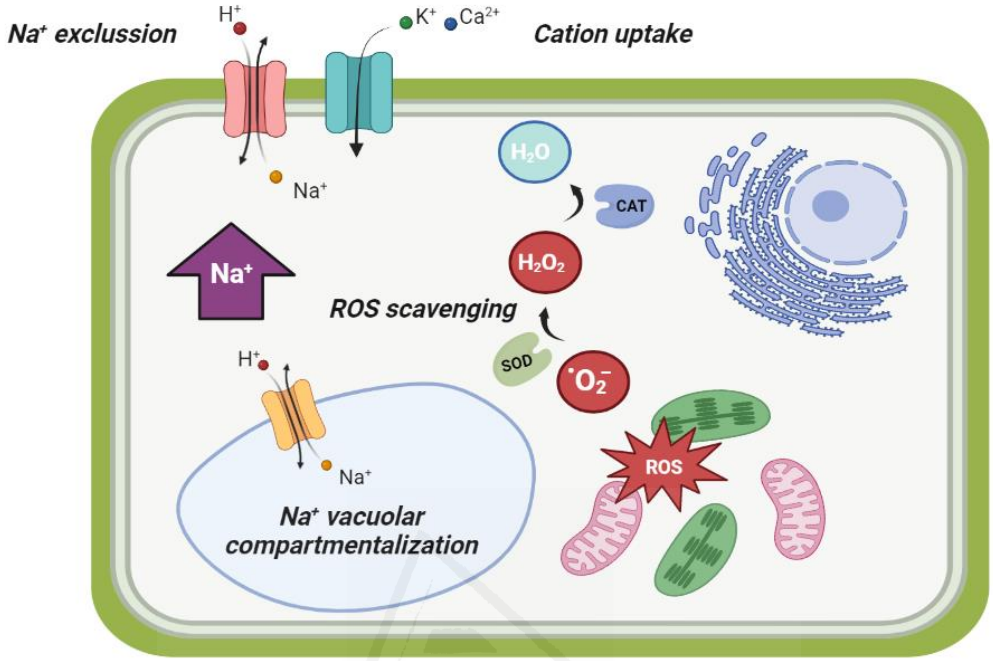

Figure 2.3: Scheme showing the main mechanisms used by plant cells to cope with salinity increments.

To effectively detect hypersaline affection and prevent seagrass decline, the use of biomarkers might arise as a useful tool. Biomarkers are measurable parameters at different levels of biological organization which can anticipate physiological damage and provide information about the responses of an organism against an environmental stressor. By studying the antioxidant capacity and osmoregulatory response of seagrasses against hypersalinity, the tolerance of a species could be assessed before physiological parameters show a clear response. In fact, in the aim of effectively protect seagrass meadows and prevent their decline, the use of more specific and early-warning indicators, or biomarkers, is needed. In this regard, molecular tools might give early information about seagrass health status before morphological of photochemical parameters (Macreadie, Schliep, et al., 2014) and thus, be used to develop effective management measures.

Moreover, considering the changing climate context and the cumulative stressors on shallow coastal ecosystems, seagrasses suffering from hypersaline stress could be more vulnerable and less resilient to other environmental changes. Regarding temperature, its potential interaction with hypersalinity, can lead to a deleterious synergy (Fernández-Torquemada et al., 2005; Salo & Pedersen, 2014; Tsioli et al., 2019, 2022), although some antagonistic behaviour has also been observed, possibly due to a cross-protection effect (Ontoria et al., 2020). Negative synergy has been also detected with pH (Fernández-Torquemada et al., 2005), nutrients (Gacia et al., 2007), and presence of pollutants (Portillo et al., 2014).

2.3 Desalination industry and its interaction with seagrass meadows

As mentioned above, human-derived impacts are the main cause of seagrasses decline (Turschwell et al., 2021), mainly in highly populated coastal areas and in temperate regions (Barragán & de Andrés, 2015). Among the environmental challenges coastal cities must deal with, water scarcity is one of the most relevant and it is even more urgent in a global change context. Lower precipitation rates, groundwater salinization and the increase of freshwater demand are the main causes of water shortage (Huang et al., 2021; Kummu et al., 2016; van Vliet et al., 2021). It is to cope with these issues that seawater reverse osmosis (SWRO) desalination plants have been installed along temperate coasts worldwide (Darre & Toor, 2018). This industry has significantly increased in the last decades, mainly in warm-temperate regions to cope with water scarcity, such as Australia, the Arabian peninsula or the Mediterranean (Jones et al., 2019; Palomar & Losada, 2010). In many cases, desalination plants coastal distribution concurs with seagrass meadows distribution (Jones et al., 2019; Short et al., 2007) and, therefore their interaction becomes a common phenomenon.

The main cause of anomalous salinity increments in the marine environment potentially affecting seagrass meadows are brine discharges from SWRO desalination plants. Desalination provides freshwater from a water source of higher conductivity, such as seawater, generating a high-conductivity disposal, known as brine, which is pumped back to the ocean (Panagopoulos & Haralambous, 2020). Because of its higher salinity, brine usually presents a higher density than the surrounding water, and therefore the discharge falls and moves along the seafloor (Fernández-Torquemada et al., 2009; Lattemann & Höpner, 2008; Portillo et al., 2014), thus mostly affecting benthic communities.

The sensitivity of seagrasses to environmental changes and their declining trend, together with the increment of desalination production worldwide during the last decades, raised awareness about the interactions between this ecological group and the industry. Some field studies revealed a relevant affection of brine discharges on seagrass meadows, even at low salinity increments (1-3 psu) (Fernández-Torquemada et al., 2005; Gacia et al., 2007; Ruiz et al., 2009). After this, many of the ecotoxicology experiments regarding hypersalinity in the Mediterranean were designed to determine seagrass vulnerability to brine discharges using artificial salinity increments (Cambridge et al., 2017; Fernández-Torquemada & Sánchez-Lizaso, 2005; Garrote-Moreno et al., 2014; Marín-Guirao et al., 2013; Sandoval-Gil et al., 2012b). Although desalination impacts have been almost exclusively focused on the osmotic stress caused by brine, desalination discharges can also alter seawater composition as it might increase concentrations of nutrients and metals naturally present in the original seawater as well as chemical compounds (antiscalants, antifoaming, biocides, etc.) added during the desalination process (Falkenberg & Styan, 2015; Fernández-Torquemada et al., 2019; Panagopoulos & Haralambous, 2020). This complex brine composition can also enhance brine toxicity on certain organisms, and have more deleterious effects on seagrasses (Cambridge et al., 2019). Apart

from the osmoprotectant responses (osmolyte synthesis, ion compartmentalization) and physiological adaptations (growth reduction, high respiration rates) demonstrated by the previous works, Capó et al. (2020) found oxidative stress and antioxidant response in a Mediterranean seagrass meadow under brine exposure. This finding revealed the activation of ROM against brine-derived stress and indicates the potential use of biochemical and metabolic parameter to detect brine affection in its first stages.

Furthermore, desalination impact has the potential to even influence seagrass population genetics at a local scale. Hypersalinity affection on seed germination and seedlings development have been previously tested in seagrasses of the *Posidonia* genus (Cambridge et al., 2019; Fernández-Torquemada & Sánchez-Lizaso, 2013), whose results may indicate a reduction of viable sexual reproduction under brine influence, thus reducing genetic diversity locally.

Considering the variability in some physiological responses of seagrasses against hypersalinity (Sandoval-Gil et al., 2023), finding specific brine-triggered metabolic markers would provide essential information about the tolerance mechanisms against this environmental stressor. This knowledge would also be the basis for the creation of novel biomonitoring tools for seagrass conservation. Sustainable desalination requires of adaptative and monitoring programs which effectively measure the affection of this hypersaline effluents on benthic communities (Sola et al., 2020). Therefore, bioindicators must be developed and considered in environmental monitoring programs (EMP) to effectively detect brine effects and prevent ecosystem degradation. In this regard, molecular and biochemical parameter could arise as suitable tools to detect early enough to act upon that causes act implement prevention or mitigation measures.

2.4 *Posidonia oceanica* and *Zostera chilensis* vulnerability to desalination brine discharges

According to an international expert survey on seagrass vulnerability to anthropogenic impacts (Grech et al., 2012), desalination was particularly concerning in the Mediterranean and Temperate Southern Pacific seagrass bioregions proposed by (Short et al., 2007). For this Ph.D. thesis two distant seagrass species have been studied: *Posidonia oceanica* (L.) Delile, the most relevant seagrass species in the Mediterranean basin, and *Zostera* (formerly *Heterozostera*) *chilensis* (J. Kuo) S.W.L. Jacobs & D.H. Les the only seagrass species in the South-American Pacific (Fig. 2.5).

In the case of the Mediterranean Sea, *P. oceanica* forms extensive meadows from 0 to 40 meters depth (Boudouresque & Meinesz, 1982; Gobert et al., 2007). This marine angiosperm is distributed along most of the Mediterranean coast, excluding part of the Adriatic and Aegean Sea as well as the region influenced by the Atlantic inflow water and in front of great rivers (Telesca et al., 2015). Along with other seagrass species, *P. oceanica* is endangered by human activities which have caused meadows regression in the last decades (Telesca et al., 2015; Waycott et al., 2009) and desalination discharges have risen particular concern as this seagrass species is considered a stenohaline organism, which tolerates a narrow salinity range (Fernández-Torquemada & Sánchez-Lizaso, 2005; Ruiz et al., 2009). Together with the turtle seagrass, *Thalassia testudinun*, *P. oceanica* is the seagrass species with more associated research regarding salinity tolerance (Sandoval-Gil et al., 2023). Its sensitivity has even led to the consideration of this seagrass species as an specific brine impact bioindicator in desalination environmental monitoring plans (EMP) (Ruiz et al., 2009; Sánchez-Lizaso et al., 2008; Sola et al., 2020).

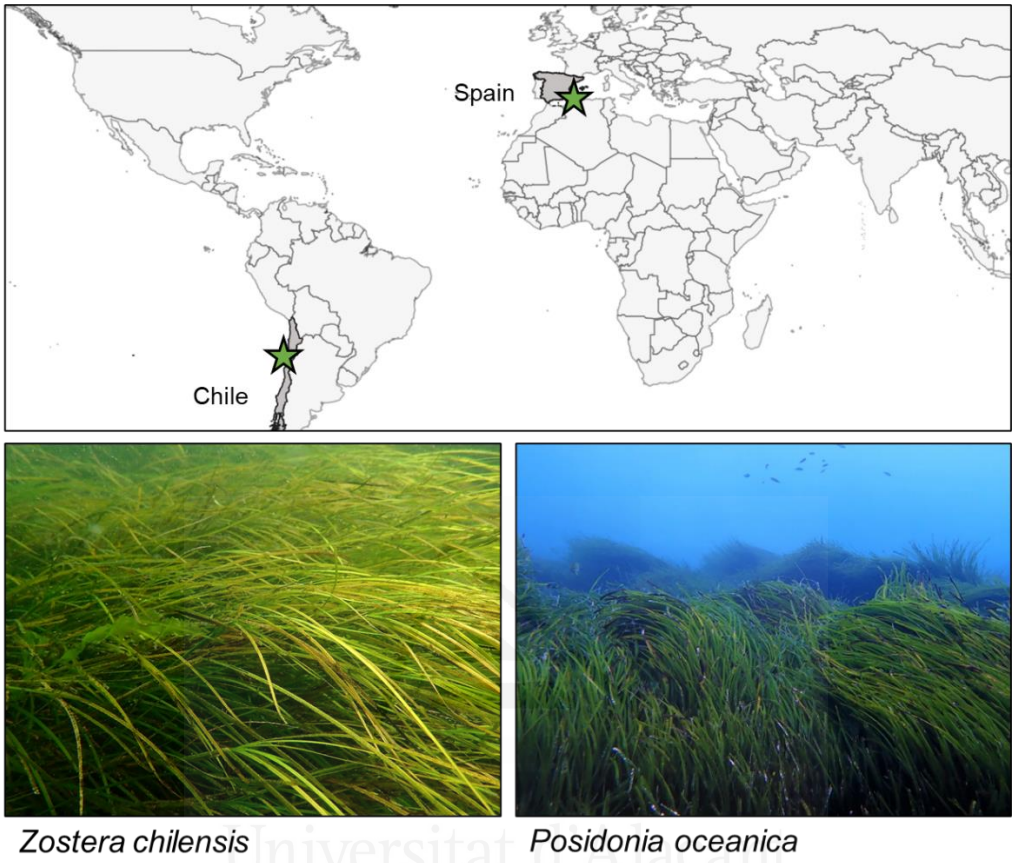

Figure 2.4: Locations and pictures of the species studied in this thesis.

Z. chilensis has been described as an endemic Chilean species (Kuo, 2005; Sullivan & Short, 2023), and is distributed between the 27°S and 30.3°S latitudes and described in only 3 semi-enclosed bays of the Chilean coast (Sandoval & Edding, 2015). Its scarce distribution and the facts that is the only seagrass species along the South-American Pacific, arises questions about the genetic origin of this species. It could be the relict presence of ancient and more extensive seagrass meadows, which only survived in certain protected areas (Vélez-Juarbe, 2014). However, genetic studies seem to relate *Z. chilensis* to the Australian *H. nigricaulis* (Coyer et al., 2013; Smith et al., 2018), indicating a more recent trans-Pacific colonization. In any case, this last representant of marine

angiosperms in this bioregion, is one of the 3 seagrass species (out of 72) listed as endangered by the IUCN Red List (Short et al., 2011; Short & Waycott, 2010) and its survival could be at risk as a consequence of desalination development in North and Central Chile. Although species of the Zostareaceae family have usually shown certain salinity tolerance range, hypersalinity has shown to reduce gross photosynthesis (Biebl & McRoy, 1971), enhance ion exclusion mechanisms (Fernández et al., 1999), or negatively affect nitrogen metabolism (Touchette & Burkholder, 2000). Moreover, in central and northern regions of Chile desalination industry has increased from 1 to 24 SWRO plants from 1997 to 2018 (Sola et al., 2019) and is expected to increase a 100% in the following decade (Herrera-León et al., 2019). In this regard, the risk assessment of *Z. chilensis* against hypersalinity tolerance becomes essential for a future sustainable desalination development in the region.

Being desalination discharges a relatively novel pollutant in the marine environment, the effectiveness of EMP measured parameters is still being assessed, both in Spain and Chile (Sola et al., 2019; 2020). In this regard, seagrasses appear as vulnerable organisms which should be protected and be used as sensitive bioindicators of brine affection.

2.5 Thesis main objectives

This Ph. D. thesis was funded by the predoctoral contract FPUUA98 from the University of Alicante and the Marie Skolowdksa-Curie Action nº888415 entitled OSMOTIC SEAGRASS, whose PI is Claudio Sáez. Moreover, this thesis took place within the framework of a convention between the University of Alicante (Spain) and the University of Playa Ancha (Valparaíso, Chile). The stays at University of Playa Ancha were funded by the University of Alicante grant number UAEEBBFPU_22 and Santander Bank-UA grants for the International Ph.D. mention.

In this thesis, the anthropogenic impact on seagrasses from medium-scale extent (km) to molecular response are assessed with specific interest in hypersalinity. The **main objective** of this work is to deepen in the knowledge of brine discharges affection on seagrasses with the aim of providing novel and specific biomarkers which effectively respond to brine exposure and might have potential as early-warning indicators of brine-derived stress. These indicators can be used as desalination impact biomonitoring tools to detect and act upon before meadow regression occurs. Furthermore, an insight into metabolic adaptations of seagrasses to the marine environment will be made by studying the tolerance responses on 2 different species and in different organs (shoot apical meristems and leaves).

To this end, this thesis focused on 6 **specific objectives**, defined as chapters:

1. Assess the global trend of shallow *P. oceanica* meadows on a wide spatial and temporal trend in Alicante (SE Spain).
2. Determine the long-term affection of a seagrass meadow submitted to a desalination discharge and other coexisting impacts in a coastal city.
3. Develop a risk assessment the tolerance of *Z. chilensis* to desalination-extrapolable hypersalinity levels at the metabolic and physiological level.
4. Assess the physiological, oxidative and osmoregulatory responses of *P. oceanica* against hypersalinity caused by brine and compare it to salinity increments achieved with artificial sea salts.
5. Test the viability of biochemical and molecular descriptors as brine-specific biomarkers under a real brine exposure on *P. oceanica* in the field.
6. Unravel hypersalinity tolerance of *P. oceanica* by comparing metabolic and oxidative responses of shoot apical meristems and leaves and test their suitability as target organs for biomonitoring applications.

Published chapters

Blanco-Murillo, F., Jimenez-Gutierrez, S., Martínez-Vidal, J., Guillén, J. E., & Sánchez-Lizaso, J. L. (2022). Spatiotemporal Trends Observed in 20 Years of *Posidonia oceanica* Monitoring along the Alicante Coast, Spain. *Water*, 14(3), 1–11. https://doi.org/10.3390/w14030274

Blanco-Murillo, F., Fernández-Torquemada, Y., Garrote-Moreno, A., Sáez, C. A., & Sánchez-Lizaso, J. L. (2022). *Posidonia oceanica* L. (Delile) meadows regression: Long-term affection may be induced by multiple impacts. *Marine Environmental Research*, 174, 1–8. https://doi.org/10.1016/j.marenvres.2022.105557

Blanco-Murillo, F., Díaz, M. J., Rodríguez-Rojas, F., Navarrete, C., Celis-Plá, P. S. M., Sánchez-Lizaso, J. L., & Sáez, C. A. (2023). A risk assessment on *Zostera chilensis*, the last relict of marine angiosperms in the South-East Pacific Ocean, due to the development of the desalination industry in Chile. *Science of the Total Environment*, 883, 163538. https://doi.org/10.1016/j.scitotenv.2023.163538

Blanco-Murillo, F., Marín-Guirao, L., Sola, I., Rodríguez-Rojas, F., Ruiz, J. M., Sánchez-Lizaso, J. L., & Sáez, C. A. (2023). Desalination brine effects beyond excess salinity: Unravelling specific stress signaling and tolerance responses in the seagrass *Posidonia oceanica*. *Chemosphere*, 341, 1–12. https://doi.org/10.1016/j.chemosphere.2023.140061

3. Spatiotemporal trends observed in 20 years of Posidonia oceanica monitoring along the Alicante coast, Spain.

Blanco-Murillo, F., Jimenez-Gutierrez, S., Martínez-Vidal, J., Guillén, J. E., & Sánchez-Lizaso, J. L. (2022). Spatiotemporal Trends Observed in 20 Years of Posidonia oceanica Monitoring along the Alicante Coast, Spain. Water (Switzerland), 14(3), 1–11.

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Posidonia oceanica meadows, known to be valuable marine ecosystems, have been reported to be in decline as a result of human activities in recent decades. However, it is still controversial if this decline is a global phenomenon or it is caused by specific disturbances related to human development at a local scale. In order to evaluate changes in *P. oceanica* meadows, in this study, monitoring data obtained at 14 stations along the Mediterranean coast near Alicante, Spain, over a 20-year period were analyzed. Field data were obtained through the citizen science project POSIMED, which had the aim of carrying out annual monitoring of both shallow and deep *P. oceanica* meadows along the coast near Alicante and determining whether their ecological status was changing over time. The percentage cover of living *P. oceanica* and dead matte and shoot density data were used to assess the ecosystem status and to determine whether there had been an overall regional decline in seagrass over the 20-year period. Both cover and density data showed a significant positive trend at most locations. However, the amount of dead matte was noted to slightly increase with time while six shallow and one deep station showed a negative *P. oceanica* cover trend, indicating that in certain locations meadow regression might be taking place. Shoot density decreased with depth and increased with the amount of rock cover;

its correlation with the dead matte percentage was unclear, which probably means that a range of different factors can result in the presence of dead plants. These results support the idea that local disturbances are the cause of seagrass decline in the Mediterranean, thus demonstrating the need for management plans that focus on local stressors of *P. oceanica* meadows at specific locations. Long-term, large-scale monitoring allows the ecosystem status in the western Mediterranean to be assessed; however, local disturbances can also affect specific locations.

water

MDPI

Article

Spatiotemporal Trends Observed in 20 Years of *Posidonia oceanica* Monitoring along the Alicante Coast, Spain

Fabio Blanco-Murillo [1,2,*], Santiago Jimenez-Gutierrez [3], Joaquín Martínez-Vidal [3], Juan Eduardo Guillén [3] and Jose Luis Sánchez-Lizaso [1]

[1] Department of Marine Sciences and Applied Biology, University of Alicante, P.O. Box 99, 03080 Alicante, Spain; jl.sanchez@ua.es
[2] Programa de Doctorado Interdisciplinario en Ciencias Ambientales, Facultad de Ciencias Naturales y Exactas, Universidad de Playa Ancha, Valparaíso 2340000, Chile
[3] Instituto de Ecología Litoral, 03560 El Campello, Spain; s.jimenez@ecologialitoral.com (S.J.-G.); j.martinez@ecologialitoral.com (J.M.-V.); j.guillen@ecologialitoral.com (J.E.G.)
* Correspondence: fabio.blanco@ua.es

Abstract: *Posidonia oceanica* meadows, known to be valuable marine ecosystems, have been reported to be in decline as a result of human activities in recent decades. However, it is still controversial if this decline is a global phenomenon or it is caused by specific disturbances related to human development at a local scale. In order to evaluate changes in *P. oceanica* meadows, in this study, monitoring data obtained at 14 stations along the Mediterranean coast near Alicante, Spain, over a 20-year period were analyzed. Field data were obtained through the citizen science project POSIMED, which had the aim of carrying out annual monitoring of both shallow and deep *P. oceanica* meadows along the coast near Alicante and determining whether their ecological status was changing over time. The percentage cover of living *P. oceanica* and dead matte and shoot density data were used to assess the ecosystem status and to determine whether there had been an overall regional decline in seagrass over the 20-year period. Both cover and density data showed a significant positive trend at most locations. However, the amount of dead matte was noted to slightly increase with time while six shallow and one deep station showed a negative *P. oceanica* cover trend, indicating that in certain locations meadow regression might be taking place. Shoot density decreased with depth and increased with the amount of rock cover; its correlation with the dead matte percentage was unclear, which probably means that a range of different factors can result in the presence of dead plants. These results support the idea that local disturbances are the cause of seagrass decline in the Mediterranean, thus demonstrating the need for management plans that focus on local stressors of *P. oceanica* meadows at specific locations. Long-term, large-scale monitoring allows the ecosystem status in the western Mediterranean to be assessed; however, local disturbances can also affect specific locations.

Keywords: seagrass long-term dynamics; environmental monitoring; citizen science; seagrass meadows; seagrass conservation; population dynamics; western Mediterranean seagrass

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1. Introduction

Seagrass meadows are identified as ecosystems that provide a wide range of both environmental and socioeconomic services in coastal areas around the world, such as primary production, supporting fisheries or sediment stabilization [1–4]. The Mediterranean Sea plays a central role the marine phanerogam *Posidonia oceanica* [5–7]. This plant, which is endemic to the Mediterranean and is an excellent bioindicator of the coastal environment due not only to its longevity, slow growth rate and wide distribution along the Mediterranean coast but also because of its sensitivity to environmental stressors [8–11]. In fact, this species has shown to rapidly respond to impacts, e.g., pollutant bioaccumulation, physiological responses to temperature and salinity variations, and low survival rate due water quality loss [5,12–16].

4. *Posidonia oceanica* L. (Delile) meadows regression: Long-term affection may be induced by multiple impacts.

Blanco-Murillo, F., Fernández-Torquemada, Y., Garrote-Moreno, A., Sáez, C. A., & Sánchez-Lizaso, J. L. (2022). Posidonia oceanica L. (Delile) meadows regression: Long-term affection may be induced by multiple impacts. Marine Environmental Research, 174(January), 1–8.

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Coastal development has an undeniable impact on marine ecosystems resulting in the detriment of the more sensible communities. *Posidonia oceanica* meadows are climax communities which offer a wide variety of ecosystem services both ecological and socio-economic. Human-derived impact on these habitats has been widely assessed although conclusions may vary depending on the area. *P. oceanica* meadow regression next to the city of Alicante (SE Spain) was analyzed on the long term (1984–2014) using bionomic cartographies and side-scan sonar images and, during the last two decades (2003–2021), using cover percentage and shoot density descriptors in the remaining meadow. Results showed a 25% colonized area reduction since 1984, this process being more rapid during the 1984–1994 period and decreasing with time. Cover and density have suffered a significant decrease in the last 20 years, mainly in the upper limit of the meadow. Dead matte cover was also assessed and have shown a significant increase in the same period following an inverse trend with the other metrics. There are several coastal impacts which have co-occurred in the area in the last few decades (port enlargement, brine and sewage discharges, industrial activity) thus resulting in the regression of the meadow. The existing negative trend of the measured descriptors indicate the necessity of implementing management actions which

focus on the present sources of impact and actively reduce their effect on *P. oceanica* beds.

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Posidonia oceanica L. (Delile) meadows regression: Long-term affection may be induced by multiple impacts

Fabio Blanco-Murillo [a, b, *], Yolanda Fernández-Torquemada [a], Aurora Garrote-Moreno [a], Claudio A. Sáez [a, c], Jose Luis Sánchez-Lizaso [a]

[a] Department of Marine Sciences and Applied Biology, University of Alicante, POB, 99, E-03080, Alicante, Spain
[b] Doctorado Interdisciplinario en Ciencias Ambientales, Facultad de Ciencias Naturales y Exactas, Universidad de Playa Ancha, 2340000, Valparaíso, Chile
[c] HUB AMBIENTAL UPLA, Centro de Estudios Avanzados, Universidad de Playa Ancha, 2340000, Valparaíso, Chile

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ABSTRACT

Coastal development has an undeniable impact on marine ecosystems resulting in the detriment of the more sensible communities. *Posidonia oceanica* meadows are climax communities which offer a wide variety of ecosystem services both ecological and socio-economic. Human-derived impact on these habitats has been widely assessed although conclusions may vary depending on the area. *P. oceanica* meadow regression next to the city of Alicante (SE Spain) was analyzed on the long term (1984–2014) using bionomic cartographies and side-scan sonar images and, during the last two decades (2003–2021), using cover percentage and shoot density descriptors in the remaining meadow. Results showed a 25% colonized area reduction since 1984, this process being more rapid during the 1984–1994 period and decreasing with time. Cover and density have suffered a significant decrease in the last 20 years, mainly in the upper limit of the meadow. Dead matte cover was also assessed and have shown a significant increase in the same period following an inverse trend with the other metrics. There are several coastal impacts which have co-occurred in the area in the last few decades (port enlargement, brine and sewage discharges, industrial activity) thus resulting in the regression of the meadow. The existing negative trend of the measured descriptors indicate the necessity of implementing management actions which focus on the present sources of impact and actively reduce their effect on *P. oceanica* beds.

1. Introduction

Human development imply the occupation of natural areas and use of its ecosystem services, changing environmental dynamics at local and sometimes global scales (Goudie, 2000). This is particularly relevant along coastal regions where economic growth have resulted in a health decline of their associated marine ecosystems (Boesch, 2001; Lotze et al., 2006; Montefalcone et al., 2012); indeed, the Mediterranean Sea is an example of this issue (Benoit and Comeau, 2012; Bianchi et al., 2012). Coastal and marine ecosystems suffer from these perturbations, and especially when they are affected by several stressors simultaneously (Halpern et al., 2008; Turner et al., 1996) (He and Silliman, 2019; Short and Wyllie-Echeverria, 1996). The Mediterranean Sea region is an example, with decades of different sources of impacts associated with industrial, social development and tourism, such as port development, marine traffic, chemical discharges, etc. (Burak et al., 2004; Gonen, 1981; Telesca et al., 2015).

Seagrasses have suffered a relevant decline along most of the world's coasts in the last few decades (Dunic et al., 2021; Green et al., 2021; Hemminga and Duarte, 2000; Orth et al., 2006; Short et al., 2011a; Waycott et al., 2009a) and among them, *Posidonia oceanica* at the Mediterranean Sea is one of the main exponents of the phenomenon (Boudouresque et al., 2009; Marbà et al., 1996). The relevance of *P. oceanica* meadows have been widely proven as an ecosystem bioengineer, sustaining a variety of fundamental habitats with ecological and economic importance (Boudouresque et al., 2012; Campagne et al., 2014). Among these ecosystems services, some of the most relevant are their support for coastal fisheries (Unsworth et al., 2019), coastline protection from erosion, sustainability of habitats for ecotourism (Barbier et al., 2011; Hemminga and Duarte, 2000) and climate change mitigation through carbon sequestration and storage (Duarte et al., 2013; Fourqurean et al., 2012; Pergent-Martini et al., 2021; Piñeiro-Juncal et al., 2021). Assessing the economic value of ecosystems is a way of simplifying and putting in value the role of their services

* Corresponding author. Department of Marine Sciences and Applied Biology, University of Alicante, POB, 99, E-03080, Alicante, Spain.
E-mail address: fabio.blanco@ua.es (F. Blanco-Murillo).

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5. A risk assessment on *Zostera chilensis*, the last relict of marine angiosperms in the South-East Pacific Ocean, due to the development of the desalination industry in Chile.

Blanco-Murillo, F., Díaz, M. J., Rodríguez-Rojas, F., Navarrete, C., Celis-Plá, P. S. M., Sánchez-Lizaso, J. L., & Sáez, C. A. (2023). A risk assessment on Zostera chilensis, the last relict of marine angiosperms in the South-East Pacific Ocean, due to the development of the desalination industry in Chile. Science of the Total Environment, 883(April), 163538.

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Seagrasses, which are considered among the most ecologically valuable and endangered coastal ecosystems, have a narrowly limited distribution in the south-east Pacific, where *Zostera chilensis* is the only remaining relict. Due to water scarcity, desalination industry has grown in the last decades in the central-north coasts of Chile, which may be relevant to address in terms of potential impacts on benthic communities due to their associated high-salinity brine discharges to subtidal ecosystems. In this work, we assessed ecophysiological and cellular responses to desalination-extrapolable hypersalinity conditions on *Z. chilensis*. Mesocosms experiments were performed for 10 days, where plants were exposed to 3 different salinity treatments: 34 psu (control), 37 psu and 40 psu. Photosynthetic performance, H_2O_2 accumulation, and ascorbate content (reduced and oxidized) were measured, as well as relative gene expression of enzymes related to osmotic regulation and oxidative stress; these, at 1, 3, 6 and 10 days. *Z. chilensis* showed a decrease in photosynthetic parameters such as

electron transport rate (ETR_{max}) and saturation irradiance (Ek_{ETR}) under hypersalinity treatments, while non-photochemical quenching (NPQ_{max}) presented an initial increment and a subsequent decline at 40 psu. H_2O_2 levels increased with hypersalinity, while ascorbate and dehydroascorbate only increased under 37 psu, although decreased along the experimental period. Increased salinities also triggered the expression of genes related to ion transport and osmolyte syntheses, but salinity-dependent upregulated genes were mostly those related to the reactive oxygen species metabolism. The relict seagrass *Z. chilensis* has shown to withstand increased salinities that may be extrapolable to desalination effects in the short-term. As the latter is not fully clear in the long-term, and considering the restricted distribution and ecological importance, direct brine discharges to *Z. chilensis* meadows may not be recommended.

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A risk assessment on *Zostera chilensis*, the last relict of marine angiosperms in the South-East Pacific Ocean, due to the development of the desalination industry in Chile

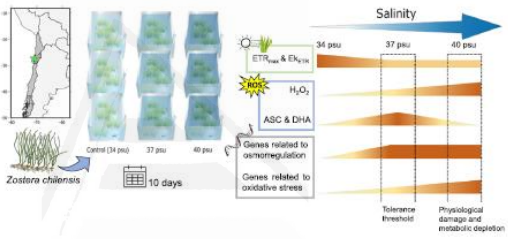
Fabio Blanco-Murillo [a,b,c,*], María José Díaz [c], Fernanda Rodríguez-Rojas [c], Camilo Navarrete [b,c], Paula S.M. Celis-Plá [c], José Luis Sánchez-Lizaso [a], Claudio A. Sáez [a,c,**]

[a] Departamento de Ciencias del Mar y Biología Aplicada, Facultad de Ciencias, Universidad de Alicante, Alicante, Spain
[b] Programa de Doctorado Interdisciplinario en Ciencias Ambientales, Facultad de Ciencias Naturales y Exactas, Universidad de Playa Ancha, Valparaíso, Chile
[c] Laboratory of Aquatic Environmental Research (LACER), HUB AMBIENTAL UPLA, Universidad de Playa Ancha, Valparaíso, Chile

HIGHLIGHTS

- *Zostera chilensis* is a relict seagrass species in the South American Pacific
- Hypersalinity triggers Reactive Oxygen Species production and antioxidant consumption
- Photosystem II electron transport rate and saturation irradiance decreased at higher salinities
- Hypersaline water activates the expression of genes related to osmotic adjustment and mainly of enzymes linked to antioxidant response
- This endemic species might be negatively affected under the influence of a desalination brine discharge

GRAPHICAL ABSTRACT



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ABSTRACT

Seagrasses, which are considered among the most ecologically valuable and endangered coastal ecosystems, have a narrowly limited distribution in the south-east Pacific, where *Zostera chilensis* is the only remaining relict. Due to water scarcity, desalination industry has grown in the last decades in the central-north coasts of Chile, which may be relevant to address in terms of potential impacts on benthic communities due to their associated high-salinity brine discharges to subtidal ecosystems. In this work, we assessed ecophysiological and cellular responses to desalination-extrapolable hypersalinity conditions on *Z. chilensis*. Mesocosms experiments were performed for 10 days, where plants were exposed to 3 different salinity treatments: 34 psu (control), 37 psu and 40 psu. Photosynthetic performance, H_2O_2 accumulation, and ascorbate content (reduced and oxidized) were measured, as well as relative gene expression of enzymes related to osmotic regulation and oxidative stress; these, at 1, 3, 6 and 10 days. *Z. chilensis* showed a decrease in photosynthetic parameters such as electron transport rate (ETR_{max}) and saturation irradiance (Ek_{ETR}) under hypersalinity treatments, while non-photochemical quenching (NPQ_{max}) presented an initial increment and a subsequent decline at 40 psu. H_2O_2 levels increased with hypersalinity, while ascorbate and dehydroascorbate only increased under 37 psu, although decreased along the experimental period. Increased salinities also triggered the expression of genes related to ion transport and osmolyte syntheses, but salinity-dependent up-

* Correspondence to: F. Blanco-Murillo, Programa de Doctorado Interdisciplinario en Ciencias Ambientales, Facultad de Ciencias Naturales y Exactas, Universidad de Playa Ancha, Valparaíso, Chile.
** Correspondence to: C.A. Sáez, Departamento de Ciencias del Mar y Biología Aplicada, Facultad de Ciencias, Universidad de Alicante, Alicante, Spain.
E-mail addresses: fabio.blanco@ua.es (F. Blanco-Murillo), claudio.saez@ua.es (C.A. Sáez).

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6. Desalination brine effects beyond hypersalinity: unravelling specific stress signalling and stress responses in the seagrass *Posidonia oceanica*

Blanco-Murillo, F., Marín-Guirao, L., Sola, I., Rodríguez-Rojas, F., Ruiz, J. M., Sánchez-Lizaso, J. L., & Sáez, C. A. (2023). Desalination brine effects beyond excess salinity : Unravelling specific stress signaling and tolerance responses in the seagrass Posidonia oceanica. Chemosphere, 341(September), 1–12.

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Desalination has been proposed as a global strategy for tackling freshwater shortage in the climate change era. However, there is a concern regarding the environmental effects of high salinity brines discharged from desalination plants on benthic communities. In this context, seagrasses such as the Mediterranean endemic and ecologically important *Posidonia oceanica* have shown high vulnerability to elevated salinities. Most ecotoxicological studies regarding desalination effects are based on salinity increments using artificial sea salts, although it has been postulated that certain additives within the industrial process of desalination may exacerbate a negative impact beyond just the increased salinities of the brine. To assess the potential effect of whole effluent brines on *P. oceanica*, mesocosm experiments were conducted within 10 days, simulating salinity increment with either artificial sea salts or brines from a desalination plant (at 43 psμ, 6 psμ over the natural 37 psμ). Morphometrical (growth and necrosis), photochemical (PSII chlorophyll a fluorometry), metabolic, such as hydrogen peroxide (H_2O_2), thiobarbituric reactive substances (TBARS) and ascorbate/dehydroascorbate (ASC/DHA), and molecular (expression of key tolerance genes) responses were analyzed in each different

treatment. Although with a still positive leaf growth, associated parameters decreased similarly for both artificial sea salt and brine treatments. Photochemical parameters did not show general patterns, although only *P. oceanica* under brines demonstrated greater energy release through heat (NPQ_{max}). Lipid peroxidation and upregulation of genes related to oxidative stress (*GR*, *MnSOD*, and *FeSOD*) or ion exclusion (*SOS3* and *AKT2/3*) were similarly incremented on both hypersalinity treatments. Conversely, the ASC/DHA ratio was significantly lower, and the expression of *SOS1*, *CAT*, and *STRK1* was increased under brine influence. This study revealed that although metabolic and photochemical differences occurred under both hypersalinity treatments, growth (the last sign of physiological detriment) was similarly compromised, suggesting that the potential effects of desalination are mainly caused by brine- associated salinities and are not particularly related to other industrial additives.

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Desalination brine effects beyond excess salinity: Unravelling specific stress signaling and tolerance responses in the seagrass *Posidonia oceanica*.

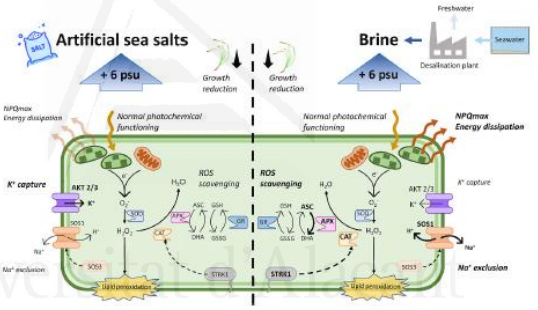
Fabio Blanco-Murillo[a,b], Lázaro Marín-Guirao[c], Iván Sola[a,d], Fernanda Rodríguez-Rojas[d,f], Juan M. Ruiz[c], José Luis Sánchez-Lizaso[a,e], Claudio A. Sáez[a,d,f,*]

[a] Departamento de Ciencias del Mar y Biología Aplicada, Universidad de Alicante, Alicante, Spain
[b] Programa de Doctorado Interdisciplinario en Ciencias Ambientales, Facultad de Ciencias Naturales y Exactas, Universidad de Playa Ancha, Valparaíso, Chile
[c] Seagrass Ecology group, Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO-CSIC), Murcia, Spain
[d] Laboratory of Aquatic Environmental Research, HUB AMBIENTAL UPLA, Universidad de Playa Ancha, Valparaíso, Chile
[e] Ciencias del Mar, Universidad de Alicante, Unidad Asociada al CSIC por el IEO, Alicante, Spain
[f] Departamento de Ciencias y Geografía, Facultad de Ciencias Naturales y Exactas, Universidad de Playa Ancha, Valparaíso, Chile

HIGHLIGHTS

- *Posidonia oceanica* the most ecologically relevant seagrass in the Mediterranean basin.
- Salinity increment (artificial salts vs desalination brine) responses were analyzed.
- Hypersalinity resulted in growth reduction, lipid peroxidation, and *SOS1* expression.
- Brine increased NPQ_{max}, ascorbate consumption (ASC) and *STRK1* and *CAT* transcription.
- Brine triggered specific responses have the potential to be used as specific biomarkers.

GRAPHICAL ABSTRACT



ARTICLE INFO

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ABSTRACT

Desalination has been proposed as a global strategy for tackling freshwater shortage in the climate change era. However, there is a concern regarding the environmental effects of high salinity brines discharged from desalination plants on benthic communities. In this context, seagrasses such as the Mediterranean endemic and ecologically important *Posidonia oceanica* have shown high vulnerability to elevated salinities. Most ecotoxicological studies regarding desalination effects are based on salinity increments using artificial sea salts, although it has been postulated that certain additives within the industrial process of desalination may exacerbate a negative impact beyond just the increased salinities of the brine. To assess the potential effect of whole effluent brines on *P. oceanica*, mesocosm experiments were conducted within 10 days, simulating salinity increment with either

* Corresponding author. Departamento de Ciencias del Mar y Biología Aplicada, Universidad de Alicante, Alicante, Spain.
E-mail address: claudio.saez@ua.es (C.A. Sáez).

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Blanco-Murillo, F., Marín-Guirao, L., Rodríguez-Rojas, F., Sánchez-Lizaso, J. L., & Sáez, C. A. Unravelling *Posidonia oceanica* (L.) Delile sensitivity to salinity increments: the role of shoot apical meristems.

7. Metabolic and transcriptomic responses to desalination brine discharges in field transplanted *Posidonia oceanica*: advances for the development of specific early warning biomarkers

7.1 Introduction

Coastal areas (*i.e.* ~100 km from the shoreline) hold 40% of world's population, with a higher human presence in tropical and temperate regions (Barragán & de Andrés, 2015). As a consequence, there are several human activities coexisting in these areas which can be harmful for the marine environment and, especially, for shallow coastal ecosystems (*e.g.* coastal development, sewage loads, plastic pollution) (Crain et al., 2009). Moreover, temperate areas are among the most vulnerable to water scarcity in a context of global warming as a result of changes in precipitation rates, groundwater salinization and increased demand of freshwater (Huang et al., 2021; Kummu et al., 2016; van Vliet et al., 2021). The development and implementation of desalination technologies, especially seawater reverse osmosis (SWRO) plants, seems promising to address water scarcity in these regions of the world. Indeed, the environmental impact that receives more attention is that associated with the discharge of brines resulting from the desalination process into coastal waters. Brines are the residue the SWRO process, and constitute mostly a concentrated seawater that, without pre-dilution, can double natural salinity levels discharge (Fernández-Torquemada et al., 2009). These discharges may also contain trace concentrations of nutrients, metals, as well as chemical compounds used in the desalination process (*e.g.,* antifouling, antifoaming agents, biocides); however, recent findings suggests that

most biological impacts associated with the brine influence area are mainly caused by the increased salinities (Blanco-Murillo et al., 2023b). In this sense, brine production, has increased globally from 11.6 million m^3/day in 2000 to 95 million m^3/day in 2018 (Jones et al., 2019), especially in mid-latitude populated regions such as the Mediterranean basin (Darre & Toor, 2018; Jones et al., 2019; Palomar & Losada, 2010). Therefore, prevalence of the desalination industry and future prospects of global growth demand increasing our knowledge on the extent of ecosystem impacts and improving current protocols for environmental monitoring.

Among the vulnerable marine ecosystems affected by brine discharges, seagrass meadows are one of the most studied (Fernández-Torquemada et al., 2019; Sandoval Gil et al 2023). This is due to their essential ecological role as habitat-forming species, the numerous ecosystem services they provide, and their particular vulnerability to environmental change (de los Santos et al., 2020; Unsworth et al., 2014). In fact, salinity is one of the most relevant factors determining the distribution, ecology and development of seagrasses (Sandoval-Gil et al., 2023). Most studies concerning the effect of saline increments on seagrasses have been limited to mesocosm experiments using artificial salts (*e.g.*, Blanco-Murillo et al., 2023; Cambridge et al., 2017; Marín-Guirao et al., 2013) while the few field studies have been conducted in seagrass meadows close to the brine discharge prior to its regression (*e.g.*, Sola et al., 2020; Capó et al., 2020; Gacia et al., 2007; Portillo et al., 2014; Ruiz et al., 2009). Moreover, the studied parameters on the field have been mainly focused on population metrics (cover %, shoot density), shoot morphometry (growth, foliar surface, necrosis marks) or physiology (nutrient concentration, carbohydrate content), while there is a lack of information about the effects of brine-associated hypersalinity at the sub-cellular and metabolic level (biomarkers) (Roca et al., 2016; Sandoval-Gil et al., 2023). In this regard, the use of experimental transplants might allow the most

reliable simulation of natural conditions while modifying the intensity of the studied stressor (Garrote-Moreno et al., 2014; Muñoz et al., 2023b; Rodríguez-Rojas et al., 2020). Moreover, it has been demonstrated that a good field transplantation approach combined with sub-cellular and metabolic responses, can be both more effective as early warning tools to address for potential further physiological and ecological effects; also, increase attributing the consequences of a response to specific stressors when several are present (Muñoz et al., 2023b; Rodríguez-Rojas et al., 2020; Sáez et al., 2015). Most of these experiences with macrophytes have been conducted with macroalgae; therefore, it necessary to address the potential usefulness of transplanted seagrasses biomarkers as environmental biotechnology tools, especially in the context of desalination discharges.

In the case of the Mediterranean Sea, *Posidonia oceanica* is the most abundant and ecologically relevant seagrass species (Sandoval-Gil et al., 2023). *P. oceanica* is a stenohaline organism with a narrow optimal salinity range of 37-38.5 practical salinity units (psu) for a normal physiological development (Fernández-Torquemada et al., 2005; Sánchez Lizaso et al 2008; Ruiz et al., 2009), although certain populations have been able to thrive under different salinity conditions (up to 51.5 psu) (Azcárate-García et al., 2023; Mancuso et al., 2023; Marín-Guirao et al., 2017; Nguyen et al., 2023). Salt increments have shown to alter ion composition in *P. oceanica* and cause physiological stress beyond certain threshold levels (photochemical depletion, growth reduction, among others) (Garrote-Moreno et al., 2015; Sandoval-Gil et al., 2012a), but the sub-cellular mechanisms to cope with osmotic stress are scarcely known. However, Blanco-Murillo et al. (2023a) under mesocosm experiments observed that *Zostera chilensis* under hypersaline exposure (+3 and +6 psu) displayed higher production of reactive oxygen species (ROS), antioxidants and in the expression of genes related with ROS metabolism (ROM) and osmotic regulation and, more

recently, similar responses were detected in *P. oceanica* exposed to real desalination brine (+6 psu) (Blanco-Murillo et al., 2023b). These results highlight the potential of these responses to be tested as environmental biotechnology tools for early detection and mitigation of environmental distress, in addition to evaluate the biomarkers to address the specificity of desalination brine effects on seagrasses.

The aim of this study is to determine responses of *P. oceanica* to mid-term (6 days) exposure to a real brine discharge, and to assess the metabolic and cellular tolerance mechanisms and thresholds. To this end, *P. oceanica* was transplanted along a salinity gradient in the area of influence of brine discharges from a SWRO desalination plant, and the response of the transplants was studied for up to 6 d. Transplantation experiments make it possible to recreate a natural environment under specific known conditions such as, for example, a *P. oceanica* meadow suddenly exposed to a brine discharge. Thus, we intended to: i) evaluate ROS production, oxidative damage, antioxidants and osmotic biochemical and molecular responses in plants at different salinity thresholds following a brine dilution plume, ii) determine the suitability of these descriptors as biomarkers to follow the extent and predict potential impacts of desalination brine discharges.

7.2 Materials and methods

Study location and experimental design

The experimental sites were selected at the same depth (3 meters) after preforming salinity measurements using a conductivity-temperature-depth meter (CTD; RBR Concerto, RBR, Canada). Different locations were chosen according to historical data (Fernández-Torquemada et al., 2005), and confirmed with a conductivity and temperature meter (CT; ALEC Infinity, Alec Electronics, Japan) measuring in continuous during the transplantation experiments: a control location (~37 psu); intermediate brine exposure (IB; ~39.5 psu); and

high brine exposure (HB; ~42psu) (Fig. 7.1a and Fig. 7.2). Experiments were conducted between November 20 and 27 2021.

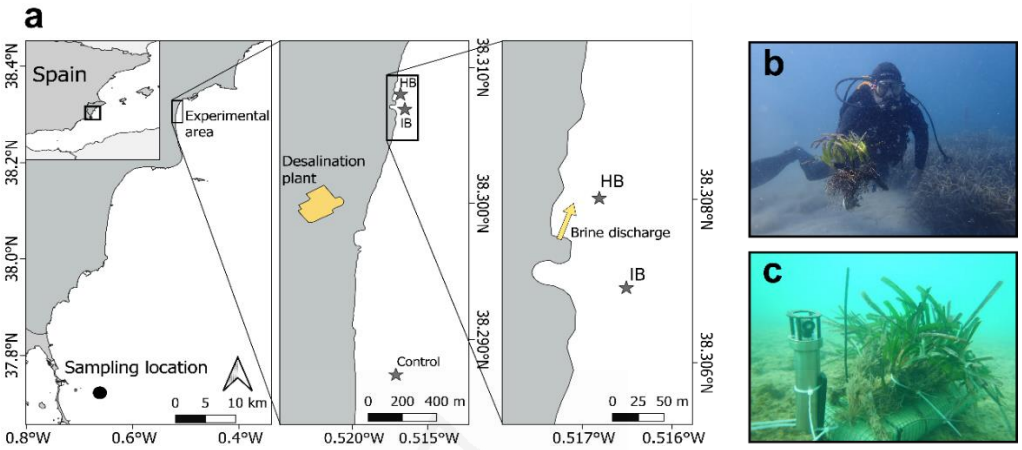

Figure 7.1: Maps showing the donor meadow (sampling location) and the experimental area (a). The arrow indicated the brine discharge point and IB and HB represent intermediate and high brine exposure sites, respectively. SCUBA diver sampling *P. oceanica* ramets (b) and sample of a transplantation unit in the field (c).

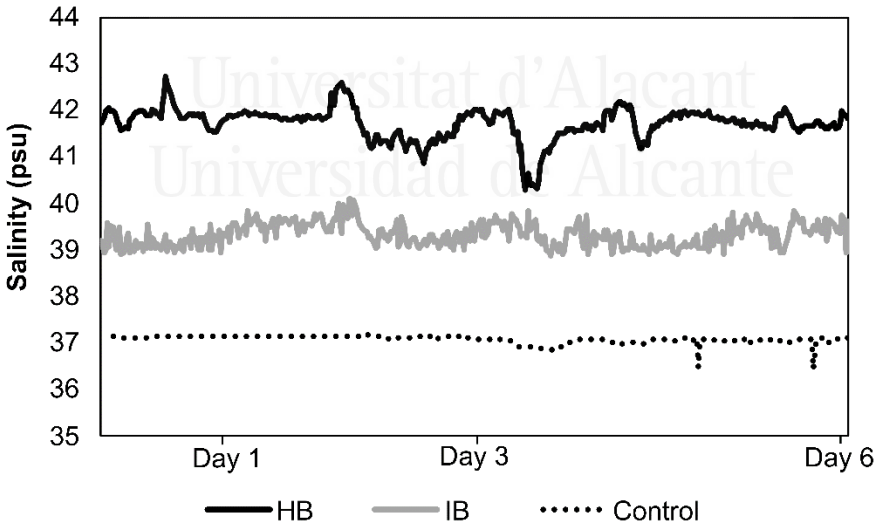

Figure 7.2: Salinity data recorded in experimental sites along the experimental period with a conductivity and temperature (CT) meter.

Plants were collected from a healthy meadow (Isla Grossa, Murcia), a Specially Protected Area of Mediterranean Importance (SPAMI) and transported in darkness and constant aeration to the experimental area (Alicante) within 4 hours (Fig. 7.1a). Two *P. oceanica* ramets consisting of a plagiotropic and 15-25 orthotropic shoots, were attached to a concrete anchor comprising a transplantation unit (TU). All transplants were closely placed in the control location for a 7 days acclimatation period and subsequently transported by divers to HB and IB experimental sites. Three TUs were installed by SCUBA divers in each site (Fig. 7.1c).

Leaf samples were collected at days 1, 3 and 6 since the start of the transplantation experiments. The first 2 mature leaves of 1 shoot from both individuals in each TU were sampled each day. The first 5 cm as well as the leaf apex were removed before sample storage to avoid response variability due to leaf age (Ruocco, Marín-Guirao, & Procaccini, 2019). Tissue destined for biochemical analyses was rapidly frozen in liquid nitrogen and subsequently transported to the laboratory and stored at -80 ºC for further analyses. Samples reserved for relative gene expression were stored in RNAlater (Invitrogen™), kept at 4 ºC for 24 h and then stored at -20 ºC, according to manufacturer's instructions.

Hydrogen peroxide (H_2O_2) determination

The determination of H_2O_2 in *P. oceanica* leaf tissues was performed by modifying the protocol described in Sáez et al. (2015). Samples were grounded using liquid nitrogen before analysis and 20 mg were added to 100 µL of 10% trichloroacetic acid (TCA), 150 µL of 10 mM potassium phosphate buffer (pH 7.0), 50 µL of FAPRB lysis buffer from FavorPrep™ Plant Total RNA Mini Kit (FAVORGEN), and 500 µL of 1 M potassium iodide. Samples were vortexed for 15

min using glass beads (3 mm) and centrifuged for 15 min at 12,000 xg and 4 ºC. Samples absorbance was measured at 392 nm using a spectrophotometer SpectroStar Nano (BMG LABTECH). Standard curves were performed using commercial H_2O_2 (Sigma Aldrich Merck, St Louis, MO, USA).

Determination of thiobarbituric acid reactive species (TBARS)

The ROS production can lead to cellular membrane lipid peroxidation. Polyunsaturated fatty acids are oxidized by hydroxyl radicals (·OH) producing malondialdehyde (MDA) which can be determined by reaction with thiobarbituric acid (TBA). Twenty mg of liquid nitrogen grounded biomass were added to 500 µL 10 % trichloroacetic acid (TCA) and vortexed for 15 min using glass beads (3 mm). Mixtures were then centrifuged at 17800 xg for 15 min at 4 °C, and 200 µL of supernatant were added to 200 µL 0.5 % TBA; the latter, was incubated for 30 min at 90 °C. Absorbance was measured by taking of 200 µL to a microplate reader (SpectroStar Nano, BMG LABTECH) at 532 nm. Commercial MDA (Sigma Aldrich Merck, St Louis, MO, USA) was used for constructing standard curves.

Ascorbate (ASC) and dehydroascorbate (DHA) content

As an indicator of *P. oceanica* antioxidant capacity, ASC (reduced) and DHA (oxidized) forms of total ascorbate were determined following a modified protocol from Benzie and Strain (1999). First, ASC was measured from 10 mg of grounded biomass using liquid nitrogen by adding 300 µL 0.1 M HCl and 300 µL of FAPRB lysis buffer from FavorPrep™ Plant Total RNA Mini Kit (FAVORGEN). Samples were vortexed for 10 min and centrifuged at 21000 xg for 10 min at 4 °C. Ten µL of the supernatant were added to 290 µL of FRAP buffer (300 mM pH 3.6 sodium acetate buffer, 20 mM $FeCl_3$ and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ)), which reacts with ASC forming a colorimetric complex than can be measure spectrophometrically at 593 nm, (BMG LABTECH). Second, total

ascorbate was obtained by reducing 250 µL supernatant with 2.5 µL of 100 mM dithiothreitol (DTT) and incubated for 1 hour at room temperature. After incubation 2.5 µL (w/v) of N-ethylmaleimide were added to stop DTT mediated-reaction and 10 µL of extract were again added to 290 µL of FRAP buffer and measured at 593 nm. DHA content was obtained by calculating the difference of Total ascorbate and ASC. L-ASC (Sigma Aldrich Merck, St Louis, MO, USA) was used for standard curves.

RNA extraction and qPCR

A set of 9 genes of interest related to antioxidant defence and osmoregulatory mechanisms were selected to analyse their response in *P. oceanica* plants under different brine exposures (Tab 6.1). These genes were also tested successfully in recent mesocosm experiments with brine-mediated increased salinities on *P. oceanica* (Blanco-Murillo et al., 2023b). Leaf samples were selected as defined by Blanco-Murillo et al. (2023b), taking the first mature leaf of each individual and selecting a 4 cm fragment after removing the first 5 cm and leaf apex. Samples were stored in RNAlater at 4 ºC for 24h and then frozen at -20 ºC. A leaf biomass of 50 mg was powdered using liquid nitrogen and RNA was extracted using Aurum™ Total RNA mini kit (BIORAD) following the manufacturer instructions. RNA purity and integrity were checked using 260/280 ratio by spectrophotometric measurements (SpectroStar Nano) and 1.2% agarose bleach electrophoresis, respectively.

For RNA quantification, Quant-iT RiboGreen RNA assay kit (Invitrogen, Waltham, MA, USA) was used to determine RNA concentration by fluorescence in a QFX fluorometer (DeNovix, Wilmington, DE, USA). Samples were then standardize using 350 ng of RNA before synthetizing cDNA with a cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fischer Scientific) with a final sample volume of 20 µL. To perform quantitative PCR (qPCR), 2 µL of cDNA were

added to 0.5 µL of each primer (forward and reverse), 10 µL of Green PCR Mix SYBR (Agilent Technologies, Santa Clara,CA, USA) and 7 µL of nuclease-free water. Samples were then taken to a qPCR Magnetic Induction Cycler (MIC; Bio Molecular Systems, Queensland, Australia) programmed with an initial denaturation at 95 °C for 5 min, and then 40 cycles of 95 °C for 10 s, 55 °C for 10 s, 72 °C for 40 s, and a final extension at 72 °C for 10 min. Quantification cycle (Cq) values of each gene were used to subsequently determine relative gene expression based in the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001):

$$\text{Fold expression change (FC)} = 2^{(|\,(-\Delta Cq\ \text{treatment}) - (-\Delta Cq\ \text{control})\,|)}$$

To measure FC, 18S rRNA was chosen as housekeeping gene according to Serra et al. (2012) and confirmed in Blanco-Murillo et al. (2023b).

Statistical analyses

A two-way ANOVA was performed with treatment (three levels: controls, HB and IB) and time (three levels: 1, 3 and 6 days) as fixed factors to determine differences in the responses of all measured parameters. Data normality and homoscedasticity were tested using a Kolmogorov-Smirnov test and a Bartlett test, respectively (Underwood, 1997). When significant differences were found, a *post-hoc* Tukey-HSD test was conducted to determine which levels were statistically different. ANOVA results are included in Supplementary data (tables S10-S11).

7.3 Results

ROS production and oxidative damage

H_2O_2 levels in *P. oceanica* leaf tissues revealed significant interaction between Treatment and time factors. HB showed significantly higher values compared to controls in all sampling days, while IB was significantly higher than control plants only at day 3 (Fig. 7.3a). Overall, values of H_2O_2 were higher on day 6 compared

to days 1 and 3, especially for HB. TBARS analysis demonstrated significantly higher lipid peroxidation in the HB treatment compared to the Controls and IB. As for the effect of time, all treatments at day 6 showed significantly higher levels compared to day 3, but not with day 1 (Fig. 7.3b).

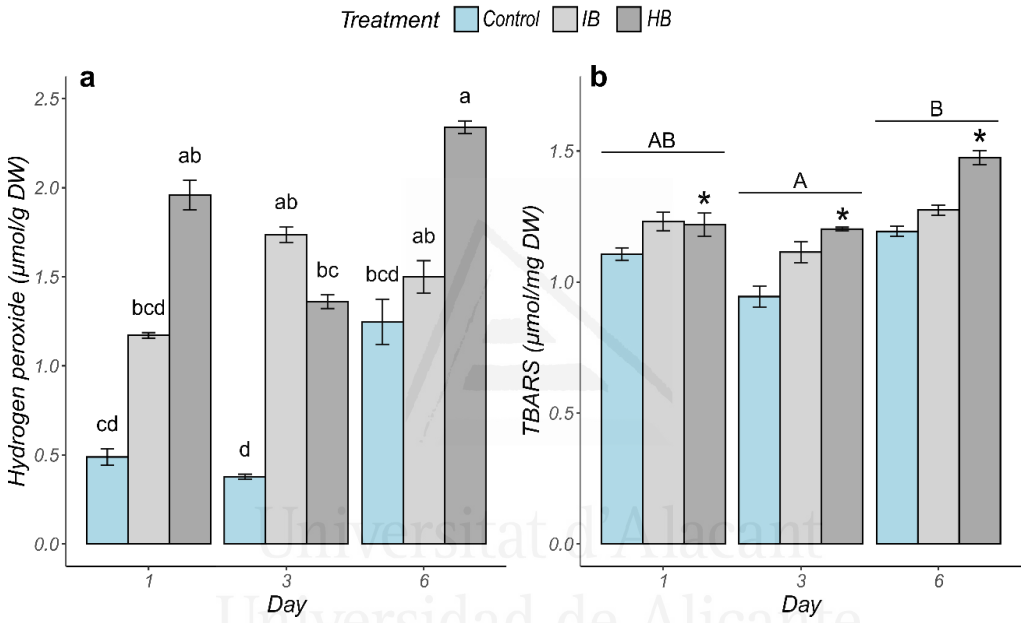

Figure 7.3: Hydrogen peroxide (H_2O_2) (a) and TBARS (b) levels contents in *P. oceanica* leaf samples from experimental treatments: Control (~37 psu), IB (~39 psu), and HB (~42 psu). Barplots represent the mean of each variable and error bars show the standard error. Upper case letters represent significant differences at 95% confidence interval ($p < .05$) between days 1, 3 and 6. Lower case letters represent significant differences between groups when the interaction factor was significant. Asterisks (*) indicate significant differences between treatments.

Antioxidant levels

Despite significant differences, total ascorbate (ASC + DHA) was lower in both brine exposures IB and HB compared to control plants; however, at day 6 controls HB did not demonstrate significant differences with controls; moreover, although total ascorbate in controls and LB displayed a trend of decrease with time, the opposite pattern was observed for HB (Fig. 7.4). ASC levels presented significant differences for the treatment factor, but with a trend of lower values in plants only under HB, and relatively constant

values between days (Fig. 7.4). DHA levels in control plants were significantly higher than in brine-exposed plants (HB and IB) on days 1 and 3, while on day 6 only IB plants showed lower values compared to control and HB; the general pattern demonstrated higher DHA than ASC levels in controls with respect to brine exposed plants across experimental times (Fig. 7.4).

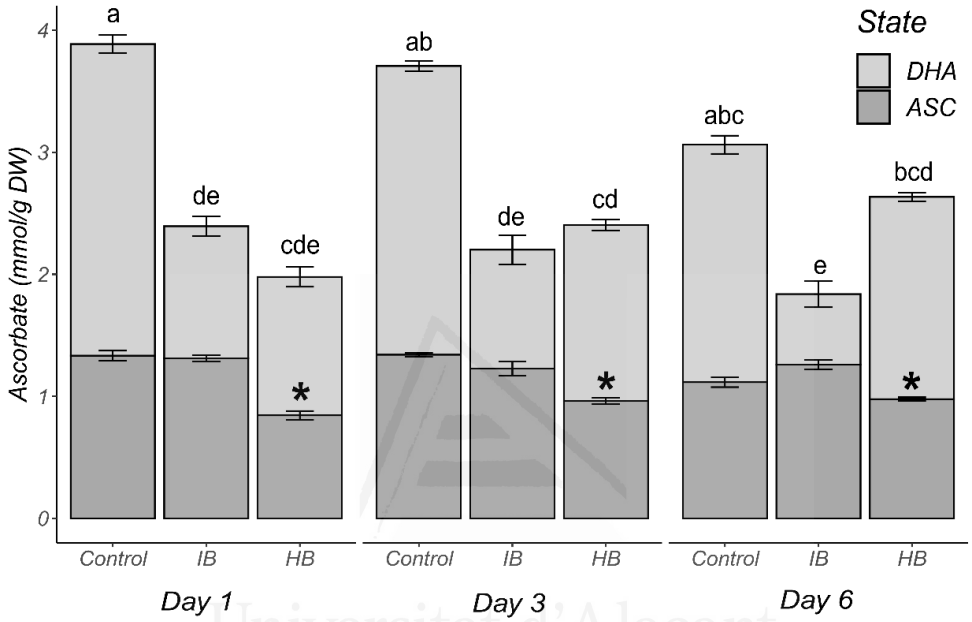

Figure 7.4: ASC and DHA levels in *P. oceanica* leaves from the experimental treatments: Control (~37 psu), IB (~39 psu), and HB (~42 psu). Barplots represent the mean of each variable and error bars show the standard error. Lower case letters represent significant differences ($p < .05$) between groups when factor interaction was significant. Asterisks (*) indicate significant differences between treatments.

Expression of genes related to osmotic and oxidative stress regulation

In relation with genes involved in oxidative stress response, a general expression structure was observed along the experimental time. In this sense, the highest levels of expression were detected at day 1 of transplantation experiments, similarly in both brine-exposed sites (IB and HB), to decrease significantly later at day 3 and 6 (Fig 7.5.a-f). Beyond general patterns, downregulation was observable at brine-influenced sites for APX and CAT only at

day 6 (Fig. 75c and 7.5f, respectively), and for GR but just in IB; indeed, the latter and STRK1 were the only oxidative response genes to present significantly higher expression in HB than IB at all experimental times (Fig. 7.5d and 7.5e).

Regarding osmotic regulation genes, in both brine exposure sites, *SOS1* showed a down-regulation throughout the experimental period, with a slight recovery on day 6 in IB plants. Although no statistical differences were detected, HB plants presented higher relative expression levels on days 1 and 3 (Fig. 7.5g). In the case of *SOS3*, plants from the HB treatment displayed higher gene expression compared to IB throughout time, even though with a trend of decline until day 6 (Fig. 7.5h). In spite no patterns were observed at days 1 and 3, *AKT* was significantly upregulated under HB compared to IB plants at day 6 (Fig. 7.5i). In case of *STRK1,* it demonstrated a significantly up-regulation in plants upon HB compared to IB along the experimental time; also, a slight decrease in the expression at day 3 and 6 compared to day 1, maintaining the pattern of upregulation in HB (Fig. 7.5d).

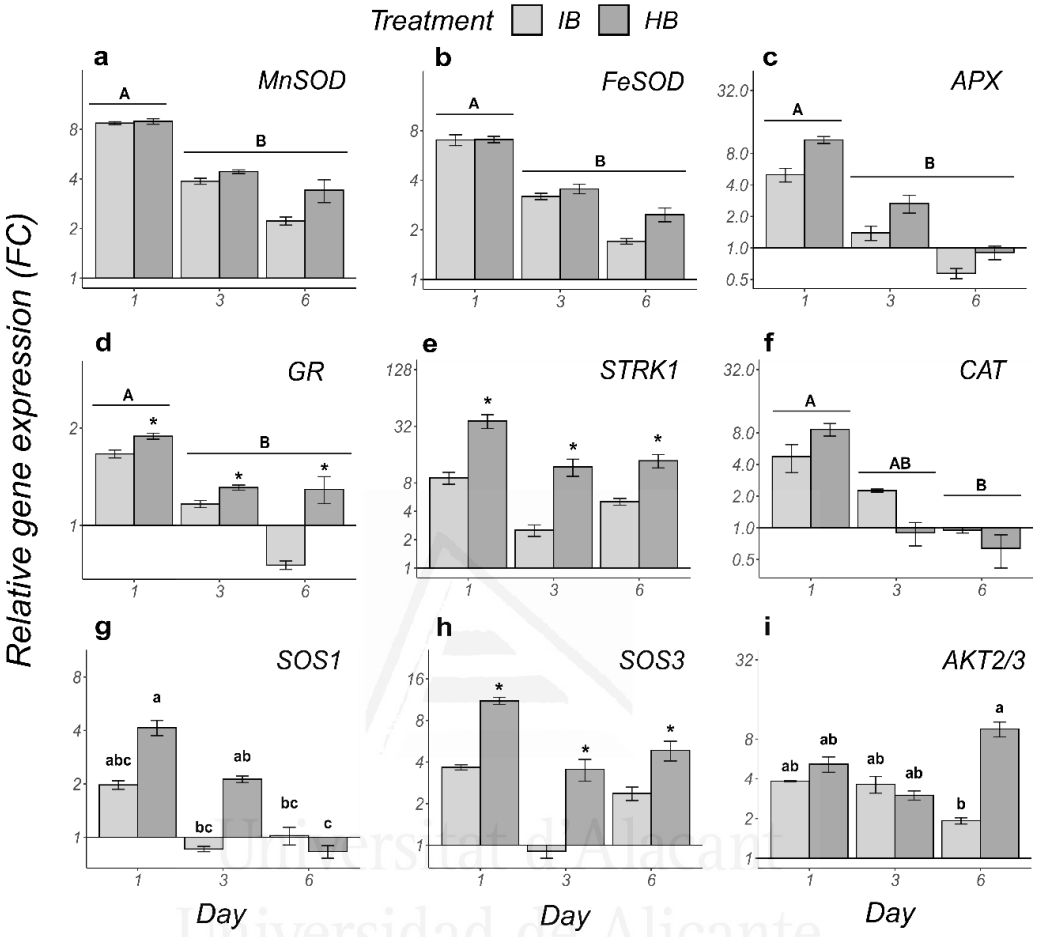

Figure 7.5: Relative levels of expression with respect to controls of osmotic regulation and oxidative stress-related genes in *P. oceanica* upon two salinity exposures in the field: IB (~39 psu), and HB (~42 psu). Genes measured were: *MnSOD* (a); *FeSOD* (b), *APX* (c), *GR* (d), *STRK1* (e), *CAT* (f), *SOS1* (g), *SOS3*(h) and *AKT2/3* (i). Upper case letters represent significant differences at 95% confidence interval ($p < .05$) between days (1, 3, and 6). Lower case letters represent significant differences between groups when factor interaction was significant. Asterisks (*) show significant differences between treatments.

7.4 Discussion

This study describes several cellular and molecular responses of *P. oceanica* exposed in the field to brine effluents from an operating seawater desalination plant (SWRO). Leaf tissues in brine influenced sites accumulated ROS, showed

signs of oxidative damage, and diminished some of their resources involved in cellular antioxidant defences. These effects were proportional to the degree of influence of the brine discharge, and therefore more pronounced in plants closer to the discharge point and exposed to higher salinity levels (~42psu vs ~39.5psu). Activation of genes related to osmoregulatory mechanisms also showed a direct relationship with brine exposure, being activated in proportion to the salinity level experienced by the plants. Transcription of oxidative stress genes was more evident after 1 day of exposure reflecting a rapid response to oxidative stress under hypersaline conditions.

In seagrasses, H_2O_2 increment as a consequence of hypersalinity had been detected in *Thalassia tesudinum* exposed to +15 psu above natural levels for 14 days (Trevathan et al., 2011) and *Z. chilensis* exposed to +3 and +6 psu over baseline salinities (Blanco-Murillo et al., 2023a). However, *P. oceanica* exposed to increased salinities (+6 psu) over natural 37 psu achieved with real brine from a desalination plant under mesocosm conditions, did not present an increment in H_2O_2 levels (Blanco-Murillo et al., 2023b). In contrast, in this field experimental study, H_2O_2 levels in *P. oceanica* were greater than controls even in sites with lower salinities (39 and 42 psu) compared to the 43 psu used in Blanco-Murillo et al. (2023b). Hydrodynamics, herbivory (which was observed during sample collection) and water turbidity could be among the main environmental factors contributing to a ROS production response in *P. oceanica* transplants. Moreover, in Alicante bay, several anthropogenic activities have historically co-occurred with the desalination plant and caused *P. oceanica* meadow decline in the last decades (Blanco-Murillo et al., 2022) and could be increasing stress responses due to the potential influence of other coastal activities (*e.g.,* sewage discharges).

Once in the cytosol, H_2O_2 can be transformed into hydroxyl radicals (·OH) via Fenton reaction (Bartosz, 1997). This reactive ROS can interact with plasmatic

membranes, which can be measured as TBARS levels as a *proxy* of lipid peroxidation (*i.e.*, oxidative damage). Lipid peroxidation was higher in HB plants, and increased at day 6, indicating a direct relationship between intensity and duration of brine exposure, ROS production and oxidative damage. Results from the mesocosm also showed higher lipid peroxidation under brine exposure of +6 psu, but stable throughout the 10 days experimental period (Blanco-Murillo et al., 2023b). On the other hand, in a local population of *P. oceanica* under the influence of a brine discharge, TBARS levels increased by 54.5% and 108.3% at +1.5 and +2.8 psu above natural levels, respectively (Capó et al., 2020). Even though laboratory experiments at exposures using brine as high as +6 psu over natural levels (Blanco-Murillo et al., 2023b), lipid peroxidation has been observed greater in *P. oceanica* in local populations (Capó et al., 2020) and transplanted (*i.e.*, this investigation) nearby desalination discharges. In this sense, Capó et al. (2020) observed shorter leaves in the site closest to the brine discharge, also observed in *P. oceanica* by Gacía et al. (2007) and attributed to enhanced herbivory by the fish *Sarpa salpa* and the sea urchin *Paracentrotus lividus*. In this sense, in spite it was not measured, shoot bites and the shortest leaves were observed in *P. oceanica* transplants of HB site (see Supplementary Fig. S2). Moreover, is important to consider that several studies have reported higher diversity and/or abundances of fish nearby desalination discharges, for reasons still not fully understood (Kelaher et al., 2020; Sola et al., 2020). Certainly, the mechanic constrains and tissue destruction caused by herbivory may be an added pressure to other anthropogenic impacts that can surely be manifested in the observed greater lipid peroxidation in local or transplanted *P. oceanica* upon desalination discharges.

To manage oxidative damage produced by exposure to harmful brine levels, *P. oceanica* cells would attempt to scavenge H_2O_2 by enzymatic and non-enzymatic antioxidant mechanisms. HB plants showed reduced levels of ascorbate (ASC),

suggesting consumption of this antioxidant enzyme to cope with brine (and other factors)-triggered ROS production. A significant consumption of ASC was also detected in *P. oceanica* under mesocosm conditions (Blanco-Murillo et al., 2023b), supporting the important role of this antioxidant in the defence of the species against ROS derived from hypersaline and stresses. The decrease of total ascorbate (ASC+DHA) in leaves of plants exposed to brine could be due to an impairment between metabolic recycling and *de novo* synthesis of this antioxidant in *P. oceanica* cells. In fact, ascorbate synthesis is highly dependent on the functioning of the electron transport chain (Millar et al., 2003), which is very sensitive to ionic stress produced by increased salinity; this was observed by lower electron transport rates (ETR) in *Z. chilensis* under +3 and +6 psu hypersaline conditions (Blanco-Murillo et al., 2023a). Therefore, and considering the presence of other potential environmental pressures, ascorbate results were consistent with brine influence in the study area.

Ion superoxide (O_2^-) is the first ROS formed in stressed plants (Bose et al., 2014), and its conversion to H_2O_2 is catalysed by FeSOD in the chloroplasts and MnSOD in the mitochondria (Van Camp et al., 1995). Our results revealed a marked up-regulation of *MnSOD* and *FeSOD* at early stages of exposure in IB and HB plants to later decrease their expression; this was similar to the results in regulation of these enzymes in Blanco-Murillo et al. (2023b) upon 43 psu in mesocosm experiments. Interestingly, Capó et al. (2020) measured the lowest SOD activity in local *P. oceanica* in the site with higher salinity within the brine-influenced area; in contrast, non-enzymatic ROS scavenging mechanisms were the strongest at the site with highest salinity. It is known that long exposure to environmental pressures, including salinity excess, can lead to epigenetic modifications in seagrasses; therefore, changing patterns of gene expression (Shen et al., 2022). Indeed, the observed intra-specific differences in response between the local population sampled by Capó et al. (2020), and subject to

desalination discharges since 1994, appear to be related to developed epigenetic adaptations compared to our transplanted *P. oceanica* with origin in the non-impacted area. The decrease in the expression of metal-substrate SODs beyond day 1 was also observed in mesocosm experiments by Blanco-Murillo et al. (2023b), and can be attributed to a sufficient enzymes stock transcribed at the earliest stage of strong gene upregulation.

STRK1 is a signalling protein which is sensitive to salinity increments, promoting CAT synthesis to cope with an increment in H_2O_2 excess (Yang & Guo, 2018; Zhou et al., 2018). *STRK1* and *CAT* observed higher relative expression in HB plants demonstrates their role as salinity-dependent mechanism to cope with H_2O_2 production as an osmotic pressure response. Therefore, H_2O_2 concentrations and STRK1 phosphorylation activity appeared to trigger *CAT* transcription, especially on day 1. Both genes followed a similar trend of upregulation throughout the transplantation period, as in laboratory experiments (Blanco-Murillo et al., 2023b), confirming their co-dependence. On the other hand, H_2O_2 scavenging is also performed by APX, which reduces H_2O_2 by oxidizing ASC to DHA. ASC can then be restored by oxidizing reduced glutathione (GSH) to glutathione disulfide (GSSG); in turn, GSSG is reduced back to GSH by glutathione reductase (GR) using NADPH as a substrate; all, part of the ascorbate-glutathione (or Foyer-Halliwell-Asada) cycle (Foyer & Noctor, 2011). In our results, *APX* was more upregulated in plants under HB influence, indicating a salinity-correlated transcription. These results also agree with lower ASC levels in HB plants, indicating APX-mediated consumption to cope with H_2O_2 excess. *GR* upregulation indicates an activation of GSH regeneration under brine exposure, which has been confirmed to occur in local *P. oceanica* nearby desalination discharges (Capó et al., 2020). GSH can be then consumed to restore ASC levels through the Halliwell-Asada cycle. It is important to mention that the patterns of upregulation observed along the transplantation period where in accord with

those observed in mesocosm experiments (Blanco-Murillo et al., 2023b), although with tendencies to lower levels of expression towards the end of the experiments in the latter; indeed, supporting the idea that more environmental pressures besides just brines induce the recorded responses.

In addition to ROM, ion balance is essential for physiological and metabolic functioning in plants cells. In particular, K^+ uptake and Na^+ exclusion are essential mechanisms for seagrasses to cope with brine-derived osmotic pressure, as intracellular Na^+ excess can be highly toxic (Garrote-Moreno et al., 2014). To respond against toxicity, plant cells have developed mechanisms of signalling prevent osmotic stress, among which the Salt Overlay System (SOS) enzymatic complexes have been demonstrated to be key. For instance, SOS3 is a Ca^{2+} binding protein which enhances the transcription of *SOS1*, which in turn encodes for a Na^+/H^+ antiporter protein; the latter mediates Na^+ extrusion through the plasmatic membrane implying H^+ extracellular intake (Hadi & Karimi, 2012; Yang & Guo, 2018). AKT2/3 is an specific transport protein which would increase K^+ capture, which is essential to keep plant metabolic functioning (Dennison et al., 2001). K^+ is used as cofactor by enzymes related to several biochemical pathways and its substitution by Na+ can cause severe metabolic impairment (Steven, 1985). All genes related with osmotic regulation *SOS1*, *SOS3* and *AKT2/3* displayed a pattern of marked upregulation at day 1, with greater expression in HB compared to IB, followed by a trend of moderate decrease at later sampling points; the exception was *AKT2/3*, which although demonstrated an upregulation at day 1, the highest levels of transcripts were detected at day 6 for the site HB. Despite the trends of regulation of these genes were similar to those observed in mesocosms experiments with *P. oceanica* under 43 psu for up to 10 days (Blanco-Murillo et al., 2023b), the decrease in regulation of *SOS1*, *SOS3* and *AKT2/3* in *P. oceanica* transplants beyond 1 day of experiments were less marked; certainly, most of the time maintaining over-expression. Therefore, these results agree and

representative of environmental salinity levels, considering that *P. oceanica* transplanted in this study was subject to an average of 39.5 psu in IB and 42 psu in HB.

To this end, the available data demonstrates there is a battery of biochemical and molecular biomarkers that represent cellular stress, which can then be extrapolated to higher levels of biological organization (*e.g*. physiology, population). Certainly, we have achieved a full description of reliable brine-monitoring biomarkers with *P. oceanica ,* an strategy that can be also applied to other habitat-forming organisms in different temperate and tropical latitudes, such as corals (*e.g*. Marques et al., 2023), macroalgae (*e.g*. Muñoz et al., 2023; Rodríguez-Rojas et al., 2020) and other seagrasses (Blanco-Murillo et al., 2023a; Capó et al., 2020) . Moreover, as observed in this investigation and comparing with previous mesocosms data, using biomarkers related to osmotic and oxidative stress, can provide information on brine-specific responses and also address other potential combined effects when more stressors are present, respectively. While osmoregulatory responses are more specific for brine discharges (Blanco-Murillo t al., 2023a,b), oxidative stress in seagrasses can be triggered by different isolated and combined environmental stressors beyond brines, such as metal pollution, invasive species or temperature increments (*e.g.* Malea et al., 2019; Sureda et al., 2008; Tutar et al., 2017). Therefore, the identified biomarkers can: address brine-derived osmotic pressure and identify its specific contribution when multiple stressors are present; and also provide early warning signs of stress to take action upon eventual further physiological, population and community affection. Future stages of development upon these findings do not only apply to desalination environmental management, but to aquatic pollution and ecotoxicology overall. Indeed, next generation sequencing (NGS) technologies open new possibilities to extend the reach of diagnosis on other stress factors contribution and preventive measures. Finally, bioengineering

application of these findings to technological solutions (i.e., biosensors) are one step forward from us to fully ascertain and sharpen aquatic environmental surveys and diagnosis.

7.5 Conclusions

The Mediterranean habitat-forming seagrass *P. oceanica* transplanted nearby desalination discharges showed signs of oxidative stress and damage. These was confirmed by the consumption of antioxidant and the patterns of gene regulation of enzymes involved in the reactive oxygen metabolism. However, the regulation of specific osmotic regulation genes (*SOS1, SOS3*, *AKT2/3*) and results under controlled conditions in previous investigations, demonstrate that stress conditions demonstrated by *P. oceanica* are not strictly related to brine discharges, but apparently more influenced by other co-existing natural and/or anthropogenic environmental pressures.

These descriptors have been successfully tested as biomarkers to follow the extent effects of desalination discharges on *P. oceanica* and are suggested to be further applied in as part of Environmental Monitoring Programs (EMPs) and in incorporated to *ad hoc* legal framework regarding the operation of desalination plants in the Mediterranean Sea.

8. Unravelling *Posidonia oceanica* (L.) Delile sensitivity to salinity increments: the role of shoot apical meristems

8.1 Introduction

Seagrasses are a unique group of angiosperms (Alismatales order) adapted to live completely submerged in seawater (Hemminga & Duarte, 2000b). The colonization of the marine environment, which occurred in at least in three different occasions (Larkum et al., 2007; Les et al., 1997), required a complex set of adaptations in which whole genome duplication events may have played a crucial role (Xiao et al., 2023). This represented one of the most relevant events in the evolutionary history of flowering plants (Davey et al., 2016; Olsen et al., 2016). The submerged lifestyle required physiological adaptations to thrive in an environment with low light, sediment anoxia and low available CO_2 (Wissler et al., 2011). But adapting to seawater did also required to deal with osmotic stress due to high salinities and modify ion and water exchange mechanisms (Touchette, 2007).

Adaptations of seagrasses to the marine environment can be found at all levels of biological organization: genetic (absence of genes related to ethylene synthesis) (Olsen et al., 2016), ultrastructural (cell wall hardening; highly invaginated plasmalemma) (Iyer & Barnabas, 1993; Jagels, 1973), physiological (ion exclusion mechanisms) (Touchette, 2007), and morphological (absence of stomata, shoot apical meristems protected by sheaths) (Gobert et al., 2007). Although some seagrass families (Zosteraceae and Cymodoceae) have a relatively wide salinity tolerance and are adapted to estuarine and coastal lagoon habitats (Fernández-Torquemada & Sánchez-Lizaso, 2011; Kuo & den Hartog, 2007),

salinity changes have been an evolutionarily determining environmental factor for seagrasses (Larkum et al., 2007) and have shaped their distribution.

Scientific interest in seagrasses lies not only in their evolutionary adaptations and their contribution to the knowledge of plant phylogeny and evolution, but also to their undeniable ecological role in tropical and temperate coasts worldwide (Larkum et al., 2007; Short et al., 2007). Carbon sequestration, ecosystem maintenance, sediment stabilization and fisheries support are among the valuable ecosystem services seagrass meadows provide (Campagne et al., 2014; Cullen-Unsworth et al., 2014; Unsworth et al., 2019). Despite their known importance, seagrasses are suffering a declining trend in highly anthropized coastal areas worldwide (Turschwell et al., 2021; Waycott et al., 2009), which can only be slowed down or reverted by scientific assessment and effective management measures (de los Santos et al., 2019). Causes of seagrass decline range from nutrient increments and water quality loss to exotic species or boat anchoring (Turschwell et al., 2021), with changes in salinity being another relevant stressor for seagrasses (Sandoval-Gil et al., 2023).

The main cause of anomalous salinity increments in the marine environment potentially affecting seagrass meadows, are brine discharges from seawater reverse osmosis (SWRO) desalination plants. Desalination provides freshwater from a higher conductivity water source, such as seawater, and generates a high-conductivity effluent, known as brine, which is pumped back to the ocean (Darre & Toor, 2018). This industry has significantly increased in the last decades to cope with water scarcity in highly populated temperate regions, such as Australia, the Arabian peninsula or the Mediterranean basin (Jones et al., 2019; Palomar & Losada, 2010). These desalination plants are usually located in areas where seagrass meadows are the main macrophyte habitats (Short et al., 2007). In the case of the Mediterranean Sea, *Posidonia oceanica* (L.) Delile is the most

ecologically relevant seagrass species, forming extensive meadows from 0 to 40 meters depth (Gobert et al., 2007). Along with other seagrass species, *P. oceanica* is endangered by human activities which have caused *P. oceanica* meadow regression in the last decades (Blanco-Murillo et al., 2022; Telesca et al., 2015) and desalination discharges have risen particular concern as this seagrass species has shown to be a stenohaline organism, tolerating a narrow range of salinity (Fernández-Torquemada & Sánchez-Lizaso, 2005; Ruiz et al., 2009). This sensitivity has led to the consideration of this seagrass species as an specific bioindicator of brine impact in desalination environmental monitoring plans (Sánchez-Lizaso et al., 2008; Sola, Zarzo, et al., 2020). Thus far, most ecotoxicological studies regarding hypersalinity and seagrasses have mainly focused on morphological and physiological variables, and mostly in leaves (Sandoval-Gil et al., 2023), which might not be completely reliable as early-warning indicators of seagrass decline (Ceccherelli et al., 2018) and indicating that novel biomarkers are needed to detect hypersaline stress in its early stages.

Despite seagrasses morphology is variable, a common feature in Posidoniaceae family is a protected meristem and leaf basis by sheaths from older leaves (Hemminga & Duarte, 2000b). This protection allows the plant to keep meristematic tissues under a modified salinity, thus generating an osmotic gradient with the tissue exposed to seawater (Tyerman et al., 1984). In fact, differential osmotic response has been detected between tissues in *Posidonia* species (Booth et al., 2022; Cozza & Pangaro, 2009). Shoot apical meristems (SAMs) contain pools of stem cells from which seagrass leaves growth and productivity depend, thus constituting a fundamental structure that ensures organogenesis throughout the life of the plant (Garcias-Bonet et al., 2012; Tomlinson, 1974). The few studies conducted to date suggest that seagrass SAMs are particularly sensitive to environmental stress factors such as heat or light limitation (Fulcher & Sablowski, 2009; Pazzaglia et al., 2022; Ruocco et al., 2019).

Considering the morphological protection of *P. oceanica* SAMs, it is hypothesized that they might also be more vulnerable to ion increments caused by hypersalinity compared to leaves, implying a higher sensitivity to desalination brine discharges. The analysis of sheath protected SAMs could provide complementary and essential information not just for *P. oceanica* tolerance to salinity increments, but also for a better understanding of the specific adaptations of seagrasses to the marine environment.

In the present study we aim to assess and compare the responsiveness and the role of *P. oceanica* leaves and SAMs under different hypersaline conditions. For this purpose, samples from two different experiments conducted in mesocosms and in the field under different salinity increments were analysed. The results from these previous experiments have shown that *P. oceanica* leaves under brine exposure exhibited indicators of hypersalinity-derived stress including oxidative damage and expression of genes related to osmotic regulation and antioxidant defence (Blanco-Murillo et al., 2023b, 2023 under review). Here, reactive oxygen species (ROS) production, lipid peroxidation and antioxidant content were measured in shoot-apical meristems to test for differences in reactive oxygen metabolism (ROM) between organs; regulation of ion homeostasis and ROM-related genes were also analysed to determine the metabolic response and tolerance capacity to increased salinity of SAMs relative to leaves.

8.2 Materials and methods

Experimental design and sample collection

Both mesocosm and field experiments, as well as *P. oceanica* sampling are described in Blanco-Murillo et al. (2023b, 2023 under review). Briefly, *P. oceanica* plants were collected from a healthy meadow located in Isla Grossa (Murcia, SE Spain). Plant fragments consisting of a plagiotropic rhizome, and 20-30

orthotropic shoots were sampled for both mesocosm experiment and field transplants. The mesocosm experiment consisted of nine 300 L aquaria where *P. oceanica* plants were exposed to different salinity conditions. Two *P. oceanica* ramets were placed in each aquarium and their position was regularly changed along the experimental time to avoid responses resulted from environmental gradients within the each mesocosm unit. Plants were kept in mesocosm conditions for 10 days as acclimatation period before performing salinity increments (+6 psu). The experiment lasted for another 10 days before collecting samples of leaves and meristems. For the field experiment, two plant fragments were attached to a concrete block forming a transplant unit (TU). Three TUs were located at 2 different salinity exposures (Intermediate exposure; IB at ~39 psu and high exposure; HB at ~42 psu) and at a control location (37 psu). Plant tissues sampling was conducted 6 days after field placement (Fig. 8.1).

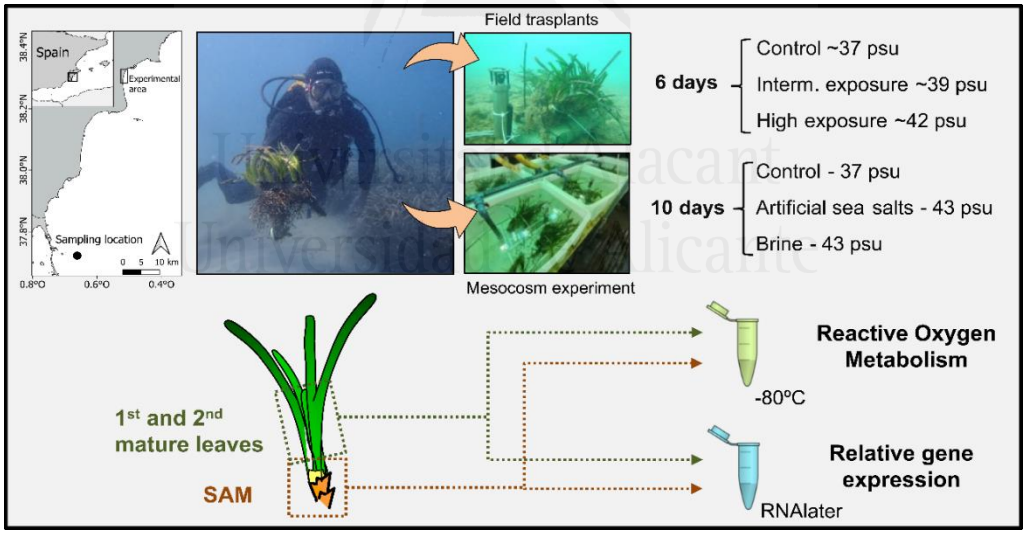

Figure 8.1: Scheme showing the experimental design of the mesocosm and field experiments.

Samples were taken from orthotropic shoots at least 3 positions away from the apical shoot, to avoid differential response due to shoot position within each

ramet (Ruocco et al., 2021). For meristem tissue sampling, whole shoots were collected, and leaves and sheaths were carefully removed to collect the rhizome tip (0.5 cm) that was subsequently washed with sterile distilled water and rapidly frozen with liquid nitrogen. First and second mature leaves were selected in each sampled shoot. A 4 cm fragment was selected after removing the first basal 5 cm and leaf apex to avoid differences due to tissue age (Ruocco et al., 2019). Leaf tissue was rapidly washed with sterile distilled water to eliminate salt excess and leaf epiphytes were carefully removed using a razor blade. Samples for biochemical analyses were immediately frozen in liquid nitrogen and then stored at -80ºC. Samples for gene expression analyses (qPCR) were stored in RNAlater, kept at room temperature for 24 h and the stored at -20ºC.

Hydrogen peroxide (H_2O_2) levels

As main ROS produced under hypersaline stress (Schmidt et al., 2013), H_2O_2 in *P. oceanica* tissues was measured by following the protocols of Sáez et al. (2015), with some modifications. Leaf and meristem biomass was grounded using a mortar and liquid nitrogen for homogenization. Twenty mg of fresh biomass were weighted and added to a mixture composed by 100 µL of 10% trichloroacetic acid (TCA), 150 µL of 10 mM potassium phosphate buffer (pH 7.0), 50 µL of lysis buffer, and 500 µL of 1 M potassium iodide. Samples were vortexed during 15 minutes after adding glass beads (3 mm) to favour cell wall rupture. The samples were taken to a centrifuge cycle of 15 min at 12000xg and 4ºC. Supernatant (250 µL) absorbance was measured at 392 nm in a SpectroStar Nano spectrophotometer (BMG LABTECH). Commercial H_2O_2 (Sigma Aldrich Merck, St Louis, MO, USA) was used to perform standard curves.

Thiobarbituric acid reactive species (TBARS)

Lipid peroxidation of cell and organelle membranes is one of the consequences of excessive ROS productions and can be measured indirectly by reaction of

peroxidation products with thiobarbituric acid (TBA). Grounded biomass (20 mg) was added to 500 µL 10 % trichloroacetic acid (TCA), vortexed for 15 min using glass beads (3 mm) and centrifuged at 17800xg for 15 min at 4°C. Two hundred µL of supernatant of each sample were added to 200 µL 0.5 % TBA and taken to a heat bath at 90ºC for 30 minutes. Once the reaction occurred, 200 µL of sample was taken for absorbance measures at 532 nm (SpectroStar Nano, BMG LABTECH). Standard curves for TBA reaction were done using commercial malonaldehyde (MDA) (Sigma Aldrich Merck, St Louis, MO, USA).

Ascorbate (ASC) and dehydroascorbate (DHA) determination

To prevent oxidative damage ASC oxidizes to DHA, becoming a first line intracellular antioxidant defence in plant cells. ASC and DHA determination was performed by modifying the protocol proposed by Benzie & Strain (1999). Ten mg of grounded leaf and SAM biomass were added to 300 µL 0.1 M HCl and 300 µL of lysis buffer to subsequently vortexed samples for 10 min and centrifuged at 21000xg for 10 min at 4°C. Supernatant (10 mg) were added to 290 µL of tripyridyl triazine (Fe III TPTZ) and absorbance was rapidly measured at 593 nm in a microplate reader (SpectroStar Nano spectrophotometer BMG LABTECH). This method allows the determination of ASC in the sample. To measure DHA levels, all ascorbate in the sample was reduced to ASC, and DHA was calculated from the difference between total ascorbate and ASC. To reduce total ascorbate, 250 µL of sample supernatant were added to 2.5 µL of 100 mM dithiothreitol (DTT) and incubated at room temperature for 1 hour. After the incubation period 2.5 µL (w/v) of N-ethylmaleimide were added to stop DTT reaction. Ten µL of extract were added to 290 µL Fe III TPTZ before measuring absorbances and determine total ascorbate content. L-ASC (Sigma Aldrich Merck, St Louis, MO, USA) was used for standard curves.

RNA extraction and qPCR

A set of 9 genes were selected to observe metabolic responses to hypersalinity in ion homeostasis and ROM (Tab 6.1). Those genes were first tested in *P. oceanica* in Blanco-Murillo *et al*. (2023b). Fifty mg of powdered Leaf and SAM biomass were sampled for RNA extraction. RNA extraction was performed by using a BIORAD Aurum™ Total RNA mini kit following the manufacturer instructions with some modifications due to high phenol content. To test RNA purity and integrity, t 260/280 ratio was evaluated and bands were visualized in a 1.2% agarose "bleach" gel (Aranda et al., 2012), respectively. RNA concentration was quantified in a QFX fluorometer (DeNovix, Wilmington, DE, USA) using a Quant-iT RiboGreen RNA assay kit (Invitrogen, Waltham, MA, USA). Using a cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fischer Scientific), 350 ng of RNA from each sampled were turned into cDNA for qPCR, adjusting all samples to a final volume of 20 µL. For each gene qPCR, 2 µL of sample cDNA were added to 10 µL of Brilliant SYBR Green PCR Master Mix (Agilent Technologies, Santa Clara,CA, USA) and 7 µL of nuclease-free water together with 0.5 µL of Forward primer and 0.5 µL of Reverse primer. These 20 µL were taken to a thermocycler (MIC; Bio Molecular Systems, Queensland, Australia) programmed with the following instructions: initial denaturation at 95 °C for 5 min; 40 cycles of 95 °C for 10 s, 55 °C for 10 s, 72 °C for 40 s; and ending with a final extension at 72 °C for 10 min. Relative gene expression, expressed as Fold Change expression (FC) was determined based on the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), using 18S rRNA as housekeeping gene, which has shown to be a reliable reference gene for *P. oceanica* exposed to salinity changes (Serra et al., 2012).

Statistical analyses

For both experiments a two-way ANOVA was performed to check for differences between treatments/ exposures and tissue type. In the case of the

mesocosm experiment, the treatment consisted of 3 levels (control, artificial salts and brine) as well as in the exposure factor in the field (control, IB and HB). In both cases the tissue factor consisted of 2 levels (Leaves and SAM). All factors were fixed and orthogonal between them. In the case of gene relative expression, Treatment/Exposure factor consisted of only 2 levels, as FC values are already referenced control samples. Before performing the ANOVA data normality and homoscedasticity were tested using a Kolmogorov-Smirnov test and a Bartlett test, respectively (Underwood, 1997). When ANOVA presented significant results, a post-hoc Tukey-HSD test was conducted to determine which levels were statistically different. ANOVA results are included in Supplementary data (tables S12-S13)

8.3 Results

H_2O_2 showed no significant differences among tissues or treatments in the mesocosm experiment. However, higher values were observed in *P. oceanica* leaves in control, while in SAMs, control values were slightly lower compared to hypersalinity treatments. Conversely, in the field experiment, significant differences were found for both factors, treatment and tissue. Regarding differences between tissues, leaves presented significantly higher H_2O_2 levels than SAMs with 141.9% of mean increment (Fig. 8.2a). Also, this ROS showed an increment following brine exposure with HB plants presenting significant differences in HB compared to control plants (Fig. 8.2b).

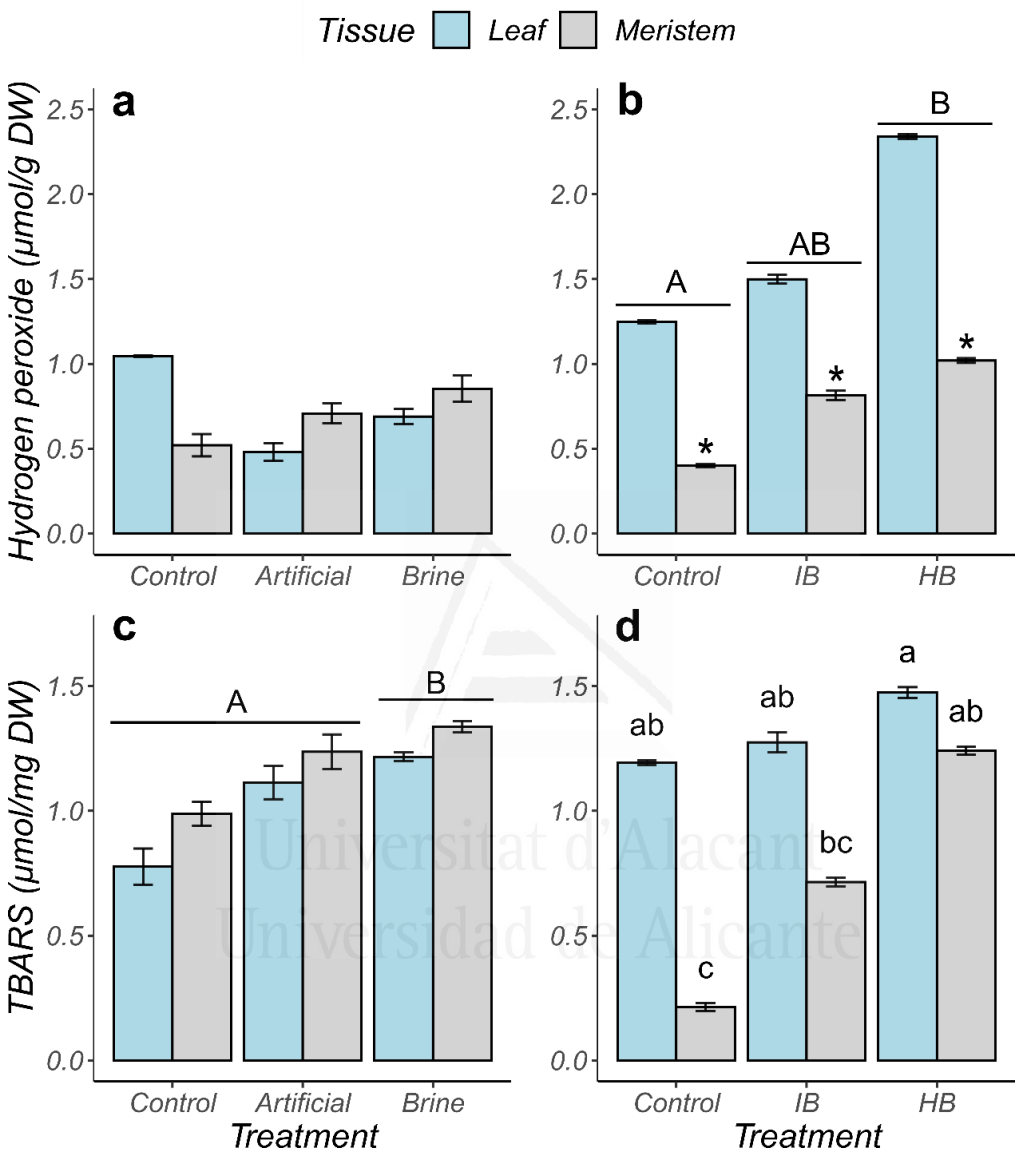

Figure 8.2: Hydrogen peroxide (H_2O_2) content in mesocosm (a) and field experiments (b) and TBARS levels in mesocosm (c) and field experiments (d) in *P. oceanica* samples under experimental salinity treatments; Mesocosm: control (37 psu), artificial sea salts (43 psu) and brine (43 psu); Field: control (~37 psu), IB (~39 psu), and HB (~42 psu). Barplots represent the mean of each variable and error bars show the standard error. Upper case letters represent significant differences at 95% confidence interval ($p < .05$) between treatments. Asterisk (*) show significant differences between tissues (leaf and meristem).

Lower case letters represent significant differences between groups when factor interaction was significant.

TBARS did not show differences among tissues in the mesocosm experiment although SAMs presented higher values in all treatments. Regarding the treatment influence on lipid peroxidation, brine plants presented significantly higher TBARS levels than control and artificial salts (Fig. 8.2c). In the field experiment, TBARS levels were higher in leaves compared to SAMs for all brine exposures, being this difference significant in control plants. TBARS in SAMs significantly (HB>Control) increased following brine exposure, while values in leaves remained relatively constant with a non-significant increment at higher brine influence (Fig. 8.2d).

ASC in the mesocosm experiment presented significantly higher levels in plants under artificial sea salts compared to brine, independently of the tissue type. Comparing leaves and SAM, ASC was significantly higher in the latter. DHA did not present significant differences although always remained higher in leaves compared to SAM and was relatively constant in between treatments. On the other hand, field transplants showed a reverse trend, with significantly lower values of ASC in SAM compared to leaves and no differences among brine exposures. DHA followed distinct trends between treatments; in leaves DHA levels significantly decreased in IB plants, while control and HB presented similar content; and SAM significantly lower levels in HB plants compared to control.

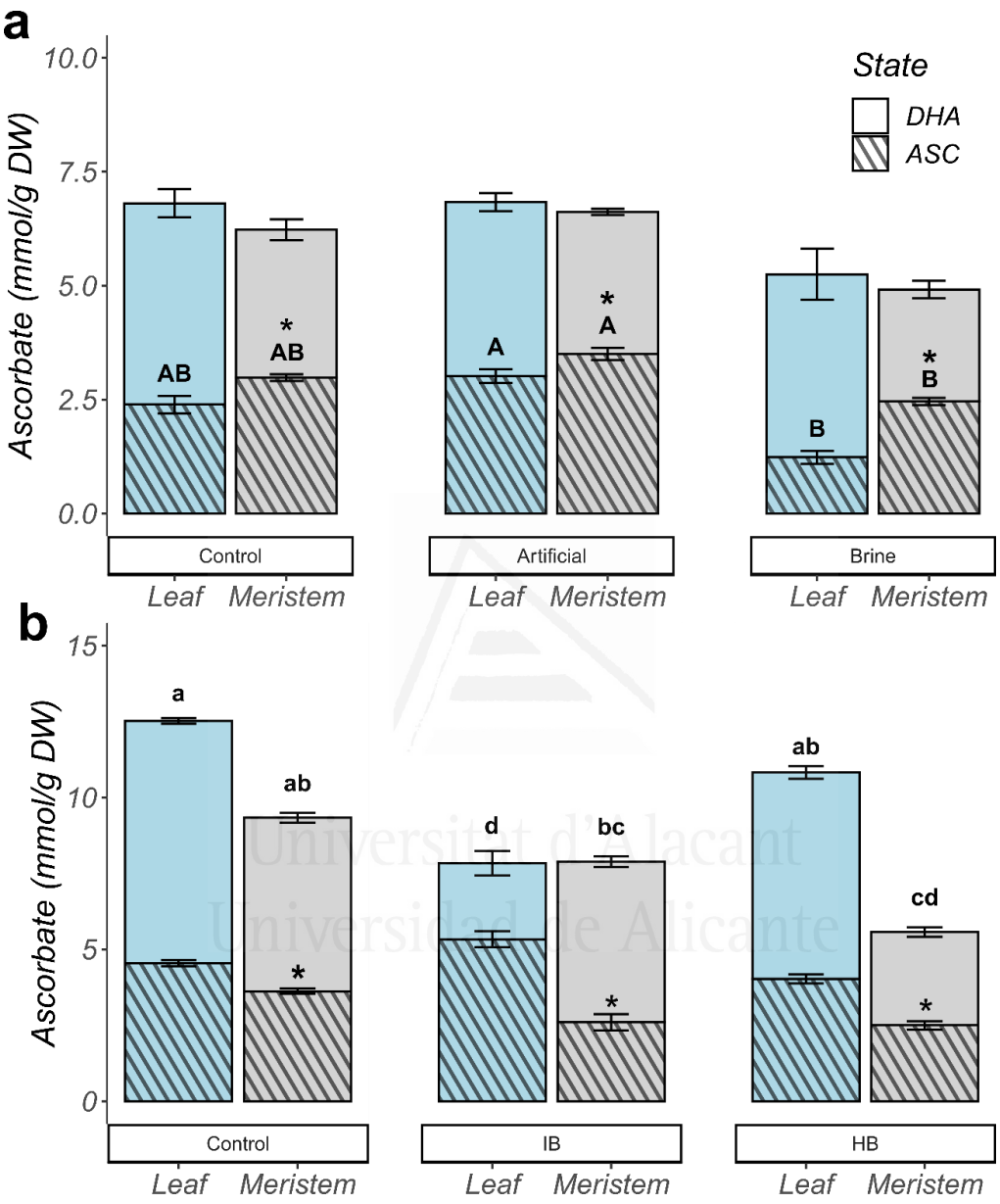

Figure 8.3: Ascorbate (ASC) and dehydroascorbate (DHA) content in mesocosm (a) and field experiments (b) in *P. oceanica* samples under experimental salinity treatments; Mesocosm: Control (37 psu), artificial sea salts (43 psu) and brine (43 psu); Field: Control (~37 psu), IB (~39 psu), and HB (~42 psu). Barplots represent the mean of each variable and error bars show the standard error. Upper case letters represent significant differences at 95% confidence interval ($p < .05$) between treatments. Asterisk (*) show

significant differences between tissues (leaf and meristem). Lower case letters represent significant differences between groups when factor interaction was significant.

SOS1 encodes for an antiporter protein which excludes Na^+ from the cytosol, in exchange for H^+. Its transcription in mesocosm conditions presented significant differences in SAM under brine exposure compared with the same tissue under artificial salts and leaves under both hypersalinity treatments. Regarding the field experiment, *SOS1* was significantly more expressed in meristems, and at higher brine exposures, reaching FC values close to 500.

SOS3 is a secondary messenger, particularly a Ca^{2+} binding protein which promotes *SOS1* transcription under increased salinity conditions (Ji et al., 2013). *SOS3* gene was downregulated in leaves under brine exposure compared to artificial salts, while a reverse trend was observed in SAM, where gene expression was significantly higher (Fig. 8.4a,b). In plants under brine exposures in the field, *SOS3* presented a similar regulation pattern in leaves and SAMs, with a non-significant increment in HB plants (Fig. 8.5a,b).

Apart from Na^+ exclusion, plants cells also deal with salinity increments by increasing K^+ uptake and AKT2/3 is a K^+-specific transport protein. *AKT2/3* relative expression was similar between laves and SAM in the artificial salts treatment but plants under brine influence showed different response between tissues; while leaves reduced its transcription, it was significantly incremented in brine-exposed SAMs (Fig. 8.4c). In the field experiment, no significant differences were found between organs; SAMs presented higher gene expression in IB plants while in HB, leaves showed higher gene expression, presenting significant differences with IB leaves (Fig. 8.5c).

STRK1 is a signalling protein which is sensitive to Na^+ increments and promotes *CAT* transcription in order to deal with hypersalinity-derived oxidative stress (Zhou et al., 2018). When comparing artificial salts and brine, leaves

showed a slight downregulation, while SAMs were upregulated, although no significant differences were found. Both organs increased their *STRK1* transcription in brine, which was significantly higher in the case of SAMs (Fig. 8.4d). Despite the absence of significant differences, plants from field transplants showed a higher *STRK1* regulation in SAMs, similar for both brine exposures, while leaves slightly increased its relative expression in HB plants (Fig. 8.5d).

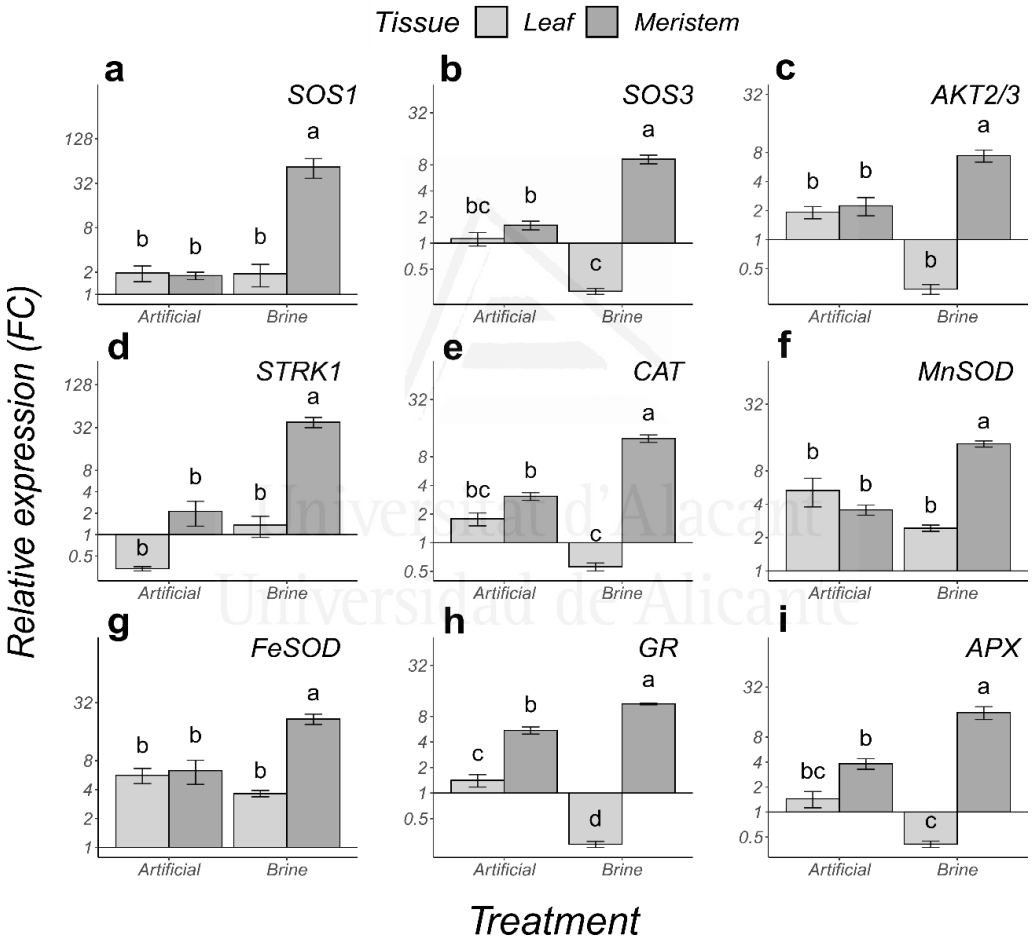

Figure 8.4: Relative expression of osmotic regulation and oxidative stress-related genes in *P. oceanica* under 2 hypersalinity treatments: artificial sea salts (43 psu), and brine (43 psu). Genes measured were: *SOS1* (a); *SOS3* (b*), AKT2/3* (c), *STRK1* (d), *CAT* (e), *MnSOD* (f), *FeSOD* (g), *APX* (h) and *GR* (i). Lower case letters represent significant differences

between groups when factor interaction was significant. Asterisk (*) show significant differences between treatments.

Catalase is one of the most relevant enzymes in ROM, as it catalyses the conversion of H_2O_2 to H_2O and O_2, thus directly counteracting ROS imbalance (Sofo et al., 2015). In *P. oceanica* plants in the mesocosm conditions, *CAT* transcription was higher in SAMs compared to leaves and leaves presented lower FC values under brine exposure, while a reverse trend was observed in SAMs (Fig. 8.4e). Plant SAMs under brine exposure showed a significantly higher up-regulation compared to the rest of samples. In the field experiment, SAMs presented a significantly higher *CAT* transcription compared to leaves for both brine exposures. For both organs a non-significant decline in *CAT* regulation was observed in HB plants (Fig. 8.5e).

ROS production occurs when electron from mitochondria and chloroplast transport chains meet free oxygen, generating the first ROS, superoxide ions (O_2^-). This ROS is transformed into H_2O_2 by superoxide dismutases (SOD). The most abundant SOD in the mitochondria is MnSOD, while FeSOD is mainly found in the chloroplasts (Pilon et al., 2011; Wolfe-Simon et al., 2006). *MnSOD* showed a slightly higher transcription in the artificial salts treatment in leaves compared to SAMs. However, under brine exposure, regulation was lower in leaves while in SAMs it significantly increased (Fig. 8.4f,g). In the case of the field experiment, *MnSOD* presented higher gene relative expression in SAMs compared to leaves and in HB plants compared to IB, but no statistical differences were found among factors. *FeSOD* presented similar gene relative expression between organs under artificial salts, but while leaves showed lower gene expression under brine exposure, SAMs presented significantly higher FC values, similarly to *MnSOD*. Regarding the plants under brine exposure in the field, SAMs presented a slightly higher regulation in IB, and gene relative expression increased for both organs in

HB, reaching significantly higher FC values in HB SAMs compared to IB samples (Fig. 8.5f,g).

GR catalyses the restoration of GSH from GSSG, which is then used either to turn DHA back into ASC or directly scavenge H_2O_2 (Foyer & Noctor, 2011). *GR* transcription was significantly higher in SAMs compares to leaves in the mesocosm experiment. Under artificial salts exposure, leaves presented a slight up-regulation while this gene was down-regulated under brine influence (Fig. 8.4h). Regarding SAMs, they showed a significantly higher gene expression in the brine treatment. Plants in the field presented a slight up-regulation in the case of SAMs and down-regulation in leaves in IB. GR regulation presented a non-significant increment for leaves, while SAMs showed a significant increment in gene expression (Fig. 8.5h).

As well as CAT, APX is one the enzymes involved in H_2O_2 scavenging. In this case, APX mediates the oxidation of ASC to DHA in order to reduce H_2O_2 to H_2O (Sofo et al., 2015). Comparing artificial salts and brine, different organs showed different transcription patterns: leaves presented a slight up-regulation in artificial salts, while this gene was down-regulated in brine (Fig. 8.4i). On the other hand, SAMs presented higher gene relative expression in brine, presenting significant differences with leaves and SAMs in artificial salts. In the field experiment, gene expression slightly increased in HB plants compared to IB, but there was a significant difference between organs, with SAMs showing higher gene expression compared to leaves in both brine exposures (Fig. 8.5i).

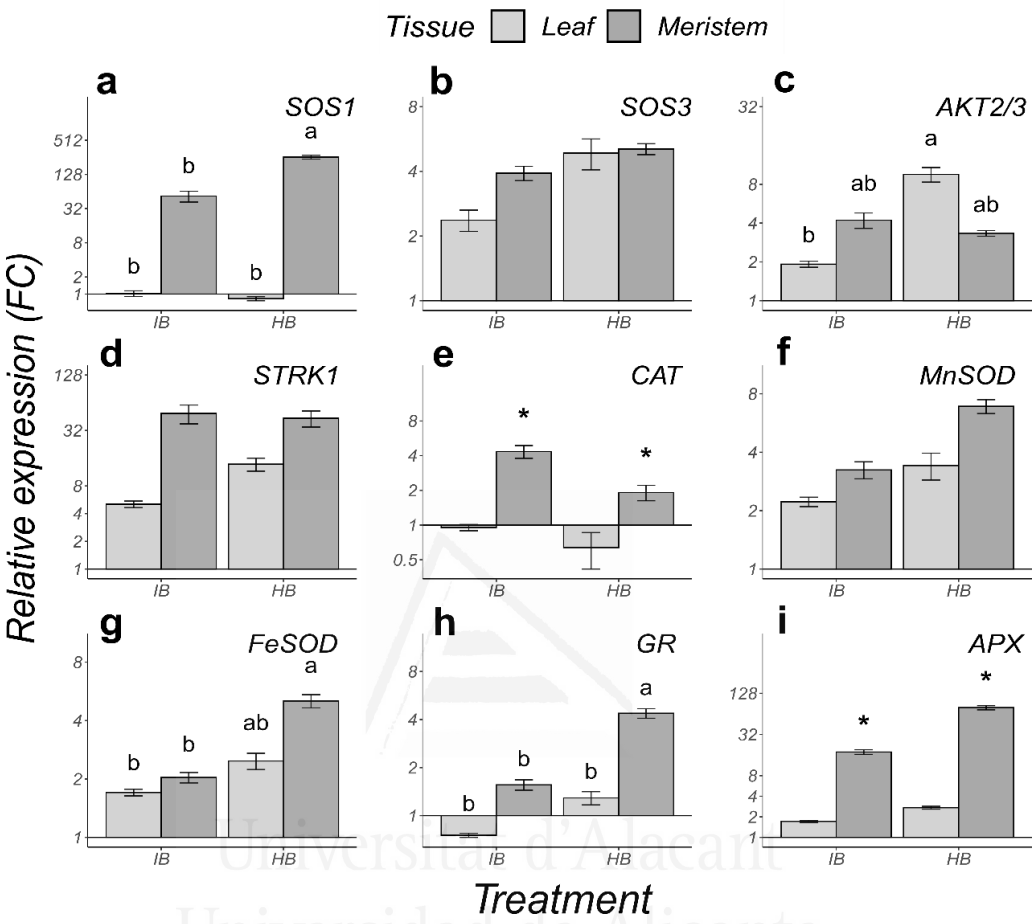

Figure 8.5: Relative expression of osmotic regulation and oxidative stress-related genes in *P. oceanica* under 2 hypersalinity exposures: IB (~39 psu), and HB (~42 psu). Genes measured were *SOS1* (a); *SOS3* (b*), AKT2/3* (c), *STRK1* (d), *CAT* (e), *MnSOD* (f), *FeSOD* (g), *APX* (h) and *GR* (i). Lower case letters represent significant differences between groups when factor interaction was significant. Asterisk (*) show significant differences between treatments.

8.4 Discussion

P. oceanica responses to hypersalinity, have shown ROS production, oxidative damage, variations in antioxidant response, and genetic regulation to cope with osmotic and oxidative stress. Despite differences between experiments, SAMs and leaves have shown different responses for several indicators, revealing a

complex tolerance strategy by *P. oceanica* to cope with salinity changes. SAMs have shown a more active gene regulation against brine-derived hypersalinity, which highlights their greater responsiveness and sensitivity, and thus behaving as sentinel organs indicators of stress associated with discharges from the desalination industry.

SAMs and leaves responses: ROS production, antioxidant capacity and gene expression

Overall, our results revealed (1) an increment in ROS production and subsequent oxidative damage in both leaf and SAM tissues at higher brine exposures, (2) a greater difference in the response of the two organs in the field experiment and (3) a higher responsiveness in SAMs than leaves, as they changed their H_2O_2 and TBARS levels with brine influence under natural conditions. These findings support the use of H_2O_2 and TBARS as indicators of salt stress in *P. oceanica*. Increased H_2O_2 production under stressful salinity conditions has been evidenced in terrestrial plants in general (Schmidt et al., 2013) and in some marine macrophytes (*Zostera chilensis*, Blanco-Murillo et al., 2023a; *Ectocarpus* sp, Rodríguez-Rojas et al., 2020). When the H_2O_2 production exceeds the antioxidant capacity of the plant, lipid peroxidation occurs in the early stages of brine affection and can be detected even after longer exposures as reported by Capó et al. (2020).

To cope with high H_2O_2 levels, *P. oceanica* SAMs up-regulated *CAT* and *APX* compared to leaves, possibly keeping H_2O_2 levels lower in the field experiment. *STRK1* also showed higher regulation in SAMs, correlating with *CAT* transcription, indicating a salinity-dependent oxidative response. Moreover, both *FeSOD* and *MnSOD* were more expressed in SAMs under brine exposure (both experiments), agreeing with the role of these enzymes to cope with salinity stress in seagrasses (Blanco-Murillo et al., 2023a; Capó et al., 2020) and confirming a

more active antioxidant response of SAMs compared to leaves. However, in the field experiment, higher *FeSOD* and *MnSOD* transcription were detected in HB plants while *CAT* was more upregulated in IB, possibly favouring H_2O_2 accumulation in HB SAMs and the subsequent increase in TBARS. Consequently, despite the active metabolic response in SAMs under brine influence, it is not efficient enough to prevent oxidative damage above certain salinity increment (~ +2 psu).

Total ascorbate and ASC declined under brine influence in the mesocosm experiment for both tissues, while this decline was more evident in SAMs in the field approach. This is consistent with the *APX* regulation in response to salinity, which would be stimulating ASC consumption to reduce oxidative stress while *GR* transcription could be favouring ASC restoration, highlighting the role of the Halliwell-Asada cycle in salt tolerance (Bose et al., 2014). Interestingly, both experiments differed in ASC/DHA contents in SAMs relative to leaves. While ASC in SAMs remained higher in plants under controlled conditions (mesocosms), it was lower in field transplants. This effect correlates with *APX* and *GR* relative expression levels; both genes were similarly expressed in the mesocosm experiment in both treatments, while in the field, *APX* presented higher FC values compared to *GR*, possibly causing an impairment between ASC consumption and restoration. This indicates that antioxidant capacity (ASC restoration) and, subsequently, the ability of *P. oceanica* tissues to prevent oxidative damage, was higher in the mesocosm, possibly overestimating the actual capacity of SAM to cope with oxidative stress under natural conditions. Finding differences between mesocosm and field studies is common (Muñoz et al., 2023a, 2023b) as the latter comprises a wider variety of environmental variable factors (temperature, wave energy, light intensity, ...) which better represent the variability and realistic responses of the proposed biomarkers. These findings support the relationship between brine exposure and ROS production, antioxidant consumption and

oxidative damage in seagrasses. Furthermore, our results complement previous works by showing how these responses are more clearly evidenced in the field experiment and a more active oxidative response was found in *P. oceanica* SAMs.

SOS1 and *SOS3* up-regulation revealed an activation of the Salt Overly System, a Na^+ exclusion mechanisms also described in leaves of *Z. chilensis* in response to hypersalinity (+3 and +6 psu, 10 days) (Blanco-Murillo et al., 2023a). The higher relative expression of *SOS1* in both experiments in SAMs supports the hypothesis of a higher metabolic effort by this organ to exclude excess Na^+ and keep salinities lower in comparison to leaves (Tyerman et al., 1984). On the other hand Garrote-Moreno et al. (2015) showed how *P. oceanica* leaves were able to increase K^+/Na^+ ratio under hypersaline exposure (+9 psu, 7 days). To do so, plants might be coupling K^+ capture with Na^+ exclusion by, for example, *AKT 2/3* up-regulation. This gene was similarly expressed in both experiments, but with higher transcription levels in leaves compared to SAMs in HB plants in the field experiment. As ion and nutrient uptake mainly occur in leaves (A. W. D. Larkum et al., 2007) and K^+ is essential for photosynthetic processes (Steven, 1985), *P. oceanica* might mainly develop this assimilation mechanisms in full seawater-exposed organs.

Most genes in leaves experienced a declining trend through the experimental period (10 and 6 days in mesocosm and field, respectively) (Blanco-Murillo et al., 2023b, 2023 under review), whereas SAMs presented higher FC values under brine-derived stress at the end of both experiments. Apart from SAMs higher sensitivity to osmotic and oxidative stress, there could also be a phase difference between leaves and SAMs responses. Longer exposures and different time sampling of both leaves and SAMs would provide useful information to understand whole plant mechanisms to cope with hypersalinity.

Gene expression in the mesocosm experiment showed a generally higher overexpression of those enzymes linked to osmoregulation and oxidative stress in SAMs under brine exposure, indicating a more active response when the source of hypersalinity came from a desalination discharge. The cause of brine higher toxicity is unclear, and might be related with certain changes in ion ratios which do not vary by increasing salinity with artificial salts. Cambridge et al. (2019) also detected higher brine toxicity in *P. australis* compared to seawater with the same increased salinity at the physiological level. Also, the higher concentration of certain metals (Fe, Cu) might be favouring Fenton reaction and increasing ROS production, and the presence of certain chemicals (antiscalants, coagulants, acidifiers, disinfectants, etc.) might also be favouring a higher metabolic response to brine (*e.g.*, Sodium metabisulphite, Portillo et al., 2014). This research opens the gateway to search for the direct relationship between toxicological indicators and specific chemical components of discharged brine in the ocean.

Adaptative strategy of P. oceanica to salinity increments

Salt stress in plants involves a complex set of morphological, physiological and metabolic adaptations aimed primarily at controlling ion homeostasis in cells, preventing oxidative damage from salinity and keeping essential cellular functioning (Van Zelm et al., 2020). These responses are particularly critical in SAMs, from which shoot growth and survival directly depends. Increasing surrounding salinity have shown an active salinity-dependent metabolic response in *P. oceanica* SAMs. As Tyerman et al. (1984) proposed, this tissue might remain partially isolated from seawater in order to keep the water surrounding SAMs under osmotically controlled conditions. Possibly, the optimal metabolic functioning of Posidoniaceae meristems occur at lower salinities than seawater, making it cost-effective to invest in keeping these modified conditions for a proper plant development. However, excessive energy investment to

maintain lower salinities around the SAM might compromise shoot growth and survival (Pazzaglia et al., 2022). SAMs also play an essential role in seagrass complex structure as clonal organisms. Seagrasses are able to tolerate at least small scale disturbances by relocating resources and investing in asexual reproduction (Macreadie, York, et al., 2014; Ruocco et al., 2021) and SAMs have a direct role in this switching mechanisms. This strategy might be essential to understand the ecological role and higher complexity of *Posidonia* meadows (Kilminster et al., 2015). However, this also highlights the vulnerability of whole-plant survival if SAMs are damaged or energy requirements under stress conditions exceed metabolic thresholds.

Regarding ion homeostasis, Na^+ exclusion and K^+ uptake mechanisms in plant tissues are an essential strategy to maintain basic metabolic functions. Among the different patterns in plant-water relations and ion content of seagrasses under hypersaline stress, keeping high K^+/Na^+ ratio is considered to be an indicator of effective salt tolerance (Sandoval-Gil et al., 2023). In our study, the increased relative expression of *SOS1* in the presence of brine, especially in SAMs, may reflect one of the adaptative responses of seagrasses to the marine environment. Rather than developing new exclusion mechanisms respect to other angiosperms, seagrasses evolved by modifying the regulation of *SOS1/NHX7* and increasing H^+-PPases like AVP1 to produce H^+ electrochemical gradients which allows more active Na^+ exclusion or vacuole storage (Xiao et al., 2023). Curiously, *SOS1* transcription seem to be partially independent from *SOS3*, a gene which is even lost in some seagrass lineages (Xiao et al., 2023), and could indicate alternative pathways for SOS activation in marine angiosperms. This process could involve other salt-sensitive pathways to cope with Na^+ toxicity and ROS production such as ENH1, which is positively selected in *P. oceanica* compared to other seagrasses (Wissler et al., 2011). In this sense, whole transcriptome studies under different

saline conditions could be useful to elucidate alternative metabolic pathways developed by seagrasses to cope with salinity increases.

Hypersalinity has shown to trigger higher respiration rates and O_2 consumption in seagrass meristems, thus becoming a potential source of ROS (Koch et al., 2022). Higher lipid peroxidation and antioxidant consumption in SAMs from brine-exposed plants in both mesocosm and field experiments, together with a more active gene transcription, possibly required higher energy consumption. In fact, seagrass SAMs have shown to activate starch and sucrose metabolism in response to increased heat (Pazzaglia et al., 2022) and salinity (Booth et al., 2022), implying higher energy consumption in this organ to cope with environmental stressors. As described by Blanco-Murillo (2023a), growth was negatively affected by hypersalinity (+ 6 psu, 10 days) in *P. oceanica* possibly due to the metabolic cost of oxidative response and osmoregulation in SAMs. However, growth reduction has also been suggested to be an adaptation to cope with hypersalinity, as *P. oceanica* populations adapted to hypersaline environments have shown lower sizes and growth rates (Marín-Guirao et al., 2017; Nguyen et al., 2023). This way plants would be reducing its ion exchange surface (leaf area) and focus resources in keeping ion homeostasis rather than shoot growth. In conclusion, *P. oceanica* SAMs responses to hypersalinity are essential to understand the tolerance of this stenohaline species to salinity stress and gives information on how this tissue actively invest in Na^+ signalling (*SOS3, STRK1*), Na^+ exclusion (*SOS1*), ROS scavenging (*CAT*, *FeSOD*, *MnSOD*) and Halliwell-Asada cycle activation (*GR*, *APX*).

SAM bioindicator potential

Most studies regarding physiological and metabolic responses of seagrasses to changing environmental conditions have been performed in leaves because of its accessibility and evident role in photosynthetic processes or nutrient uptake.

However, considering the sensitivity showed by SAMs to environmental stressors, which has led it to be proposed as a bioindicator organ of seagrass health (Bruno et al., 2009), certain responses could have been missed and tolerance capacity overestimated. For example, ROS overproduction in SAMs has the potential to alter redox homeostasis, thus affecting metabolic and hormonal processes related to vegetative development, flowering and germination (Mhamdi & Van Breusegem, 2018) and therefore alter meadow health and development.

In *P. oceanica*, the few studies on SAM metabolic responses have focused on light limitation (Ruocco et al., 2019), temperature and nutrients (Pazzaglia et al., 2022; Santillán-Sarmiento et al., 2023), or metal toxicity (Greco et al., 2012). For example, Ruocco et al. (2019) exposed *P. oceanica* plants to light limitation (80% light reduction for 40 days) and found a more active and sensitive transcriptomic response in SAMs compared to leaves, thus generating direct affection in shoot growth. This differential metabolic response was also showed by Pazzaglia et al. (2022) in *P. oceanica* plants under heat stress (+5ºC for 14 days), where SAMs presented higher up-regulation of genes related to DNA methylation or starch metabolism compared to leaves, while the latter invested more transcription effort on genes belonging to defence mechanisms and stress responses. In both cases responses were variable depending on the plant origin, highlighting the role of local adaptation in the tolerance to environmental stressors.

SAMs do not only reveal a more active gene expression response to environmental stressors but also its metabolic alterations and subsequent energy consumption have a more direct relationship with plant development and survival. *P. oceanica* plants under nutrient and temperature stress for 5 weeks with higher SAM transcriptomic response also presented higher shoot mortality (Pazzaglia et al., 2020, 2022; Santillán-Sarmiento et al., 2023). Regarding the

effect of salinity, *Thalassia testudinum* meristems exposed to hypersalinity (+25 psu, 4 hours) revealed higher O_2 consumption and H_2S production caused by salt increments (Johnson et al., 2020). This is particularly concerning as H_2S increments have proven to be highly harmful in seagrasses and might compromise its survival (Garcias-Bonet et al., 2008) and this effect might be causing massive die-off events in Florida Bay (Koch, Schopmeyer, Kyhn-hansen, et al., 2007). More recently, Booth et al 2022 investigated how different tissues (leaves, meristems, and roots) responded to natural salinity increments (40-50 psu) in *P. australis* meadows in Shark Bay (Australia). Their research showed SAMs transcriptome to be linked to key processes like photosynthesis, growth and salt exclusion compared to leaves or roots and this tissue was proposed as effective indicator of plant response to hypersaline stress.

In our case, the responsiveness of SAMs in the field experiment described above makes it an ideal target plant organ to address brine affection on *P. oceanica* meadows at an early stage. Although the use of SAMs involves a more destructive sampling technique than leaves, as it requires the sampling of whole shoots (Gobert et al., 2020), this is offset by the fact that the responses of leaves is more variable and dependent of factors such as position and age, and are subject to biotic factors like epiphytes and herbivory, while gene expression of SAMs shows a more stable pattern (Booth et al., 2022; Ruocco, et al., 2019).

Considering the threatened status of seagrass ecosystems (Turschwell et al., 2021; Waycott et al., 2009), the search for effective biomarkers of biological stress is essential to prevent these valuable ecosystems decline. These biomarkers should allow the detection of metabolic/physiological damage early enough to act upon the source of impact before the 'point of no return' were population regression is unavoidable (Macreadie et al., 2014). In addition, restoration strategies of slow growing species like *P. oceanica* are still being

evaluated (Pansini et al., 2022), highlighting the importance of management measures and conservation strategies to focus in prevention rather than restoration in this particular seagrass species. In this regard, the use of molecular tools and sampling stressor-sensitive organs such as SAMs might be a reliable strategy to detect stress responses and act upon its sources in *P. oceanica* meadows prior to irreversible damage.

8.5 Conclusions

Although H_2O_2 and TBARS values were lower in SAMs, they responded more actively to salinity increments in the field experiment. ASC/DHA ratio decreased with brine exposure but showed different organs responses in each experiment.

There is a general pattern of higher gene regulation in SAMs compared to leaves at higher salinities, supporting the existence of different physiological responses between seagrass organs, a key feature in seagrass adaptation to the marine environment.

Hypersalinity, especially brine derived, triggered ion exclusion mechanisms and oxidative stress response genes more actively in SAMs highlighting the sensitivity of this organ to salinity changes. The responsiveness and essential role of SAMs arise the as suitable sampling organ in *P. oceanica* to assess brine affection in its early stages.

9. General discussion

9.1 Seagrasses under desalination and other cumulative impacts: impact discrimination and environmental management

Seagrass meadows have been reported to suffer significant regression processes worldwide (Short et al., 2011; Waycott et al., 2009). However, it has been in discussion if this declining pattern was a global phenomenon (Duarte, 2002; Short et al., 2011; Waycott et al., 2009) or regression processes occurring at local scale (González-Correa et al., 2007; Guillén et al., 2013). Results from Chapter 3 revealed a stable trend in shallow *P. oceanica* meadows along 200 km of Western Mediterranean coastline. Conversely, Chapter 4 showed a severe regression in the *P. oceanica* meadow close to a coastal city in the same region. These apparently contradictory results indicate that meadow regression processes do occur, but are mainly associated to located coastal stressors, such as ports, sewage loads or desalination plants, although seagrass meadows in relatively well-preserved areas are not suffering a declining trend. However, these results should be considered carefully as the deep limit in seagrass meadows is poorly studied due to logistic issues and regression processes might be underestimated.

Environmental changes or perturbations cause physiological stress in seagrasses, triggering a variety of responses to cope with the new conditions (Duarte & Hemminga, 2000). Usually, stress if firstly detected at the lower levels of biological organization and follows its way up to the higher levels if the stressor intensity is not reduced and physiological defences are not able to counteract its effects (Osmond et al., 1987). The results from this thesis have

shown how salinity increments triggered a set of responses starting by molecular signalling and gene expression, to plant growth. Considering previous works regarding seagrass salinity tolerance (Sandoval-Gil et al., 2023), we can assume that hypersaline effects can scale up to population and community levels. This scaling is, of course, dependent on the stress intensity and duration (Lam, 2009). Furthermore, seagrass meadows under the influence of a brine discharge are presumably susceptible to be under other potential stressors, both natural and anthropogenic (Griffiths et al., 2020; Lefcheck et al., 2017). In this regard long term brine exposures, with the addition of other environmental stressors can cause deleterious effects on marine communities (Crain et al., 2008; Gissi et al., 2021), compromising seagrass survival and leading to whole habitat loss as shown in Chapter 4. Hypersalinity might trigger certain metabolic and physiological responses which could be worsen by the existence of other stressors which might overcome *P. oceanica* capacity to cope with. However, hypersalinity synergies with other stressors has been poorly studied compared to other environmental impact combinations (Stockbridge et al., 2020), but it has shown to decrease decrease photochemical performance and increase mortality at high temperatures and presence of anoxic sediments in *Thalassia testudinum* (Koch, Schopmeyer, Holmer, et al., 2007; Koch & Erskine, 2001), and increased oxygen consumption in the presence of nutrients (Kahn & Durako, 2006). Conversely, *Halophila ovalis* presented antagonistic responses when studying the interaction of hypersalinity and increased temperatures (Ontoria et al., 2020), certain salinity increments induced nutrient uptake in *Z. marina* (Lv et al., 2018) and *T. testudinum* was more resistant to wasting disease under hypersaline conditions (Bishop et al., 2017; Trevathan et al., 2011). Due to the complexity of hypersalinity relation with other stressors, further studies are needed to unravel which mechanisms could be positively or negatively affected by these interactions.

In this investigation, the metabolic responses observed in Chapter 7, demonstrated more responsive oxidative stress (H_2O_2 and TBARS levels) and gene expression (*SOS3, AKT2/3, STRK1, MnSOD, FeSOD, APX*, and *GR*) compared to even higher salinities in the laboratory (Chapter 6). Garrote-Moreno et al. (2014) demonstrated how *C. nodosa* under the influence of a brine discharge presented more severe physiological damage compared to mesocosm experiments, even at higher salinities (Fernández-Torquemada & Sánchez-Lizaso, 2011). These results agree with the ones obtained in Chapter 7 (field; +2 and +4 psu) compared to Chapter 6 (mesocosm; +6 psu) with *P. oceanica*. This effect might be caused by variable salinity pulses under real brine exposure (Fig. 27), the presence of natural environmental factors (waves, turbidity, herbivory), or even the interaction of other human derived stressors (sewage discharge) which might increase metabolic stress responses. Moreover, brine discharges can also change water temperature (Lattemann & Höpner, 2008), which was a controlled parameter in mesocosm experiments and could potentially generate higher stress on *P. oceanica*. Therefore, if growth was already reduced with hypersalinity (Chapter 6), it would presumably be more affected in the field and, after longer exposures, *P. oceanica* plants would start to perish, triggering a meadow declining process (Chapter 4). Further studies should be taken to fully understand the interaction of hypersalinity with stressors at different levels of biological organisation and longer exposures to ascertain their effect on seagrass meadows in the Anthropocene.

Co-occurring industries and environmentally harmful activities are a common feature in coastal cities in temperate regions (Holon et al., 2018) and other massive P. *oceanica* meadow losses have been reported in the Mediterranean in highly anthropized areas (Holon, Boissery, et al., 2015; Marbà et al., 2014). The existence of several impact sources and causes of meadow regression present a complex challenge for environmental management (Griffiths et al., 2020).

Considering the consequences of cumulative stressors through the years, highlights the need of effective monitoring programmes which properly evaluate the health status of seagrass meadows in a multiple-stressor context. Moreover, considering the stable pattern showed in healthy meadows in the last decades (Chapter 3), special focus should be given to highly impacted areas and develop monitoring tools which allow stress detection and impact discrimination.

The specific responses should be analysed in presence of a desalination discharge coexisting with other pollutants to test their responsiveness and the capability of these biomarkers to be used in impact discrimination. By developing a set of impact-specific indicators, like the ones proposed in Chapter 7, this discrimination might allow the development of local management measures which act upon the most harmful sources of impact. In fact, proper management is currently slowing down seagrass decline to a great extent (de los Santos et al., 2019), and reliable biomonitoring tools are needed to effectively prevent ecosystem degradation, allow seagrass recovery and promote a sustainable desalination industry.

9.2 Seagrass physiology under short-term hypersaline stress

One of the most used techniques to assess the physiological status of photosynthetic organisms is the measuring of their photochemical performance. To do so, PAM fluorometry has served as reliable indicator of photosynthetic functioning, which is commonly altered by environmental stress (Ralph & Gademann, 2005). This technique allows the determination of the Photosystem II chlorophyll a (Chla) effective capture of light. Activated reaction centres release fluorescence when transferring electrons to the thylakoidal transport chain. These fluorescence can be measured and by using different light pulses and

intensities and allows the measuring of several indicators of photochemical and non-photochemical quenching (Schreiber et al., 1995). This technique had been used to assess seagrass hypersaline affection (Cambridge et al., 2017; Kahn & Durako, 2006; Marín-Guirao et al., 2011; Sandoval-Gil et al., 2012a), revealing different affections depending mainly on the species and the stress intensity and duration.

PAM fluorometry was used to measure phosynthetic performance in *Z. chilensis* (Chapter 5) and *P. oceanica* (Chapter 6) in this thesis. Photochemical response was variable between experiments and species, although NPQ increment against hypersalinity (especially brine-derived) seemed to be a common response. *Fv/Fm* and α_{ETR} did not vary in any hypersalinity treatment for both species, indicating there were no signs of severe photoinhibition. Photoinhibition (*i.e*. *Fv/Fm* decline), seems to occur only at high salinities or long exposures such as measured in *C. nodosa* (+13 psu, 20 days) (Tsioli et al., 2022) and *P. oceanica* (+22.5 psu, 7 days) (Garrote-Moreno et al., 2015). In fact Marín-Guirao et al. (2013) demonstrated that *P. oceanica* under hypersaline levels similar to the tested in this thesis (43 psu) did not present photochemical depletion after 1 month and were capable to physiologically recover after the stress, whereas plants exposed to this hypersaline stress for 3 months showed photosynthetic rates dropping and a limited capacity to recover. These results might indicate that, at least for *P. oceanica,* photochemical parameters are indicators of severe physiological damage.

Z. chilensis did show a decline in ETR_{max} and Ek_{ETR}, revealing an earlier achievement of photochemical saturation, which might be linked to a decline in gross photosynthesis (Chapter 5). The decline of ETR_{max} and Ek_{ETR} coupled with an increase of NPQ at 40 psu on day 3 could indicate a physiological stress maximum, and an eventual acclimatation process afterwards. On the other hand,

in *P. oceanica* these parameters also kept constant independently of salinity. This might be caused by the short duration of the proposed experiments which did not cause severe damage in the photosynthetic apparatus, supporting the idea that photochemical parameters might not be a useful tool to measure physiological damage at the first stages of brine affection under desalination extrapolable salinity levels. This lack of response in chlorophyll fluorescence-derived parameters in seagrasses was also detected in previous works (Sandoval-Gil et al., 2012a). This might be the reason of NPQ increment under hypersaline conditions, *P. oceanica* could be increasing photoprotection by heat release to keep photosystem II normal functioning. Energy dissipation through heat (NPQ) helps the photosynthetic apparatus to deal with energy excess, preventing photodamage and the formation of ROS, usually through the activation of photosystems with xanthophylls instead of Chla (Demmig-Adams & Adams, 1996). The activation of the xanthophyll cycle under hypersaline conditions has been measured in *P. oceanica* (43 psu, 2 months) (Marín-Guirao, Ruiz, et al., 2013), *T. testudinum* (45 psu, 7 days) (Trevathan et al., 2011) and *C. nodosa* (41.5 psu 15 days) (Garrote-Moreno et al., 2015) indicating this response to be a relevant strategy to cope with hypersalinity even in the mid-term in seagrasses. Photochemical responses are also variable depending on the species and even the population studied, as different salinity regimes might induce local adaptations (Sandoval-Gil et al., 2023). Because of this inter and intraspecific variability, local measurements should be taken to test seagrass responsiveness to altered environmental conditions against a brine discharge.

However, considering the oxidative and osmotic stress detected by the metabolic responses in *P. oceanica* and *Z. chilensis* (discussed below), a more severe photochemical depletion is predicted to occur, based on previous evidence (Marín-Guirao et al., 2013). Oxidative damage, higher respiration rates and lower photosynthetic rates could eventually lead to a metabolic impairment

and depletion due to energy unbalance, causing growth reduction, like the one observed in Chapter 6. Further stress can then cause a physiological collapse ending in shoot death and meadow regression in the long term (Sandoval-Gil et al., 2012b). Some species ecotype could be able to tolerate the hypersaline conditions of a brine discharge influence area, or population plasticity and epigenetic changes might allow the survival of certain meadows (Nguyen et al., 2023; Tomasello et al., 2009). However, growth and primary production would be significantly lower, having an impact in the ecosystem services provided by these seagrass meadows (Mancuso et al., 2023). In this sense, PAM fluorometry can be used for long-term exposed seagrasses as a basic physiological health indicator which should remain mainly stable under tolerable salinity conditions.

9.3 Osmoregulatory responses of seagrasses (*Posidonia oceanica* and *Zostera chilensis*) to hypersaline conditions

Increased salinities cause a reduction in the osmotic potential of surrounding water and plants cope with this issue by activating mechanisms to exclude ion excess while keeping a low osmotic potential to avoid dehydration. In the case of seagrasses, marine adaptation required a set of mechanisms to maintain ion homeostasis and prevent tissue dehydration (Touchette, 2007). These adaptations include specialized water uptake, ion exclusion and the synthesis of organic osmolytes (soluble sugars, amino acids) (Munns & Tester, 2008). In this thesis, Na^+ exclusion and K^+ uptake systems were studied under different salinity increments. *Z. chilensis* presented an upregulation of NHX1 genes, indicating Na^+ storage in vacuoles as mechanism to avoid high levels in the cytosol, a common strategy in terrestrial halophytes (Flowers & Colmer, 2008). In seagrasses, Garrote-Moreno et al. (2015) found that *P. oceanica* was able to keep controlled

Na^+ levels for 7 days until 50.5 psu, revealing an efficient Na^+ exclusion from leaf cells in the short term. However after longer exposures (30-90 days) at 43 psu *P. oceanica* leaves presented increased Na^+ contents, which could only return to normal values if hypersaline exposure lasted for 30 days, with irreversible damage detected at 90 days (Marín-Guirao et al., 2013).

Ion excess can be toxic to plant cells, mainly due to Na^+. High Na^+ levels can cause a K^+ displacement as cofactor of several enzymes causing a metabolic impairment in essential processes, such as photosynthesis (Maathuis & Amtmann, 1999; Munns & Tester, 2008). Salt Overly System (SOS) genes were upregulated under hypersaline conditions both in *Z. chilensis* (Chapter 2) and in its distant cousin, *P. oceanica* (Chapters 5-8). *SOS1* higher relative expression indicate a metabolic investment in Na^+ exclusion from the cytosol as common response in seagrasses under hypersaline conditions in exchange for H^+. These responses are consistent with previous works in which *Z. marina* also reported pH-dependent Na^+ exclusion mechanisms under hypersaline conditions (Fernández et al., 1999) and this gene was recently detected in the genome of the same species (Xiao et al., 2023). The upregulation of *SOS1* in both seagrasses (*P. oceanica* and *Z. chilensis*) indicates that this response against hypersalinity is an essential and common strategy in seagrasses and it seems to be able to keep Na^+ content stable at least for moderate salinity increases and short periods of time.

Regarding salinity origin, SOS1 presented higher relative expression in brine compared to artificial salts, indicating that Na^+ content might not be the only stimulus triggering the transcription of this antiporter protein. Under a real brine discharge (chapter 7), *P. oceanica* plants presented higher *SOS1* upregulation under higher brine exposure. Moreover, when comparing leaf regulation levels with SAMs, they were significantly overexpressing this Na^+ exclusion gene,

highlighting the role of ion homeostasis in this essential organ under brine-derived stress.

In *P. oceanica* Na^+ exclusion was complemented with K^+ active transport by AKT2/3. Although this gene was mostly downregulated in the mesocosm experiment, it was expressed in the field under brine exposure (Chapter 7) and increased its transcription on day 6, when SOS1 presented its lowest FC values. This could indicate a complex temporal response against salinity increments, where Na^+ toxicity appears as a more urgent stress and the response to other ions depletion occurs after a longer exposure period. Carpaneto et al. (2004) found highly selective K^+ channels, which were impermeable to other ions, in *P. oceanica* protoplasts, supporting the active K^+ uptake of this cation as essential response of this seagrass to maintain normal metabolic functioning. In the case of SAMs, AKT 2/3 relative expression did not present such as different results as SOS1 compared to leaves possibly indicating this response to be common among the different plant organs.

These results agree with the analyses made by Garrote-Moreno et al. (2015), in which K^+/Na^+ ratio increased in *P. oceanica* leaf tissue under 46 psu for 7 days, although this ion ratio could not be maintained at higher salinities (>55 psu). In fact, maintaining a high K^+/Na^+ ratio is an adaptative strategy of seagrasses to cope with high salinities in the natural environment (Garrote-Moreno et al., 2016). More euryhaline species such as *C. nodosa* are able to keep constant ion ratios at higher salinities or longer exposures compared to *P. oceanica*, possibly by using different tolerance mechanisms, such as cell wall hardening, thus preventing turgor pressure loss due to a decrease in osmotic potential (Garrote-Moreno et al., 2015; Sandoval-Gil et al., 2012b).

Ion homeostasis control is complemented by other processes, such as water uptake or changes in organelle organization (Fig. 9.1). For example, aquaporins

are membrane proteins designed to stimulate water uptake and aquaporin genes being upregulated in response to hypersalinity in seagrasses have been previously reported. *Z. muelleri* showed higher relative expression of aquaporins, highlighting its role in water balance and nutrient uptake (Shivaraj et al., 2017) and PIP aquaporins have proven to be up-regulated in *P. oceanica* under hypersalinity stress (+8 psu, 2 days) (Serra et al., 2011, 2012). In addition, the ultrastructural modifications found in seagrass species such as *Z. capensis* and *T. testudinum* under hypersaline showed an accumulation of mitochondria next to the plasmatic membrane conditions (Iyer & Barnabas, 1993; Jagels, 1983), indicating an increase in energy demand next to this structure. This could possibly be related with ion and water transport, which might require energy consumption. The energy demand of this ion exclusion to a hypertonic medium after long exposures could be after the causes of energetic impairment and further cellular damage in seagrass cells.

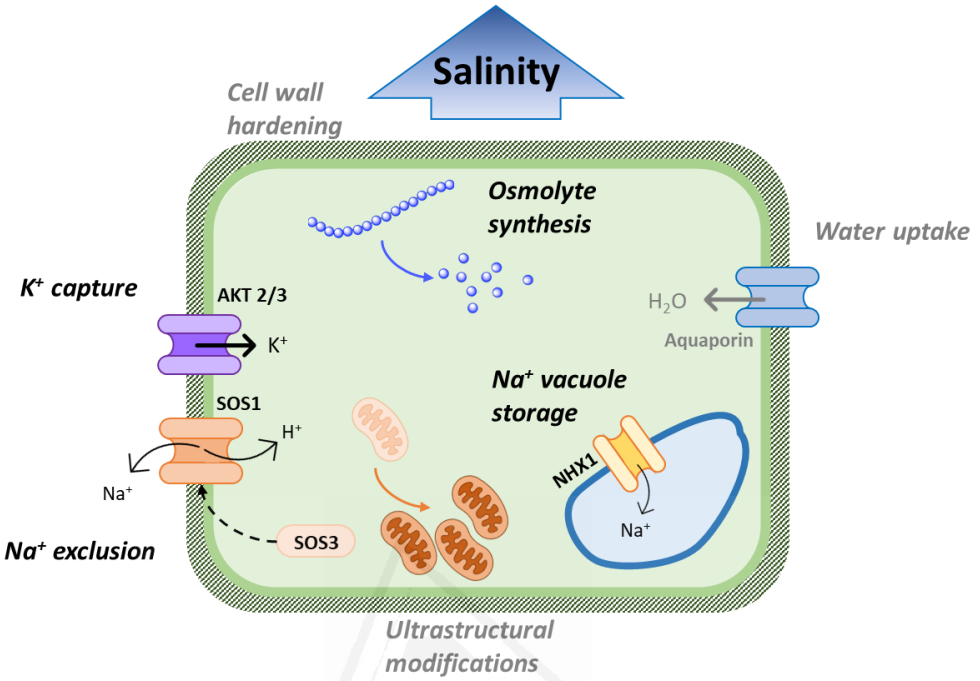

Figure 9.1: Scheme showing osmoregulatory mechanisms used by seagrasses to cope with salinity increments. Responses analysed in this thesis (black) and previous works (grey) are shown.

Another mechanism to keep low osmotic potential in seagrasses is the synthesis and accumulation of organic osmolytes (soluble sugars, free amino acids). In Chapter 5, *Z. chilensis* showed an increment in *OC* and *P5CR* which codify for enzymes directly related to proline synthesis from ornithine and pyrroline-5-carboxilic acid, respectively. These enzymes have essential roles in glutamate metabolism and are directly related to plant's nitrogen metabolism and subsequently to plant development. This increment in proline levels could be helping to keep osmotic balance. The tested primers for genes related to osmolyte synthesis in *P. oceanica* did not work, however, several works have shown how this endemic plant increases osmolyte content to cope with osmotic stress (proline, glycine, soluble sugars) (Marín-Guirao, et al., 2013; Sandoval-Gil et al., 2012b, 2014). The use of organic monomers as osmoprotectants is,

therefore, a complementary response to ion homeostasis and both remain active in seagrasses exposed to increased salinities.

In conclusion, this thesis complements previous studies by demonstrating how *Z. chilensis* and *P. oceanica* actively respond to increased salinities (both artificial salts and brine) with osmoregulatory mechanisms involving regulation of genes related to Na^+ exclusion and storage, K^+ uptake and osmolyte (proline) synthesis. However, despite the efficiency these mechanisms might have under short-term exposures, the higher metabolic cost of keeping these mechanisms active in the long term and the existence of oxidative damage (discussed below) might compromise seagrass survival under a desalination brine discharge.

9.4 Hypersalinity-derived oxidative stress on seagrasses (*Posidonia oceanica* and *Zostera chilensis*)

Hypersalinity can be a cause of ROS production as ion unbalance can alter electron transport chains potential and induce the transference of free electrons to O2 molecules present in the cytosol (Bose et al., 2014; Luo & Liu, 2011). ROS excessive formation have toxic effects in plant cells and trigger scavenging mechanisms to avoid cell structures to be damaged (Foyer & Noctor, 2011). In this thesis, indicators of ROS production, oxidative damage and antioxidant response were measured in response to hypersalinity (Fig. 9.2).

Hypersalinity induced the formation of ROS as detected by H_2O_2 levels in Chapters 5, 6, 7 and 8, correlating with previous works of seagrasses and salinity stress (Trevathan et al., 2011). However, in chapter 6, H_2O_2 levels were higher in control plants compared to *P. oceanica* leaves under the artificial salts and brine treatment. In this case, *CAT* and *APX* transcription together with ASC consumption, could be causing a highly effective H_2O_2 scavenging, although this did not prevent lipid peroxidation to increase under brine exposure. Conversely,

both H_2O_2 levels and TBARS increased with salinity in chapters 7 and 8 in *P. oceanica*, being these responses more sensitive in SAMs compared to leaves.

Regarding seagrass oxidative response, ASC/DHA ratio was used as indicator of antioxidant capacity and differences were detected between species. *Z. chilensis* did not show ASC consumption as a response to salinity increments (Chapter 5), whereas in *P. oceanica* ASC consumption was detected both under brine exposure in the mesocosm and under HB exposure in the field, both in leaves and meristems (Chapters 6-8). This could be explained by higher *APX* expression in *P. oceanica* during the first days of brine exposure, which could have increased ASC consumption, while *Z. chilensis* ASC restoration due to *GR* upregulation could have been more efficient. A common response was detected in both mesocosm approaches (Chapter 5, 6) as total ascorbate declined during the experimental period for both species in all treatments. This effect could possibly be caused by experimental conditions which might influence seagrass metabolism and limit ascorbate *de novo* synthesis, thus reducing total antioxidant capacity under controlled conditions.

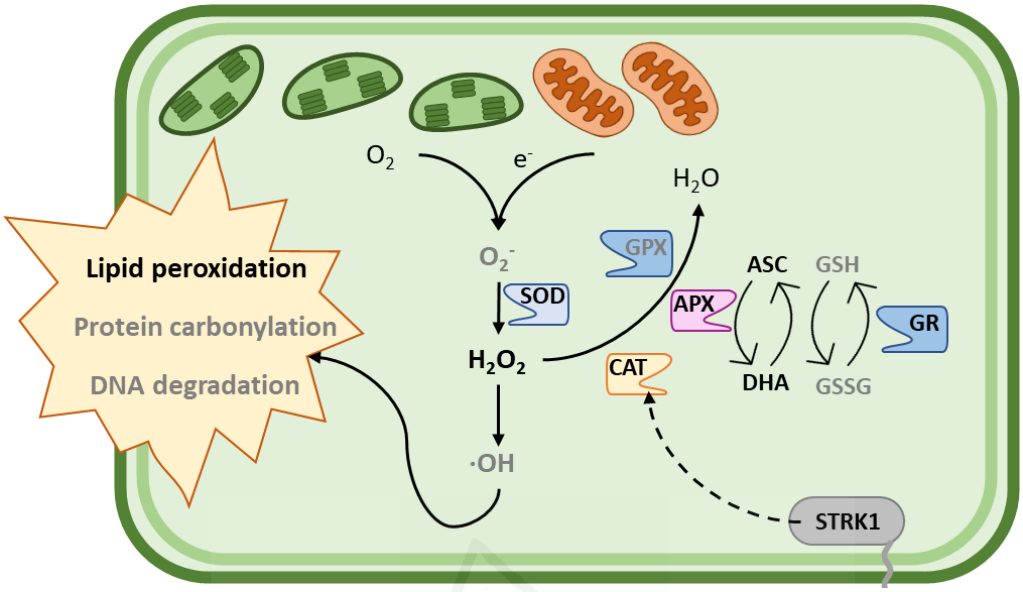

Figure 9.2: Scheme showing reactive oxygen species metabolism (ROM) in seagrasses to cope with salinity increments. Responses analysed in this thesis (black) and previous works (grey) are shown.

Both seagrasses responded to increased salinities by expressing genes codifying for enzymes linked to ROS scavenging (*SODs*, *CAT*, *APX* and *GR*), however, while *Z. chilensis* gene regulation tend to increase with time (Chapter 5), in *P. oceanica* higher FC values were detected during the first hours and declined during the experimental period (Chapter 6, 7). This could be due to an efficient response by *P. oceanica* which produces enough enzymes during the first hours of stress exposure or, on the contrary, by a higher capacity of *Z. chilensis* to invest energy in antioxidant enzymes synthesis on a longer term. In addition, despite the downregulation pattern in P. oceanica leaves in Chapters 6 and 7, SAMs showed and upregulation all these genes at the end of both experiments, revealing a longer-lasting oxidative response in SAMs. This higher oxidative response in SAMs agrees with the osmoregulation results, supporting the higher sensitivity of this organ to brine exposure and its active capacity to respond against it.

Salinity tolerance depends on the physiological plasticity of an organism, and it is usually related to the salinity range an species or population is adapted to (Sandoval-Gil et al., 2012b), and both species (*P. oceanica* and *Z. chilensis*) are mostly found in relatively constant salinity environments. This supports the sensitivity and responsiveness of these 2 species against salinity increments found in this thesis. In agreement with the previously set salinity threshold (Ruiz et al., 2009; Sánchez-Lizaso et al., 2008), field results (Chapter 7) might suggest that the maximum hypersaline exposure *P. oceanica* can withstand (at least in the sampled population) keeping ROS homeostasis and metabolic functioning is close to 39 psu, while higher salinities trigger higher oxidative damage which cannot be prevented by *P. oceanica* ROM. In the case of *Z. chilensis*, this salinity maximum in the short term is possibly close to 37 psu, where ROS production is lower and antioxidant capacity is higher. These levels might be used in each of these seagrass species meadows close to a brine discharge as limit levels to be considered in EMP to ensure seagrass survival within their tolerance capacities.

9.5 Seagrass metabolic indicators for desalination brine biomonitoring

Biomarkers, early warning indicators and environmental management

Biomarkers in monitoring programmes are measurable parameters which might be able to detect the presence and affection of a particular stressor, such as brine exposure, in an organism and allow the management measures taking to prevent ecosystem degradation (Lam, 2009). Regarding seagrasses and hypersalinity, most studies have used parameters related to morphology and physiology, which are not always reliable indicators to prevent seagrass meadows regression (Ceccherelli et al., 2018; Macreadie et al., 2014). In this

regard the biochemical and molecular indicators analysed in this thesis could provide a more anticipated and complete response to brine discharges in seagrasses.

The use of molecular analysis for environmental monitoring has been previously addressed (Graczyk & Conn, 2008; Lam, 2009). Despite a certain technical complexity, biochemical or molecular analyses provide physiological information which would not be addressed by more traditional methods (Makiola et al., 2020). Moreover, the specificness and early awareness of this potential indicators might compensate the invested cost and effort and arise as novel tools which might allow the prevention of severe damage in vulnerable ecosystems and even their suitability for restoration (Makiola et al., 2020; Pazzaglia et al., 2021; Wood et al., 2013). In fact, the use of bioindicators related to different biological levels of organization, allows a more complete idea of the magnitude of an environmental impact on seagrasses, and the stage of physiological stress (Roca et al., 2016). In this case, by completing the journey of hypersalinity ecotoxicology in seagrasses through the different levels of biological organization, a link between molecular and ecological processes can be made (Mazzuca et al., 2013), and these biomarkers can be also linked to ecosystem services seagrass provide and their potential loss due to brine discharges and other cumulative stressors. As shown in Chapter 6, the energy investment in ROM and osmoregulation, coupled with a constant photochemical functioning, resulted in a reduced growth rate, *i.e.* lower primary production. In fact, lower size shoots, rhizomes and roots have been found in *P. oceanica* meadows subjected to natural hypersaline conditions (Mancuso et al., 2023). Brine discharges might change seagrass morphological and physiological traits which can subsequently alter the ecosystem services they provide (Teichberg et al., 2023). For example, metabolic impairment and subsequent growth reduction would reduce the secondary production dependent on seagrass meadows, limit

their shelter capacity and reduce their potential role in coastal erosion mitigation.

Although Zosteraceae and Posidoniaceae families diverged approximately 75 million years ago (Anderson & Janssen, 2009), their lineages have followed parallel evolutionary processes regarding marine adaptation and they show common metabolic responses to salinity increments (Wissler et al., 2011). This is supported by this thesis' results, in which several parameters, i.e., photochemical (NPQ), biochemical (H_2O_2), and molecular (*SOS1*, *CAT*, *SOD*s, *APX*, and *GR*), responded similarly for both species to similar salinity increments (between +2 and +6 psu). These biomarkers have thus the potential to become applicable biomonitoring tools in other seagrass species subjected to hypersaline desalination discharges. When comparing the responsiveness of these biomarkers, relative expression of target genes and parameters related to ROS metabolism reacted more to salinity increments (Fig. 9.3), arising as specific and highly responsive biomarkers to detect brine affection of seagrasses in the short term. On the other hand, photochemistry resulted in a very constant pattern in most cases, except for NPQ_{max}. In this regard, gene expression and metabolic biomarkers are have the potential to be used as early-warning indicators (Ruocco et al., 2019), allowing the detection of osmotic and oxidative stress in its first stages. These parameters could allow the application of management measures (reduction of desalination production; brine dilution) which prevent physiological damage which can be irreversible and lead to a loss of seagrass meadows.

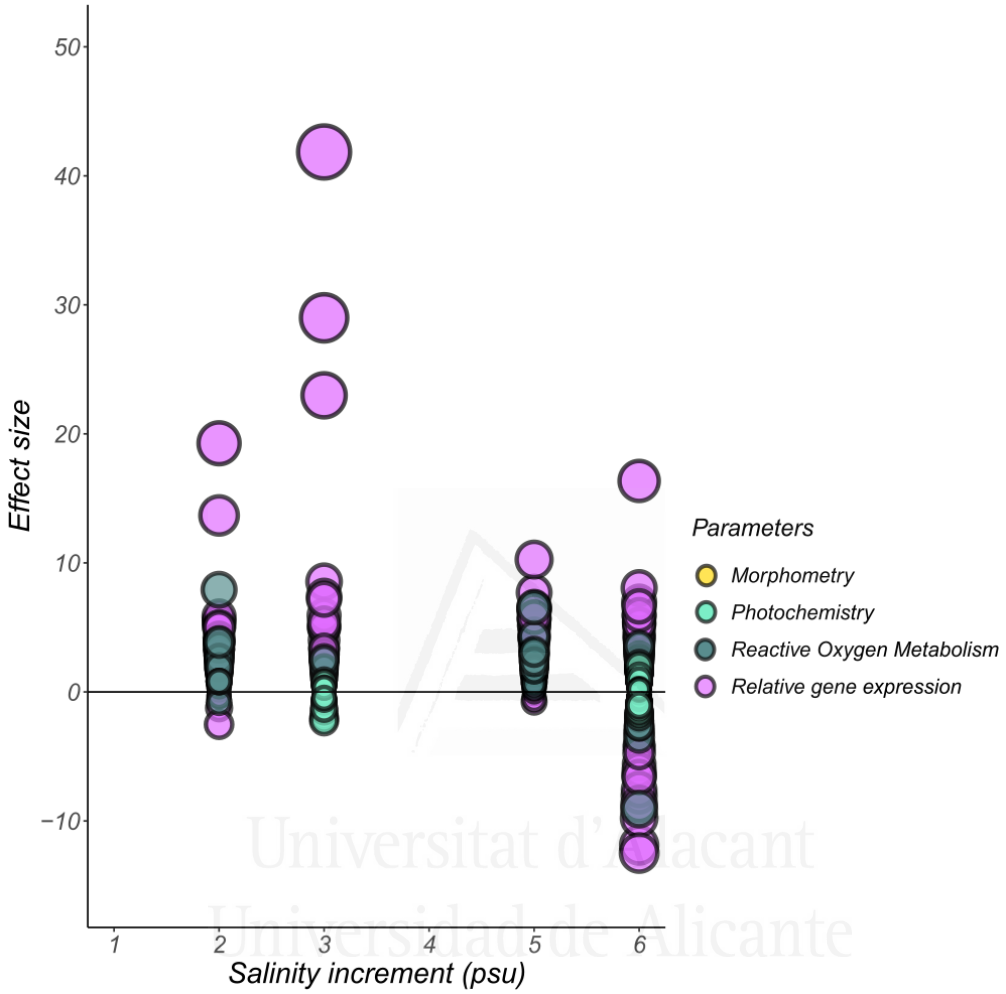

Figure 9.3: Effect size (Cohen's D) of the different parameters measured in chapters 5-8

As firstly proposed by Cambridge et al. (2019), brine exposure showed a higher and earlier detrimental effect than the same salinity reached by using artificial salts. Our results supported this hypothesis by presenting an effective response by osmoregulation and ROM genes, antioxidant consumption, excess energy dissipation and growth reduction under brine influence compared to salinity increments with artificial salts. Moreover, several metabolic descriptors in SAMs (H_2O_2, TBARS, regulation of the 9 tested genes) also responded more actively to

hypersalinity when it was originated by brine. In this regard, these biomarkers appear as suitable to detect not just hypersaline stress but especially when it is caused by a brine discharge.

To assess the environmental impact of brine discharges and other coexisting stressors on seagrasses, proper monitoring plans should be developed. These plans should apply specific descriptors or biomarkers to determine the grade of affection on sensitive communities. More unspecific descriptors (like oxidative responses) could allow the detection of cumulative impacts affecting seagrass metabolism, while salinity-dependent (genes related to osmoregulation) could detect the specific response caused by brine discharge. However, the effectiveness of the different biomarkers mainly depends on the hypersalinity level and time exposure and metabolic, physiological, morphometrical and population responses differ in time (Fig. 9.4). Because of this, biomonitoring should be adapted depending on the experimental time scale, focusing on gene expression and metabolic parameters in the short term (days) and photochemistry, physiology, and population metrics for longer exposures (months, years), thus combining different sampling techniques adapted to characteristics of the discharge and its time scale.

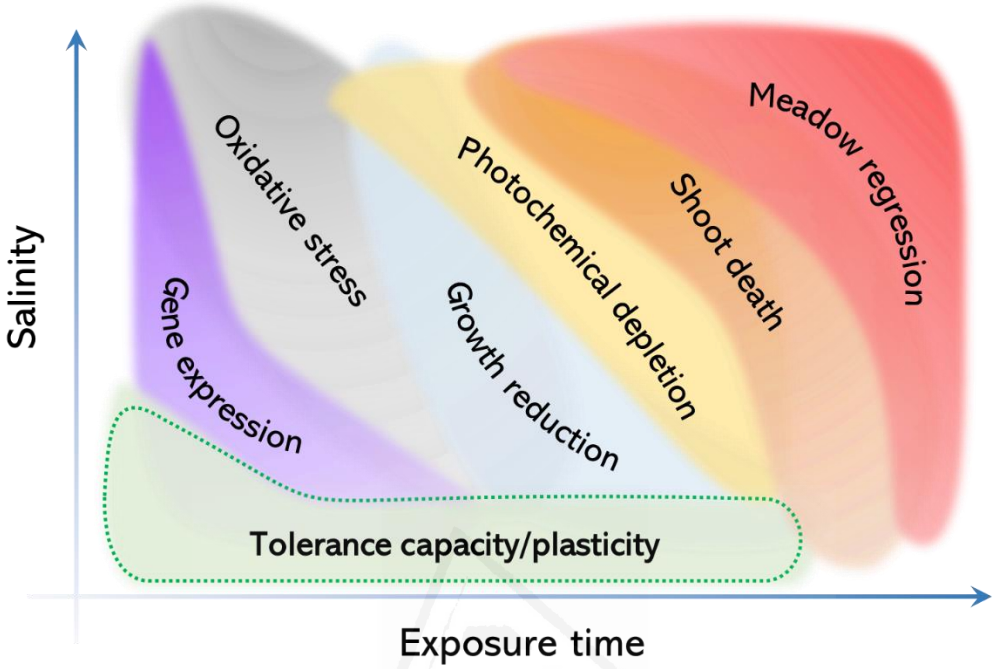

Figure 9.4: Scheme showing the relationship between salinity stress duration and intensity and the response range of each level of biological organization.

While restoration viability and success is still being studied, especially in slow-growing species such as *P. oceanica* (Pansini et al., 2022), seagrass conservation should focus in preventing its decline. If we are to find nature-based solutions to cope with climate change and increase ecosystem resilience against it, seagrass meadows conservation should be imperative. These marine ecosystems play an essential role as carbon sinks by storing 18% of world's Blue Carbon while only accounting for 1% of ocean's surface (Duarte et al., 2010; Pergent-Martini et al., 2021). This is why seagrass conservation and recovery are unrefusable tools in climate change mitigation. Apart from the role in the global change context, the ecosystem services and biodiversity associated to seagrass meadows demand urgent and efficient measures to protect these ecosystems, starting by managing and mitigating human-derived stressors.

The aim of this Ph.D. thesis was to provide advance knowledge and novel biomonitoring tools regarding seagrasses and desalination discharge effects. These results are expected to improve impact management and move forward in the development of a sustainable desalination industry.

9.6 Future approaches

From the results derived from this thesis, it can be concluded that, although different experimental approaches might provide complementary information, future ecotoxicological assays related to desalination impact should: (1) use actual brine instead of artificial sea salts, (2) focus on field approaches to properly assess seagrass vulnerability under natural conditions, and (3) consider SAM sampling for metabolic biomarkers instead of leaves due to their higher responsiveness.

Moreover, all the experimental approaches of this research have been made with adult plants and, therefore, we might underestimate the vulnerability on seagrass seeds, seedlings and young plantets by brine discharges. In fact, different life stages show different tolerances and physiological performances against hypersalinity (Cambridge et al., 2019; Kahn & Durako, 2006). This fact would reinforce the previously detected lower genetic diversity in hypersaline seagrass habitats by compromising seagrass survival in their first development stages.

In this regard, studies related to genetic diversity at a local scale would be interesting in brine affected meadows. Although some studies have reported an increment in seagrass flowering under some stressful conditions, potentially increasing genetic diversity (Ruiz et al., 2018), recent population genetic analyses have revealed a lower genetic richness in seagrass populations under extreme salinity conditions (Nguyen et al., 2023; Tomasello et al., 2009). This implies that, even though some resilient seagrass ecotypes might withstand in a hypersaline

environment (Mancuso et al., 2023; Marín-Guirao et al., 2017), they might be suffering from this genetic impoverishment. This genetic diversity loss might be a serious concern in a context of global change and increasing anthropogenic pressures on marine communities because more genetically diverse meadows are more resilient to disturbances (Hughes & Stachowicz, 2004) and therefore, low genetic richness indicate higher vulnerability to a changing environment. This could be particularly concerning in the case of *Z. chilensis*, which has only 3 described populations and genetic pool is thus limited by its actual distribution.

To further ascertain the molecular responses of seagrasses against hypersalinity, the use of Next generation sequencing (NGS) could allow a more complete view of the subcellular mechanisms affected by this stressor. By performing RNAseq analyses, a complete view of the metabolic pathways affected could provide new potential genes, or regions to be selected and used as biomarkers of brine affection.

Also, the application of brine-specific and more generalist stress responses in seagrass meadows submitted to desalination discharges and other cumulative stressors might allow the developments impact discrimination assessments, which allow the determination of the most harmful stressor potentially affecting seagrasses in highly anthropized areas. These novel techniques, together with physiological, population and community descriptors could allow a complete meadow health assessment and the development of locally adapted management measures which ensure seagrass conservation and future recovery.

10. Main conclusions

o The upper limit of *P. oceanica* meadows in the province of Alicante has remained stable for the last 20 years according to populational descriptors.

o *P. oceanica* meadows submitted to multiple and cumulative impacts in the Bay of Alicante have suffered an important regression in their extension.

o Regression events in *P. oceanica* meadows mainly occur at local scale, due to specific disturbances and their interaction, rather than global processes.

o *Z. chilensis* showed a reduction in its photochemical performance under hypersaline conditions, higher ROS production and antioxidant consumption.

o Salinity increments triggered an active transcriptomic response in *Z. chilensis* by upregulating genes related to osmolyte regulation and ion exclusion and ROS scavenging, however, osmotic stress could compromise this endemic plant's physiology and survival in the long term.

o Brine effects on *P. oceanica* at morphometrical and metabolic scales are mainly caused by hypersalinity.

o Certain responses such as NPQmax, ASC consumption and regulation of genes such as STRK1 and CAT, were incremented in *P. oceanica* plants exposed to brine compared to the same hypersaline conditions reached using artificial sea salts.

o Molecular and metabolic biomarkers tested in *P. oceanica* have shown to be efficient and specific to brine discharges as they actively responded to brine exposure in the field and could be used to assess the contribution of this stressor where multiple environmental pressures exist.

o Molecular descriptors are proposed as early warning indicators in *P. oceanica* plants under brine discharge exposures with the aim of preventing further physiological damage and potential meadow regressions on the long term.

- ROS production and lipid peroxidation responded more to brine exposure in *P. oceanica* SAMs compared to leaves.
- Meristems presented higher relative expression in genes related to osmoregulation and oxidative response compared to leaves, indicating a higher sensitivity to hypersalinity and an essential role in P. oceanica salinity tolerance.
- SAMs have the potential to be target organs to be sampled to detect brine affection by using metabolic biomarkers as early warning indicators and be representative of seagrass physiology and response to this environmental stressor.
- In future studies regarding brine discharges and seagrasses it is recommended: The use of descriptors from different levels of biological organization (like complete transcriptomes through RNAseq), the use of real desalination brine instead of artificial salts, natural conditions simulation when possible and the use of SAMs as biomonitor organ of brine-derived stress.

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ANEX 1 – Supplementary data

Table S1: ANOVA results of photochemical parameters in Chapter 5. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01

Variable	Factor	Df	Mean Sq	F	p-value
Effective Yield*	Treatment	2	0.0003996	2.808	0.0802
	Day	3	0.0000315	0.221	0.8808
	Treatment:Day	6	0.0000832	0.585	0.7391
	Residuals	24	0.0001423		
Maximal quantum Yield	Treatment	2	0.0000175	0.079	0.92379
	Day	3	0.0012998	5.902	**0.00109**
	Treatment:Day	6	0.0002896	1.315	0.26005
	Residuals	80	0.0002202		
ETR_{max}	Treatment	2	8.65	7.639	**0.000921**
	Day	3	37.75	33.346	**4.40E-14**
	Treatment:Day	6	1.01	0.894	0.503352
	Residuals	80	1.13		
α_{ETR}*	Treatment	2	0.017476	3.487	0.0353
	Day	3	0.006387	1.274	0.2888
	Treatment:Day	6	0.006104	1.218	0.3058
	Residuals	80	0.005011		
Ek_{ETR}	Treatment	2	2654	10.877	**6.63E-05**
	Day	3	5250	21.515	**2.58E-10**
	Treatment:Day	6	371	1.522	0.182
	Residuals	80	244		
NPQ_{max}	Treatment	2	0.07168	1.055	0.35316
	Day	3	0.06606	0.972	0.4103
	Treatment:Day	6	0.22911	3.37	**0.00516**
	Residuals	80	0.06798		

Table S2: ANOVA results of biochemical parameters in Chapter 5. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01.

Variable	Factor	Df	Mean Sq	F	P-value

H_2O_2	Treatment	2	1.1747	3.921	**0.03362**
	Day	3	1.4954	4.991	**0.00786**
	Treatment:Day	6	0.3267	1.09	0.39635
	Residuals	24	0.2996		
DHA	Treatment	2	10094	5.223	**0.0131**
	Day	3	37272	19.285	**1.39E-06**
	Treatment:Day	6	3162	1.636	0.1805
	Residuals	24	1933		
ASC	Treatment	2	0.419	3.185	**0.0485**
	Day	3	2.8038	21.316	**1.61E-09**
	Treatment:Day	6	0.0774	0.588	0.7383
	Residuals	60	0.1315		

Table S3: ANOVA results of relative gene expression (FC) in Chapter 5. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01.

Variable	**Factor**	**Df**	**Mean Sq**	**F**	**p-value**
SOS1	Treatment	1	1.743	3.559	0.0837
	Day	3	14.416	29.439	**8.14E-06**
	Treatment:Day	3	1.593	3.253	0.0598
	Residuals	12	0.49		
P5CR	Treatment	1	0.338	0.206	0.658049
	Day	3	29.247	17.828	**0.000102**
	Treatment:Day	3	5.163	3.147	0.064869
	Residuals	12	1.64		
*NHX1**	Treatment	1	0.4643	0.848	0.3754
	Day	3	2.1097	3.851	**0.0384**
	Treatment:Day	3	0.3001	0.548	0.6591
	Residuals	12	0.5478		
OC	Treatment	1	14.911	8.433	0.01323
	Day	3	15.579	8.811	0.00232
	Treatment:Day	3	15.375	8.695	**0.00245**
	Residuals	12	1.768		
Mn/Fe SOD	Treatment	1	6.237	1.23	0.289
	Day	3	10.416	2.055	0.16

	Treatment:Day	3	8.83	1.742	0.212
	Residuals	12	5.07		
Cu/Zn SOD	Treatment	1	17.894	10.259	**0.00759**
	Day	3	15.259	8.748	**0.00239**
	Treatment:Day	3	2.691	1.543	0.2542
	Residuals	12	1.744		
*CAT**	Treatment	1	3151.3	17.69	0.00122
	Day	3	3129.3	17.56	0.00011
	Treatment:Day	3	2597.2	14.58	**0.000264**
	Residuals	12	178.2		
APX	Treatment	1	0.51	0.382	0.5481
	Day	3	11.123	8.332	**0.0029**
	Treatment:Day	3	2.361	1.769	0.2066
	Residuals	12	1.335		
GR	Treatment	1	68.66	5.089	**0.04353**
	Day	3	91.02	6.745	**0.00644**
	Treatment:Day	3	10.73	0.796	0.51966
	Residuals	12	13.49		

Table S4: Results of seawater chemical analysis performed at the Institute of Water and Environmental Sciences, (University of Alicante).

Parameter	**Methodology**	**Detection limit**	**Result**	**Unit**
Chlorophyll a	Absorption spectroscopy	1	<1	µg/L
Chlorophyll b	Absorption spectroscopy	1	<1	µg/L
Chlorophyll c	Absorption spectroscopy	1	<1	µg/L
Turbidity	Nephelometer	0.02	2.99	UN
Suspension solids	Gravimetry	1	<1	mg/L
Amonium	Absorption spectroscopy	0.05	<0.05	mg/L
Ntrates	Absorption spectroscopy	0.1	<0.1	mg/L
Nitrintes	Absorption spectroscopy	0.02	<0.02	mg/L
Total nitrogen	Absorption spectroscopy	2	<2	mg/L
Orthophosphates	Absorption spectroscopy	0.01	<0.01	mg/L

Total phosphorus	Inductively coupled plasma mass spectrometry (ICP-MS)	60	3.4	µg/L
Iron	Inductively coupled plasma mass spectrometry (ICP-MS)	6	9.2	µg/L
Niquel	Inductively coupled plasma mass spectrometry (ICP-MS)	3.1	0.8	µg/L
Copper+	Inductively coupled plasma mass spectrometry (ICP-MS)	3	11	µg/L

Table S5: Results of desalination brine chemical analysis performed at the Institute of Water and Environmental Sciences, (University of Alicante).

Parameter	**Methodology**	**Detection limit**	**Result**	**Unit**
pH	Electrochemistry	0.01	7.66	pH units
Biological oxygen demand (BOD)	Manometric	0.5	<0.5	mg/L
Kjedahl nitrogen	Absorption spectroscopy	0.04	<0.04	mg/L
Nitrates	Absorption spectroscopy	0.4	<0.4	mg/L
Nitrites	Absorption spectroscopy	0.03	<0.03	mg/L
Total nitrogen	0.15	<0.15	mg/L	
Suspension solids	Gravimetry	1	4	mg/L
Total phosphorus	Inductively coupled plasma mass spectrometry (ICP-MS)	0.2	65	µg/L
Iron	Inductively coupled plasma mass spectrometry (ICP-MS)	0.9	16	µg/L
Niquel	Inductively coupled plasma mass spectrometry (ICP-MS)	0.1	1.8	µg/L
Coppper	Inductively coupled plasma mass spectrometry (ICP-MS)	0.2	14	µg/L
Total residual cloride	Absorption spectroscopy	0.1	<0.1	mg/L
Chemical oxygen demand (COD)	Absorption spectroscopy	5	<5	mg/L
Anionic detergents	Absorption spectroscopy	0.1	<0.1	mg/L

Table S6: ANOVA results of morphometrical parameters in Chapter 6. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01

	Factor	Df	MS	F-value	p-value
Growth biomass	Treatment	2	0.0005572	5.682	**0.00592**
	Residuals	51	0.0000981		
Growth length	Treatment	2	0.1104	3.707	**0.0314**
	Residuals	51	0.02978		

Table S7: ANOVA results of photochemical parameters in Chapter 6. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01

	Factor	Df	MS	F-value	p-value
Fv/Fm	Treatment	2	1.34E-05	0.837	0.445253
	Day	3	1.71E-04	10.707	**0.000117**
	Treatment:Day	6	8.32E-06	0.522	0.786165
	Residuals	24	1.60E-05		
ETR_{max}	Treatment	2	2.751	0.886	0.4255
	Day	3	14.401	4.637	**0.0107**
	Treatment:Day	6	4.84	1.558	0.2025
	Residuals	24	3.106		
α_{ETR}	Treatment	2	0.0000341	0.289	0.751
	Day	3	0.0004728	4.015	**0.019**
	Treatment:Day	6	0.0002279	1.935	0.116
	Residuals	24	0.0001178		
Ek_{ETR}	Treatment	2	20.14	0.704	0.5045
	Day	3	126.03	4.405	**0.0132**
	Treatment:Day	6	44.83	1.567	0.2
	Residuals	24	28.61		
NPQ_{max}	Treatment	2	0.19036	7.6	**0.00277**
	Day	3	0.13132	5.243	**0.00632**
	Treatment:Day	6	0.0116	0.463	0.82862
	Residuals	24	0.02505		

Table S8: ANOVA results of Reactive Oxygen Metabolism (ROM) parameters in Chapter 6. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01.

	Factor	Df	MS	F-value	p-value
H_2O_2*	Treatment	2	0.0024365	13.049	**0.000146**
	Day	3	0.0003905	2.091	0.127944
	Treatment:Day	6	0.0004346	2.327	0.06507
	Residuals	24	0.0001867		
TBARS	Treatment	2	0.3244	8.056	**0.00211**
	Day	3	0.0247	0.612	0.61363
	Treatment:Day	6	0.0392	0.974	0.4636
	Residuals	24	0.0403		
ASC	Treatment	2	2.7224	31.235	**2.09E-07**
	Day	3	0.2316	2.657	0.0712
	Treatment:Day	6	0.1295	1.485	0.2254
	Residuals	24	0.0872		
DHA	Treatment	2	1.1829	1.326	0.284
	Day	3	0.7178	0.804	0.504
	Treatment:Day	6	0.1733	0.194	0.975
	Residuals	24	0.8922		

Table S9: ANOVA results of relative gene expression (FC) values in Chapter 6. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01.

	Factor	Df	MS	F-value	p-value
SOS1/NHX7	Treatment	1	1.8538	8.712	0.00938
	Day	3	1.9792	9.301	0.000854
	Treatment:Day	3	1.3416	6.305	**0.004996**
	Residuals	16	0.2128		
*SOS3**	Treatment	1	0.025	0.018	0.895
	Day	3	20.951	15.015	**6.51E-05**
	Treatment:Day	3	0.42	0.301	0.824
	Residuals	16	1.395		
CAT	Treatment	1	1.2412	6.858	0.018619
	Day	3	1.9678	10.872	0.000386

	Treatment:Day	3	0.8356	4.617	**0.01644**
	Residuals	16	0.181		
AKT2/3	Treatment	1	0.9675	27.3	8.34E-05
	Day	3	0.6472	18.26	2.03E-05
	Treatment:Day	3	0.1701	4.8	**0.0143**
	Residuals	16	0.0354		
*SODMn**	Treatment	1	0.779	2.145	0.1624
	Day	3	20.493	56.443	**9.90E-09**
	Treatment:Day	3	1.757	4.838	0.0139
	Residuals	16	0.363		
SODFe	Treatment	1	0.803	2.862	0.11
	Day	3	16.988	60.562	5.92E-09
	Treatment:Day	3	1.008	3.595	**0.037**
	Residuals	16	0.281		
STRK1	Treatment	1	3.682	20.743	**0.000325**
	Day	3	0.23	1.298	0.309456
	Treatment:Day	3	0.132	0.743	0.542057
	Residuals	16	0.177		
GR	Treatment	1	0.082	1.054	0.32
	Day	3	14.8	190.02	**1.02E-12**
	Treatment:Day	3	0.196	2.518	0.0949
	Residuals	16	0.078		
APX	Treatment	1	0.061	0.493	0.492
	Day	3	5.621	45.285	**4.83E-08**
	Treatment:Day	3	0.288	2.317	0.114
	Residuals	16	0.124		

Table S10: ANOVA results of Reactive Oxygen Metabolism (ROM) parameters in Chapter 7. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01.

	Factor	**Df**	**MS**	**F-value**	**p-value**
ASC	Treatment	2	0.34	10.034	**0.00118**
	Day	2	0.0087	0.255	0.7773
	Treatment:Day	4	0.0309	0.912	0.47809
	Residuals	18	0.0339		
DHA	Treatment	2	4.562	55.558	1.99E-08

	Day	2	0.118	1.441	0.2627
	Treatment:Day	4	0.295	3.592	**0.0254**
	Residuals	18	0.082		
H_2O_2	Treatment	2	3.235	28.074	2.93E-06
	Day	2	0.795	6.904	0.00595
	Treatment:Day	4	0.425	3.687	**0.02315**
	Residuals	18	0.115		
TBARS	Treatment	2	0.10709	4.184	**0.0322**
	Day	2	0.11695	4.57	**0.0248**
	Treatment:Day	4	0.0108	0.422	0.7908
	Residuals	18	0.02559		

Table S11: ANOVA results of relative gene expression (FC) values in Chapter 7. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01.

	Factor	**Df**	**MS**	**F-value**	**p-value**
SOS1	Treatment	1	0.4502	12.195	0.004446
	Day	2	0.7672	20.784	0.000126
	Treatment:Day	2	0.1846	5.002	**0.026307**
	Residuals	12	0.0369		
SOS3	Treatment	1	78.96	16.787	**0.00148**
	Day	2	42.74	9.086	**0.00396**
	Treatment:Day	2	11.87	2.523	0.12172
	Residuals	12	4.7		
CAT	Treatment	1	0.0049	0.013	0.9112
	Day	2	2.3449	6.214	**0.0141**
	Treatment:Day	2	0.8066	2.138	0.1607
	Residuals	12	0.3773		
AKT 2/3	Treatment	1	0.8343	7.087	0.0207
	Day	2	0.1274	1.083	0.3696
	Treatment:Day	2	0.7241	6.151	**0.0145**
	Residuals	12	0.1177		
SODMn	Treatment	1	1.92	1.284	0.279
	Day	2	59.82	40.001	**4.92E-06**
	Treatment:Day	2	0.37	0.25	0.783
	Residuals	12	1.5		

SODFe	Treatment	1	0.7	0.456	0.512
	Day	2	39.79	25.969	**4.37E-05**
	Treatment:Day	2	0.2	0.128	0.881
	Residuals	12	1.53		
STRK1	Treatment	1	4.228	7.614	**0.0173**
	Day	2	1.741	3.135	0.0803
	Treatment:Day	2	0.08	0.144	0.8674
	Residuals	12	0.555		
GR	Treatment	1	0.4105	6.523	0.025276
	Day	2	0.9011	14.318	**0.000663**
	Treatment:Day	2	0.065	1.033	0.385657
	Residuals	12	0.0629		
APX	Treatment	1	0.847	4.108	0.065487
	Day	2	3.65	17.708	**0.000263**
	Treatment:Day	2	0.142	0.688	0.521556
	Residuals	12	0.206		

Table S10: ANOVA results of Reactive Oxygen Metabolism (ROM) parameters in Chapter 8. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01.

Experiment	Variable	Factor	Df	MS	F-value	p-value
Mesocosm	H_2O_2	Treatment	2	0.1339	1.2	0.3349
		Tissue	1	0.0088	0.157	0.6985
		Treatment:Tissue	2	0.5209	4.667	**0.0317**
		Residuals	12	0.6698		
	TBARS	Treatment	2	0.5025	4.73	0.0306
		Tissue	1	0.1037	1.952	0.1877
		Treatment:Tissue	2	0.008	0.076	0.9276
		Residuals	12	0.6375		
	ASC	Treatment	2	6.067	9.148	**0.00386**
		Tissue	1	2.645	7.975	**0.01535**
		Treatment:Tissue	2	0.479	0.722	0.50583
		Residuals	12	3.98		
	DHA	Treatment	2	1.069	0.33	0.725

Field		Tissue	1	5.873	3.629	0.081
		Treatment:Tissue	2	0.557	0.172	0.844
		Residuals	12	19.419		
	H_2O_2	Treatment	2	2.237	8.052	**0.00606**
		Tissue	1	4.067	29.27	**0.00015**
		Treatment:Tissue	2	0.327	1.177	0.341438
		Residuals	12	1.667		
	TBARS	Treatment	2	1.2902	14.597	0.000611
		Tissue	1	1.5709	35.548	6.59E-05
		Treatment:Tissue	2	0.4197	4.749	**0.03025**
		Residuals	12	0.5303		
	ASC	Treatment	2	0.02353	1.95	0.184811
		Tissue	1	0.13468	22.318	**0.00049**
		Treatment:Tissue	2	0.02528	2.095	0.16585
		Residuals	12	0.07242		
	DHA	Treatment	2	0.2689	14.909	0.000558
		Tissue	1	0.0514	5.701	0.03428
		Treatment:Tissue	2	0.3495	19.38	**0.00017**
		Residuals	12	0.1082		

Table S13: ANOVA results of relative gene expression (FC) values in the field experiment in Chapter 8. Bold p-values indicate the existence of statistical differences in the studied factor. *Cases in which data homogeneity was not achieved and significance level was set in 0.01.

Experiment	Variable	Factor	Df	MS	F-value	p-value
Mesocosm	*SOS1*	Treatment	1	4.628	8.585	0.019
		Tissue	1	5.544	10.283	0.0125
		Treatment:Tissue	1	5.384	9.988	**0.0134**
		Residuals	8	4.313		
	*SOS3**	Treatment	1	0.576	8.482	0.019517
		Tissue	1	3.847	56.656	6.75E-05
		Treatment:Tissue	1	2.512	36.985	**0.00029**
		Residuals	8	0.543		

CAT	Treatment	1	0.31	4.735	0.061241
	Tissue	1	4.805	73.456	2.65E-05
	Treatment:Tissue	1	2.267	34.661	**0.00036**
	Residuals	8	0.523		
AKT 2/3	Treatment	1	0.0303	0.243	0.635
	Tissue	1	2.6098	20.96	0.00181
	Treatment:Tissue	1	2.2986	18.461	**0.00263**
	Residuals	8	0.9961		
SODMn	Treatment	1	43.24	4.311	0.07153
	Tissue	1	72.52	7.23	0.02754
	Treatment:Tissue	1	134.18	13.377	**0.00642**
	Residuals	8	80.24		
SODFe	Treatment	1	134.7	3.984	0.081
	Tissue	1	263	7.777	0.0236
	Treatment:Tissue	1	226.6	6.701	**0.0322**
	Residuals	8	270.5		
STRK1	Treatment	1	7.303	19.12	0.00237
	Tissue	1	8.867	23.22	0.00132
	Treatment:Tissue	1	3.837	10.04	**0.01321**
	Residuals	8	3.056		
GR	Treatment	1	0.001	0.025	0.87863
	Tissue	1	8.057	165.566	1.26E-06
	Treatment:Tissue	1	1.245	25.582	**0.00098**
	Residuals	8	0.389		
APX	Treatment	1	0.38	2.433	0.1574
	Tissue	1	7.113	45.494	0.000146
	Treatment:Tissue	1	2.123	13.579	**0.00617**
	Residuals	8	1.251		
*SOS1**	Treatment	1	31489	12.65	0.007443
	Tissue	1	71951	28.9	0.000665
	Treatment:Tissue	1	31607	12.69	**0.00737**
	Residuals	8	19920		
SOS3	Treatment	1	10	2.509	0.152
	Tissue	1	2.35	0.59	0.464
	Treatment:Tissue	1	1.33	0.334	0.579
	Residuals	8	31.89		
CAT	Treatment	1	5.629	2.825	0.131

Field

	Tissue	1	16.344	8.202	**0.021**
	Treatment:Tissue	1	3.344	1.678	0.231
	Residuals	8	15.941		
AKT 2/3	Treatment	1	0.9319	6.575	0.0334
	Tissue	1	0.0797	0.562	0.4749
	Treatment:Tissue	1	1.3064	9.216	0.0162
	Residuals	8	1.134		
SODMn	Treatment	1	17.61	5.3	0.0503
	Tissue	1	15.2	4.572	0.0649
	Treatment:Tissue	1	4.53	1.363	0.2766
	Residuals	8	26.59		
SODFe	Treatment	1	10.707	10.179	**0.0128**
	Tissue	1	6.302	5.991	0.0401
	Treatment:Tissue	1	3.747	3.562	0.0958
	Residuals	8	8.415		
STRK1	Treatment	1	0.492	0.357	0.567
	Tissue	1	4.202	3.046	0.119
	Treatment:Tissue	1	0.279	0.202	0.665
	Residuals	8	11.036		
GR	Treatment	1	8.436	15.451	**0.00435**
	Tissue	1	11.357	20.799	**0.00185**
	Treatment:Tissue	1	3.891	7.126	0.02838
	Residuals	8	4.368		
APX	Treatment	1	1.779	5.096	0.053942
	Tissue	1	10.511	30.108	**0.00058**
	Treatment:Tissue	1	1.089	3.118	0.115408
	Residuals	8	2.793		

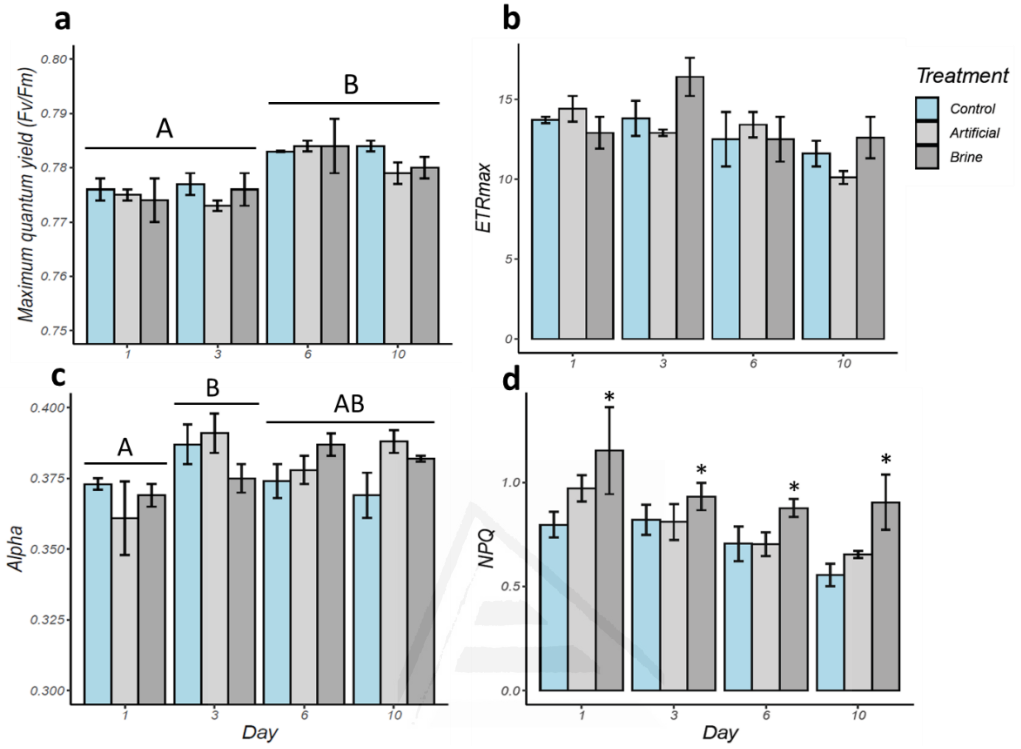

Figure S1: Maximum quantum yield (a), electron transport rate (b), photosynthetic efficiency (c) and non-photochemical quenching (NPQ) in *P. oceanica* leaves experimental salinity treatments: Control (37 psu), Artificial Salts (43 psu), and Brine (43 psu) at 4 sampling times (Days 1, 3, 6, 10). Bars represent the mean of each variable with their respective standard errors (SE). Upper case letters represent significant differences at 95% confidence interval ($p < .05$) between days. Asterisks (*) show significant differences between treatments.

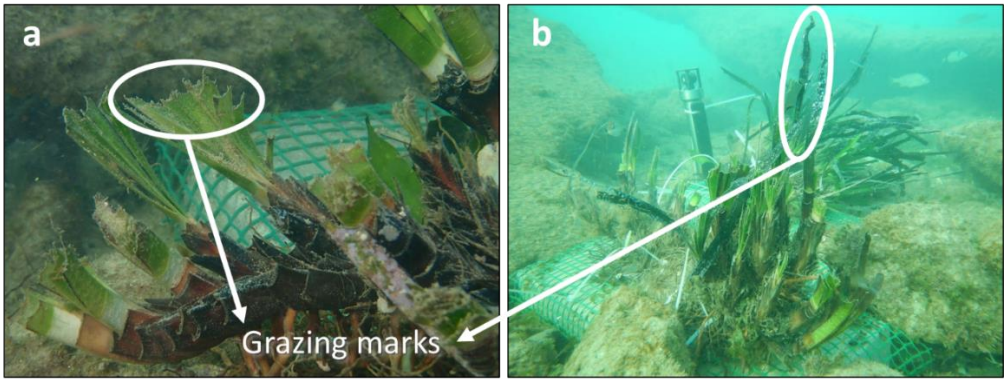

Figure S2: Detailed picture of grazing marks on HB *P. oceanica* plants.