UNRAVELING THE SUCCESS OF INVADERS: BIOTIC AND ABIOTIC FACTORS DETERMINING THE INVASIBILITY OF MEDITERRANEAN BENTHIC ASSEMBLAGES BY *Caulerpa cylindracea*

Jorge Santamaría Pérez

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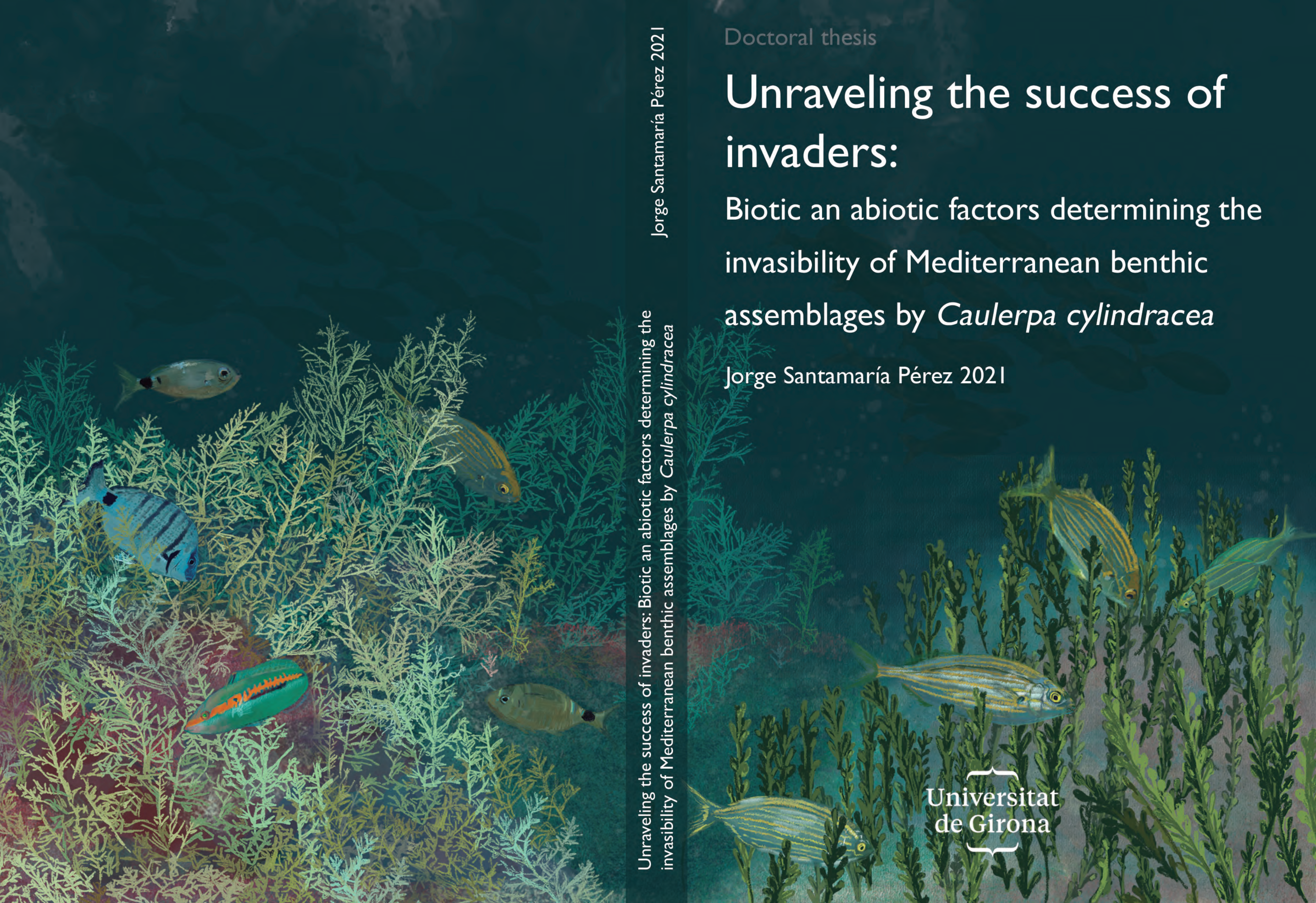

Doctoral thesis

Unraveling the success of invaders:

Biotic an abiotic factors determining the invasibility of Mediterranean benthic assemblages by *Caulerpa cylindracea*

Jorge Santamaría Pérez 2021

Universitat de Girona

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DOCTORAL THESIS

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2021

Programa de Doctorat en Medi ambient

Dirigida i tutoritzada per:

Dra. Emma Cebrian Pujol

Memòria presentada per optar al títol de Doctor per la Universitat de Girona

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A mis abuelos Paulino y Josefina,
a mis padres y a mi tía Fina,
a mi familia y amigos,
a Tere.

Por enseñarme a ser,
por hacerme creer.

Agradecimientos

Después de varios días sentado frente a una hoja en blanco, sin saber muy bien como empezar a escribir esta parte, hoy, por fin, he encontrado el coraje y espero que la "inspiración" para escribir estas líneas de agradecimiento y reconocimiento a todos aquellos que me han acompañado a lo largo de este viaje. Sois muchos, los que de un modo u otro, habéis contribuido a que haya llegado hasta aquí y me habéis inspirado a lo largo del camino; y espero no dejarme a ninguno en el tintero. Así pues, no tengo otra forma más sincera de comenzar que dándoos las gracias a todos por el apoyo incondicional, las risas, la ayuda desinteresada, los momentos de desconexión y las horas de aprendizaje que me habéis regalado. Esta tesis es, en parte, un reflejo de todo lo que hemos compartido a lo largo de estos años.

A medida que escribo estas líneas, no puedo evitar tener la sensación de que el tiempo ha pasado demasiado rápido, y esa percepción acelerada del tiempo, sin lugar a dudas, es indicativa de que esta etapa la he disfrutado desde el primer día y tengo muy presente de que la recordaré día tras día como una de las aventuras más emocionantes e inolvidables que me ha dado la vida. Y es en este momento cuando no puedo evitar que me invadan sensaciones encontradas, que supongo les son familiares a todos los que han pasado antes por esta situación. Por un lado, me llena de alegría y satisfacción haber llegado hasta este punto y estar a punto de conseguir cumplir la meta de terminar el doctorado; pero por otro lado, me da mucha pena y me llena de tristeza y nostalgia aceptar que esta etapa está a punto de acabar y que ha llegado el momento de dar el último paso para cerrarla.

Aquí sentado, frente a las vistas que me han acompañado durante los últimos meses de "retiro", recuerdo como si fuese ayer, el cúmulo de casualidades que me llevaron hasta Blanes y hasta el CEAB… Todo empezó durante unas vacaciones de verano pasando por delante de la puerta del centro e imaginándome trabajando allí; y continuó con una conversación telemática con Emma y una invitación para venir unos días a conocer el centro. Eso dio paso a un coche cargado de maletas, trastos y hasta la cesta de coger setas; y el sentimiento de que venía a quedarme "para siempre".

Emma, sin ti y sin tu apoyo esta tesis no hubiese sido posible. Mil gracias por darme esta oportunidad, por confiar en mi cuando no me conocías de nada y por abrirme las puertas de tu equipo. Desde el primer momento que intercambiamos emails siempre has mostrado una gran empatía y cercanía, cosa que no es habitual y que se agradece en un mundo tan egoísta como puede ser la investigación. A lo largo de estos años siempre has tenido palabras de ánimo cuando parecía que las cosas se torcían y has hecho que mi pasión por la investigación haya ido creciendo exponencialmente. Gracias por ser la capitana del GRMAR-Algae, por ser un referente para todos y por hacer lo imposible por ayudarnos. Jamás olvidaré los momentos que hemos compartido entre reuniones, campañas de muestreo, congresos y viajes post-congreso, y veladas de ocio. Tenerte cerca ha supuesto innumerables consejos y comentarios constructivos que me han ayudado a crecer tanto personalmente como profesionalmente. Gracias por ser y por hacernos unos inconformistas, por unirte a todas nuestras batallas y luchar a nuestro lado, y por empujarnos y animarnos a que nuestra imaginación e inquietudes vuelen alto y libre. Sin miedo a equivocarme puedo decir que has sido la mejor guía y maestra que podría haber tenido durante esta etapa; y espero que la vida nos mantenga cerca para continuar aprendiendo de ti y seguir compartiendo éxitos y alegrías.

Jana, que hubiese hecho yo sin ti! Es una suerte tenerte como amiga y compañera. Tú fuiste la persona que, literalmente, me abrió las puertas del CEAB y desde ese primer momento, te has convertido en alguien imprescindible en mi vida. Hemos sido uña y carne desde que nos conocimos, yendo de muestreo y de campañas juntos por "todo" el Mediterráneo, siendo mi apoyo en mi primer congreso y compartiendo alegrías y penas a lo largo del camino. Realmente creo que sin ti, esto hubiese sido infinitamente más difícil, menos ameno y más aburrido. De hecho, en este tiempo hemos conocido los mejores AirBnbs y restaurantes desde Canadá hasta Formentera y las mejores inmersiones desde Kotor hasta Sharm al Sheikh; y es que has sido una compañera de viajes inmejorable. Gracias por tu sinceridad, por contar conmigo para todo, por involucrarme en tus éxitos y en tus experimentos y por enseñarme a valorar y apreciar las "Cystos". Tú has sido la parte de locura y alegría de esta tesis, tú has sido mi sombra durante una gran parte de este camino, y ahora que estás más lejos te echaré mucho de menos. GRACIAS por todo compi.

Y como no hay dos sin tres, apareció Raül para mejorar y alborotar las cosas. Contigo puedo decir que he encontrado un hermano y un apoyo incondicional a lo largo de este viaje. Desde que llegaste has estado dispuesto a ayudar en todo y has sido una de las personas con la que más horas he pasado en los últimos 3 años. Incluso Marta y Tere se ponen celosas de vez en cuando… Gracias por toda la ayuda, por tener siempre una palabra de ánimo y por poner el punto de optimismo cuando parece que todo está oscuro. Tú has amenizado las innumerables horas de laboratorio, entre experimentos de termotolerancia y extracciones de ADN; y has sido un inmejorable maestro de genética. Y con la modelización… menos mal que llegaste para no dejarme solo, y es que nuestras conversaciones sin duda nos han permitido ir un paso más allá siempre. Y como no podía ser de otra manera, gracias por embarcarte en esta nueva aventura, por ser partícipe y cómplice de mis ideas locas, y por contribuir con tu locura. Esto no ha hecho más que empezar y es una suerte y un orgullo compartir este viaje contigo. GASSS.

Jana y Raül gracias por tanto. Podéis estar seguros de que seguiremos juntos por la "road-trip" de la vida.

Y el último fichaje junior del equipo, la persona que ha llegado para revolucionarnos a todos con sus experimentos. Cristina, gracias por ser un soplo de alegría y energía cada día y por transmitirnos a todos tu inocencia e inquietud. Cuando más necesitaba desconectar, apareciste para hacerme partícipe de tu campaña de campanas y revolucionaste mis días con los preparativos. Es un orgullo verte trabajar y tenerte como amiga, siempre dispuesta a escuchar y a ayudar, y en esta última etapa has sido un apoyo fundamental. Espero mantenerte cerca para verte crecer, para compartir tus éxitos y para seguir aprendiendo de ti.

Jordi: la última incorporación del grupo y la persona más optimista y sonriente que conozco! Desde que me conociste te interesaste por todo lo que estaba haciendo, y tu curiosidad y espíritu crítico han contribuido a que esta tesis sea mejor. Gracias por esas preguntas siempre oportunas, por el entusiasmo con el que afrontas todo y por aportar todo tu optimismo. Es un verdadero regalo que me hagas partícipe de tus éxitos capitaneando Hidden Desserts y es un orgullo que cuentes conmigo para las campañas de muestreo y me hagas sentir un poco "influencer". Ya sabes, puedes contar conmigo para lo que necesites.

Por último y no menos importantes, Alba y Sonia. Vosotras sois las otras "mamis" del grupo, las que aportan el equilibrio tan necesario para que todo funcione de manera balanceada. Gracias por la confianza que habéis depositado en mí desde el principio y por darme la oportunidad de participar como profesor en las prácticas de botánica, lo que sin duda ha sido una grandísima experiencia. Alba, gracias por todas las lecciones sobre microscopía y cómo hacer cortes para el microscopio, por tu sinceridad y tu punto de perfeccionismo que tanto me han ayudado a lo largo de este camino. Sonia, gracias por ser un apoyo constante, por afrontar todo con una sonrisa y por mostrarme la importancia de la positividad. Eres un ejemplo de constancia y detallismo.

Todos vosotros, componentes del GRMAR-Algae, os habéis convertido en una familia para mí y es una suerte teneros cerca para aprender de vosotros. Este grupo, demuestra que la unión hace la fuerza y es que juntos, ¡somos invencibles! Que orgullo ver hasta donde hemos llegado, los éxitos que hemos conseguido y lo que queda por llegar. Esta tesis es un reflejo del grupo tan maravilloso que hemos construido y todos vosotros la habéis hecho posible; así que sin lugar a dudas la podéis tomar como vuestra también.

Kike, gracias por tus consejos siempre acertados, por tus críticas constructivas y por ayudarme a ser más conciso escribiendo. Nunca olvidaré esa campaña de Cabrera. Gracias por ejercer de anfitrión, de maestro y de guía de buceo. María García, que suerte conocerte y compartir campañas contigo, siempre dispuesta a todo con tal de ayudar. Gracias a ti conozco el mejor curry de la Costa Brava y tú me inculcaste gran parte de mi amor hacia la *Caulerpa*. Echo de menos nuestros viajes en furgoneta, compartiendo confidencias y preocupaciones. Gracias por todo el cariño, la complicidad y por recibirme con los brazos abiertos. María Elena, tú fuiste mi anfitriona cuando aterricé aquí y me abriste las puertas de tu casa. Gracias por la ayuda, por las conversaciones de sofá, por ser la probadora oficial de mis primeros pinitos en la cocina y por todo el cariño con el que me trataste. ¡Sin ti hubiese estado perdido! Por supuesto, no puedo olvidarme de Ramón y Xavi, nuestro "Media Team" en la campaña de Formentera. Que bien lo pasamos y que suerte haber compartido esa aventura con vosotros. Y tú, Xavi, espero que algún día sepas agradecerme que te pusiese las orcas en bandeja… Gracias por tus clases de fotografía y tus grandísimas fotos, tus vaciles y piques, y tu alegría. ¡Que gran viaje compartimos por Patagonia!

En el inicio y en el final de esta tesis he pasado muchas horas en el CEAB, donde he tenido la suerte de coincidir con gente maravillosa que me ha acompañado y que me ha ayudado, de un modo u otro, a lo largo de este camino. Ibor, Jordi Pagés, Macpherson, Nuria, Rafel Coma, Simone, Teresa, Xavi Torras, Xavi Turón, Xevi y alguno más que seguramente me olvide… Gracias a todos por amenizar los cafés, por hacer que las comidas fuesen un punto de encuentro para las risas, y en ocasiones fuente de inspiración, y por todas esas actividades extra-laborales que han sido esenciales para desconectar. Vosotros hacéis del CEAB un sitio más humano, cálido y mejor. Rudi y Mirco, compañeros inseparables de fatigas en nuestras tardes de crossfit, noches de billar y dardos, y veladas de cine. Gracias por todo el cariño que me mostrasteis desde el principio. Una mención especial merecen los molonguis, ese grupo de locos y grandes amigos que tantos buenos momentos me han regalado. Desde los Alpes hasta Andorra, desde el Lapu-Lapu hasta Garrigolas, desde Barcelona hasta la vinya. Suerte la mía de haberos conocido y haberos tenido durante este viaje. Adri, Arnau, Buñuelito, Celia, Jan, Joan, María, Mario, Marta García, Mateu, Roger, Turón, Vicente, gracias por todo. Mateuet, gracias por salvarme en varias ocasiones, por actualizar la cámara de buceo, por ser mi compañero de batallas en Blanes, y por estar siempre dispuesto a ayudar. Que bueno que viniste al principio e hicimos piña, que bueno que sigues ahí para seguir sumando y catando. Elena, eso va para ti también. Turón, tú eres la energía del grupo y la que nos enreda a todos para hacer planes. Es una suerte haberte encontrado por el camino y contar con tu ayuda y apoyo. Tengo la sensación de que seguiremos cerca para continuar compartiendo aventuras, como la del Mar Rojo o mejores… Roger, gracias por haber sido un apoyo, por ser una fuente de risas y desconexión y por el cariño que siempre me muestras. El grupo no sería lo mismo sin tus despistes y tu espontaneidad. Buñuelito, contigo he compartido parte del proceso, y muchos días de risas y resacas. ¡Es una pena que ahora no coincidamos tanto y es un orgullo ver hasta donde has llegado! Por último, Miquel, tú has sido el gran descubrimiento de esta etapa. Aún no sé muy bien si actúas como padre o como hermano, lo que tengo claro es que te has convertido en alguien esencial para mí. Gracias por contar conmigo para todo, por estar ahí para escuchar y por transmitir esa positividad y alegría. Gracias a ti conozco los mejores sitios de bolets y los mejores restaurantes. Te has convertido en un compañero inseparable de este viaje.

Una parte importante de esta tesis se la debo a la UdG y a su gente, que me abrió las puertas desde el principio y me ha visto crecer a lo largo del camino. Y aunque es cierto que la mayor parte de las horas las he pasado entre el laboratorio y el despacho, en los ratos que he podido compartir con los doctorandos ha podido sentir una inmensa calidez y empatía. Jordi Bou, Alex, Carlos, David, Irene, Lauras, Lorena, Cesc, Julia, Joan Pere, Anna, Marías, Nuria, … Gracias a todos por amenizar las comidas y por ese viaje tan maravilloso por la Cerdanya. Suerte a todos en vuestros proyectos y mucho ánimo. Por supuesto, no puedo olvidarme de la otra parte del GRMAR: Marta Muñoz, Xènia y Marta. Es un orgullo compartir grupo y aprender de peces con vosotras, y estoy seguro de que conseguiréis lo que os propongáis. Alba Serrat, gracias por tu humanidad, por liderar el proyecto de cooperación en las Islas del Rosario y por ser una fuente constante de optimismo y energía. Espero conocer a Carlota pronto. Ander, gracias por tu vitalidad y por ese entusiasmo con el que inundaste Colombia. Por último, Silvia, gracias por estar siempre dispuesta a ayudar, por preocuparte de que no falte nada, por estar pendiente de que todo funcione correctamente y por facilitar la logística. Sin ti, hacer experimentos sería mucho más complicado.

No puedo olvidarme de los miembros del MedRecover, que han sido una fuente de inspiración y un espejo al que mirarse. Gracias Quim, David, Cristina, Paula, Pol, Alba, Dani; y al resto de miembros con los que he coincidido a lo largo de estos años. Compartir ideas, reuniones y retiros con vosotros ha sido una experiencia realmente enriquecedora. Y a ti Eneko, gracias por su mi último compañero de piso en

Blanes y por esas conversaciones de sofá tan constructivas. Estoy tranquilo porque sé que el Mediterráneo está en buenas manos con vosotros.

Esta tesis hubiese sido muy diferente sin el apoyo de la Fundación La Caixa. Gracias por confiar en mí y por darme esta oportunidad. Sobre todo quiero agradecerle al equipo humano la gran labor que hace, para hacernos sentir como en casa y solucionar todos los problemas de la manera más eficiente posible. Gracias Gisela, Elisabeth, Ana Julia y Emilia. Estos años de formación y experiencias los llevaré siempre grabados. Gracias también a todos los becarios, por los momentos de risas, preocupaciones y enseñanzas que hemos compartido. Y hablando de oportunidades, mi más sincero agradecimiento va para Manuel y Manu. Gracias por apostar por nuestro proyecto, por enseñarnos a desenvolvernos en un mundo que nos es tan ajeno y por vuestro apoyo e ilusión constante. Juntos sacaremos esto adelante, y lo más importante, nos divertiremos por el camino.

El paso de biólogo terrestre a biólogo marino lo di gracias al máster EMBC+, al que estaré eternamente agradecido por las grandes experiencias que me regaló, los grandes lecciones que me enseñó y las grandes amistades que me ha dejado. Gracias a todos por contribuir a que esos dos años fuesen maravillosos y asentasen en mi la pasión por el mar y el convencimiento de apostar por esta vía. Especialmente quiero agradecerles a mis tres hermanos su apoyo constante y que siempre estén ahí a pesar de la distancia. Yeray, Usa y Rodrigo, que grandes momentos nos han regalado Faro, Irlanda, Gante y Azores… pero que grandes momentos nos regalará la vida. Es una suerte teneros en la vida y cuánto os echo de menos! E de Açores apareceram Zé e Manel, que junto com Rodrigo formam minha família portuguesa. Faial não teria sido o mesmo sem vocês. Obrigado por sua hospitalidade, as risadas e o caminho percorrido juntos. 2030 nos espera em Calheta, Porto, Lisboa, o além dos mares!

Salamanca es la ciudad que me vio nacer, crecer y formarme como biólogo; y el lugar en el que he pasado la mayor parte de mi tiempo. De entre todas las personas que he conocido desde la infancia son tres los pilares que han estado ahí siempre y que me esperan con los brazos abiertos cuando el hijo pródigo vuelve a casa. Andrés, Plaza y Rodri gracias por ser los tres mejores amigos que podría tener, por entenderme tan bien como lo hacéis y por complementarme con vuestras virtudes. Es una suerte haber crecido junto a vosotros y haberos tenido a mi lado durante esta etapa ha sido una fuente de energía y apoyo esencial. Las noches eternas, las partidas interminables, las charlas sinceras y las escapadas improvisadas se han vuelto una parte fundamental de mi vida. Y en Salamanca también aparecieron los biólogos, Nacho, Jose, Sierro y Guille. Hacer la carrera junto a vosotros fue un regalo pero lo es aún más teneros cerca para compartir éxitos, locuras y viajes. Gracias por ser fuente de conocimiento e inspiración y por el apoyo durante estos últimos años. Ánimo Nacho y Sierro que estáis en la recta final.

Los que me conocen, saben que una parte fundamental de mi vida es el pueblo y de eso son en gran parte culpables, Jaime, Alba, Víctor y Marta. Que suerte teneros, que rabia que estéis tan lejos. Gracias por el cariño, las risas y las escapadas compartidas. Los cuatro os habéis convertido en una parte fundamental de mi vida. Víctor, gracias por amenizar las noches de pandemia, por haber estado al pie del cañón, pendiente de cómo iba todo y por siempre estar dispuesto a ayudar. Jaime, gracias por tu apoyo, por las charlas de ánimo, las risas cuando nos juntamos y sumar siempre con tu positivismo y perfeccionismo. Gracias por la portada tan maravillosa que has hecho y por tu paciencia infinita durante el proceso. Te debo una amigo.

I cannot forget my Californian family. Thanks Wendy, Hans, Melinda and Brad. Even being miles away from me, you have always been encouraging and supporting, and I have always felt you close. I am sure that we will see each other very soon.

Adriana y Albert, aunque habéis llegado de rebote, conoceros y teneros cerca durante estos últimos años ha sido una gran suerte y un gran apoyo. Gracias por aparecer.

Y ha llegado el momento de esa persona que ha estado a mi lado cada día, durante estos últimos años. Tere, que suerte que viniste, que suerte que te quedaste, que suerte tenerte! Tú has sido mi mayor regalo, el apoyo incondicional, la mano de la que aferrarme y el refugio al que volver. Gracias por dar rienda suelta a mis sueños, por acompañarme en todo momento y por seguirme hasta el fin del mundo. Sin ti nada de esto hubiese sido posible, porque tu me empujas a creer, tú me invitas a soñar y me permites volar. Gracias por aguantar mis días malos, por escucharme siempre y por transmitirme paz y serenidad. A ti, que tanto me has sufrido en los días de decepciones y fracasos; y tú que tanto has celebrado mis éxitos y alegrías, te debo una gran parte de todo en lo que me he convertido. Gracias de corazón, por que sin ti y sin tu cariño sería la mitad de lo que soy. Gran parte de esta tesis es más tuya que mía.

Y con Tere vinieron los Rodríguez… mi segunda familia, una parte fundamental de mi vida. Trini y Flore gracias por adoptarme como un hijo y por recibirme siempre con los brazos abiertos, por preocuparos por mi y por estar siempre dispuestos a todo. Lourdes, Miguel y Aarón, os habéis convertido en unos hermanos para mí. Gracias por el cariño, por las risas contagiosas y por nuestras escapadas que se han convertido en un refugio para desconectar. Hablar con vosotros es un soplo de energía en los días de bajón y teneros en mi vida es una suerte. Ojalá poder vernos más.

Y esta parte de la tesis no podía cerrarla de otra forma que no fuese agradeciendo a mi familia el apoyo constante, el inmenso cariño y las facilidades que me han dado para todo. Mamá y papá, gracias por la vida que me habéis dado, por haberme acompañado en cada paso y por empujarme a seguir siempre. Sois mi mayor fuente de inspiración y a vosotros os debo mi pasión por la naturaleza y mi amor por la biología. Vosotros me habéis enseñado a ser quien soy, me habéis inculcado la filosofía del esfuerzo y me habéis mostrado que nunca hay que dejar de creer en uno mismo. Gracias por apoyarme en todo momento y vivir como vuestra esta aventura que me llevó tan lejos de vosotros. Gracias por estar siempre ahí para escuchar, ayudar y sumar, sin vosotros nada de esto hubiese sido posible. Fina, gracias por ser como mi segunda madre, por tus consejos siempre apropiados, por tu apoyo constante y por el cariño infinito. Eres la mejor tía que podría tener. A mis otros tíos: Luisa, Santi, Javier, Pauli y César. Gracias por vuestros consejos y por la paciencia para escuchar y para enseñar. Vosotros me habéis enseñado a ser mejor persona y el valor de la familia. A mis primos: Jesús, Carlos y Luis. Gracias por haber estado siempre ahí, por vuestro inmenso cariño y por ser maestros en diferentes aspectos de la vida. Y por último a mis abuelos, Isabel y Santiago, Paulino y Josefina; 4 pilares que se han ido demasiado pronto y han dejado un hueco demasiado grande. Os echo mucho de menos y a veces caminar sin vuestro apoyo es muy duro. A vosotros os dedico esta tesis, especialmente a Paulino y Josefina, que vivieron conmigo el inicio de esta etapa pero que no la han podido ver terminar. Cuanto me gustaría compartir el resultado final con vosotros para haceros sentir orgullosos de lo que he conseguido con vuestro apoyo. Os estoy eternamente agradecido por vuestro amor incondicional, por enseñarme a creer en mí mismo y por empujarme a perseguir mis sueños. Gracias por todo lo que me habéis dado, gracias por todo lo que me habéis enseñado, gracias por la persona en la que me habéis convertido.

Pineda de Mar, 23 de junio de 2021

La Dra. Emma Cebrian Pujol, investigadora del Centre d´Estudis Avançats de Blanes (CEAB-CSIC),

DECLARO:

Que el treball titulat "**Unraveling the success of invaders: Biotic and abiotic factors determining the invasibility of Mediterranean benthic assemblages by *Caulerpa cylindracea***", que presenta el Sr. Jorge Santamaría Pérez per a l'obtenció del títol de doctor, ha estat realitzat sota la meva direcció.

I, perquè així consti i tingui els efectes oportuns, signo aquest document.

Dra. Emma Cebrian Pujol

Blanes, 25 de Juny del 2021.

Universitat
de Girona

La Dra. Emma Cebrian Pujol, investigadora del Centre d´Estudis Avançats de Blanes (CEAB-CSIC), directora i tutora de la Tesi Doctoral titulada "**Unraveling the success of invaders: Biotic and abiotic factors determining the invasibility of Mediterranean benthic assemblages by *Caulerpa cylindracea***"

INFORMO:

Que els treballs de recerca duts a terme pel Sr. Jorge Santamaría Pérez com a part de la seva tesi doctoral se han organitzat en quatre capítols principals, que es corresponen amb els quatre articles científics llistats en la següent secció. De aquests articles, un ja està publicat, un altre està acceptat, un està enviat a la revista i un altre està en preparació per ser enviat a una revista d'àmbit internacional; per tant, aquest treball és idoni per ser presentat com a compendi de publicacions per a l'obtenció del títol de doctor.

A més, CERTIFICO que el doctorant ha participat activament en el desenvolupament del treball de recerca associat a cadascun d'aquests articles, així con en la seva elaboració. En concret, la seva participació en cadascun dels articles ha estat la següent:

- Plantejament de les hipòtesis i objectius.
- Planificació i execució dels experiments, tant pel que fa a la feina de camp com al laboratori., participant a més en els seguiments biològics i censos.
- Processat i anàlisi de les dades obtingudes.
- Redacció dels articles i seguiment del procés de revisió dels mateixos.

Per tant, la seva rellevant contribució a cada una de les publicacions queda demostrada; i perquè així consti i tingui els efectes oportuns, signo aquest document.

Directora i tutora de la tesi doctoral
Dra. Emma Cebrian Pujol

Blanes, 25 de Juny de 2021.

List of publications derived from this thesis

This PhD thesis is presented following the format of a compendium of publications. It includes four publications: one already published, one accepted for publication, one submitted and the other one in preparation.

The list of manuscripts is here presented; organized by chapters and indicating the publication stage, impact factor (IF) and quartile (Q). A copy of the published and accepted manuscripts, following the format of the journal, can be found at the end of the thesis, in the last appendix.

CHAPTER 2: Jorge Santamaría[1], Fiona Tomas[2], Enric Ballesteros[3], Juan M. Ruiz[4], Jaime Bernardeau-Esteller[4], Jorge Terrados[2], Emma Cebrian[1,3]. (2021). The role of competition and herbivory in biotic resistance against invaders: A synergistic effect. Accepted in *Ecology*. IF (2019): 4.7, Q: Q1 (Ecology).

CHAPTER 3: Learning takes time. Biotic resistance by native herbivores increases through the invasion process. In preparation.

CHAPTER 4: Jorge Santamaría[1], Fiona Tomas[2], Enric Ballesteros[3], Emma Cebrian[1,3]. Herbivory on the invasive alga *Caulerpa cylindracea*: The role of omnivorous fishes. Submitted to *Frontiers in Marine Science*. IF (2019): 3.661, Q: Q1 (Aquatic Science).

CHAPTER 5: Jorge Santamaría[1], Raül Golo[1], Emma Cebrian[1,3], María García[3], Alba Vergés[1]. (2021). Stressful conditions give rise to a novel and cryptic filamentous form of *Caulerpa cylindracea*. Published in *Frontiers in Marine Science* 8:548679. doi: 10.3389/fmars.2021.548679. IF (2019): 3.661, Q: Q1 (Aquatic Science).

Authors affiliations:

[1] Grup de Recerca en Biodiversitat i Recursos Marins (GRMAR), Institut d´Ecologia Aquàtica, Facultat de Ciències, Universitat de Girona, Girona, Spain.

[2] Department of Marine Ecology and Resources – Instituto Mediterráneo de Estudios Avanzados (IMEDEA), Universitat de les Illes Balears (UIB) – Consejo Superior de Investigaciones Científicas (CSIC), Esporles, Balearic Islands, Spain.

[3] Centre d´Estudis Avançats de Blanes, Consejo Superior de Investigaciones Científicas (CEAB-CSIC), Blanes, Spain.

[4] Seagrass Ecology Group – Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), San Pedro del Pinatar, Murcia, Spain.

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List of Abbreviations

3-D: Three-dimensional
ANOSIM: Analysis of Similarities
bp: base pair
C: degree Celsius
cm: centimeters
Df: Degrees of freedom
DNA: Deoxyribonucleic Acid
dNTP: Deoxyribose Nucleotide Triphosphate
E: East
E: Ivlev´s Electivity Index
e.g.: *exempli gratia* (“for example”)
FO: Frequency of Occurrence
g: grams
GII: Geometric Index of Importance
GLM: Generalized Linear Model
GSHHG: Global-Self-consistent, Hierarchical, High-Resolution Geography Database
h: hours
i.e.: *id est* (“in other words”)
IAN: Integration & Application Network
IUCN: International Union for Conservation of Nature
IPBES: Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services
km: kilometers
K-W: Kruskall-Wallis
L: liters
L:D: Light:Dark
LM: Linear Model
LMM: Linear Mixed Effect Model
m: meters

MHW: Marine Heatwave

ml: milliliters

mM: millimolar

MPA: Marine Protected Area

n: sample size

N: North

NJ: Neighbor Joining

NMDS: Non-metric Multi-Dimensional Scaling

NW: North-Western

OISST: Reynolds Optimally Interpolated Sea Surface Temperature

PCR: Polymerase Chain Reaction

Q: Combined Index

RMPQ: Relative Measures of Prey Quantity

S.E.: Standard Error of the mean

SIMPER: Similarity Percentage Analysis

SST: Sea Surface Temperature

vs.: *versus* ("against")

χ^2: Chi-square

Abstract

Biological invasions are currently considered one of the main drivers of anthropogenic change and have become one of the most pervasive threats to native ecosystems worldwide, causing species extinctions, disrupting the functioning of ecosystems, altering the provision of critical ecosystem services and causing great economic costs. In the future, due to the intensification in trade and transport and to climate change, the number of invasions is expected to continue increasing, something that inevitably will go associated with greater impacts to native ecosystems. Therefore, finding effective ways for the management of invasive species has become a global priority. Unfortunately, once invasive species establish in the new habitat, their control and eradication becomes really difficult, especially in marine systems, where most management interventions have proven to be unsuccessful due to the open nature of these systems. However, during the invasive process, several mechanisms and characteristics from the receiving habitat can influence the success of non-native species, causing many invasions to fail and limiting the abundance and the negative impacts of already established invaders. Thus, there is an increasing interest in understanding how these mechanisms influence the success of invaders in order to find successful management interventions that could foster the resilience of native habitats towards invaders. Surprisingly, this knowledge is still quite limited, particularly in marine systems, where invasions have been largely understudied. Therefore, given this lack of knowledge and considering that invasions will further increase in number and impacts in the coming future, there is an urgent need for studies that shed light on the mechanisms influencing the success and dynamics of well established invaders to find the ways of hampering, or at least lessening the impacts of present and future invasions.

In this thesis, given that marine algae are one of the most conspicuous and successful invaders, we aim to unravel some of the mechanisms influencing the success and the invasiveness of algae species by using *Caulerpa cylindracea*, one of the most successful and harmful marine invaders worldwide, as a model organism. Specifically, we intend: i) to determine whether biotic mechanisms can limit the success of the invader and influence its long-term dynamics, and ii) to assess the role of abiotic factors and disturbance events on the invasiveness of the species, something particularly relevant considering the current scenario of global change.

In the first three chapters of this thesis (**Chapters 2, 3 and 4**), we assess the influence of biotic mechanisms on the success of the invader by using a combination of long-term monitoring, chronosequences, *in-situ* manipulative experiments and field sampling. In **Chapter 2**, our findings from the benthic assemblage sampling show that the dominance of canopy-forming and erect algae species limits the abundance of the invader through competition mechanisms, while results from the *in situ* exclusion experiment show that herbivory by *Sarpa salpa* further limits the abundance of the invader in shallow habitats, where herbivory pressure is high. Therefore, as also confirmed by the long-term monitoring of the invader at the study site, the interaction between both biotic mechanisms influences the success of the invader at the local scale, with the abundance-depth distribution of the invader being shaped by the strength of the biotic mechanisms here assessed. Consequently, there are the habitats where the intensity of competition and herbivory is highest, the ones that sustain the lowest abundances of *C. cylindracea*. Moreover, by following the dynamics of *C. cylindracea* at the study site, we observed a decline in the abundance of the invader mainly in shallow habitats, thus we hypothesized that it could be due to an increase in the intensity of herbivory through time. Therefore, in **Chapter 3** we followed a chronosequence methodology to assess whether the predator-prey interaction between *S. salpa* and *C. cylindracea* develops from the beginning of the invasion or whether it needs certain time to flourish. Remarkably, our findings revealed that *S.*

salpa needs time to start targeting the invader and to become an effective biotic resistance mechanism, thus confirming our previous hypothesis that herbivory increases its intensity through time. Particularly, an exposure time of at least 6 years was necessary for *S. salpa* to include *C. cylindracea* as a preferred food item, while the highest *per capita* consumption rates and the greatest proportions of herbivores feeding on *C. cylindracea* were found in places historically invaded compared to recently invaded locations. Interestingly, the electivity of *S. salpa* for the invader increased constantly through the invasion, even when the abundance of the invader had decreased, highlighting herbivory as an effective long-term biotic resistance mechanism that is maintained throughout the invasion. In previous chapters, herbivory was assessed in the herbivorous fish *S. salpa*, however, in the Mediterranean Sea other fish species have been occasionally observed feeding on *C. cylindracea*. Therefore, in **Chapter 4** we assessed whether four of the commonest fish species in the Western Mediterranean Sea can feed on the invader and to what extent they elect to feed on it. Stomach content analysis revealed that three of the four assessed species: *Diplodus annularis*, *Diplodus vulgaris* and *Spondyliosoma cantharus*, were feeding on *C. cylindracea*, although, being these species omnivorous, the importance of the invader for the diet was generally low, except for *D. annularis*. Actually, the low values in the electivity index suggest that all the species are avoiding to feed on the invader. However, despite this avoidance, high amounts of *C. cylindracea* were found in several specimens, which together with the numerical importance of these species in the Mediterranean rocky habitats, might suggest that the consumption of *C. cylindracea* by omnivorous fishes can complement the stronger impact of the herbivory by *S. salpa*, and contribute to limit the abundance of the invader in shallow habitats.

In the last chapter of this thesis (**Chapter 5**), we performed an *ex situ* thermotolerance experiment to assess whether acute disturbance events in the form of a marine heatwave (MHW) can affect the performance of the invader and to understand the influence of extreme temperatures in the plasticity of *C. cylindracea*.

We found that long exposures to high temperatures reduced the survival of the species, however, once the harsh conditions disappeared, *C. cylindracea* showed an outstanding capacity to recover. Actually, the exposure to stressful conditions triggered the morphological plasticity of the invader and gave rise to a novel filamentous morphology that had been previously observed in the field after a disturbance event. Therefore, these findings highlight the tolerance of the invader to disturbance and its remarkable capacity to adapt to environmental change by developing a morphology that improves the persistence, resistance and dispersion of the invader. Interestingly, the novel morphology also transforms the invader into a cryptic species, which compromises easy identification, thus affecting the management of the species.

Overall, this thesis provides an integrated view of some of the mechanisms and factors influencing the success and the long-term dynamics of *C. cylindracea* in the Mediterranean Sea. In this sense, we provide evidence on the role that biotic resistance mechanisms have on the history of the invasion, demonstrating that in well-preserved habitats, where populations of fishes are healthy, the habitat itself has the mechanisms to limit the success of the invader. Also, we demonstrate that *C. cylindracea* has the ability to withstand acute disturbance events and to suffer drastic morphological changes that compromise its identification. Therefore, to lessen the impacts of invaders, a successful management intervention will be to focus efforts on the conservation of native ecosystems, in order to promote the natural resistance and resilience of the system, while complementary improving the early detection of invaders by implementing monitoring tools that take into account the potential plasticity of invasive species. Particularly, given that global change will make things easier for the establishment of invasive species, promoting the maintenance of the mechanisms that limit invader success will be increasingly necessary to hinder the impacts of present and future invaders.

Resum

Les invasions biològiques són una de les causes principals del canvi antropogènic actual i representen un dels impactes més generalitzats als ecosistemes d'arreu del món, ja que comporten l'extinció d'espècies, pertorben el funcionament dels ecosistemes, alteren importants serveis ecosistèmics i provoquen grans pèrdues econòmiques. A més, a causa de l'augment del comerç, el transport i el canvi climàtic, en un futur s'espera que el nombre d'invasions segueixi creixent, fet que inevitablement va associat a un increment dels impactes als ecosistemes natius. Per aquests motius, trobar eines efectives per la gestió d'espècies invasores ha esdevingut una de les principals prioritats a nivell global. Malauradament, quan una espècie invasora s'estableix en un nou hàbitat, el seu control i eradicació esdevé complex, particularment en ecosistemes marins, on la majoria d'intents de gestió no han prosperat principalment a causa de la naturalesa oberta d'aquests sistemes. No obstant això, durant el procés d'invasió, l'èxit de les espècies no natives ve determinat per diversos mecanismes i característiques de l'hàbitat receptor, provocant que moltes invasions fracassin i podent limitar l'abundància i els impactes negatius de les espècies invasores ja establertes. Per aquest motiu, cal entendre com aquests mecanismes determinen l'èxit dels invasors, per tal de dissenyar eines de gestió que fomentin la resiliència dels hàbitats natius enfront les espècies invasores. Així doncs, tenint en compte la sorprenent manca de coneixement i donat que les invasions seguiran augmentant en nombre i impactes en el futur, apareix una necessitat urgent de generar estudis que aportin llum sobre els mecanismes que afecten l'èxit i la dinàmica de les espècies invasores ja establertes, amb l'objectiu de trobar formes per eliminar, o mitigar, els impactes de les invasions.

Les algues marines representen un dels invasors més exitosos, és per això que l'objectiu d'aquesta tesi consisteix en descobrir alguns dels mecanismes que

influeixen en l'èxit i la capacitat d'invasió d'aquests organismes. Per això, utilitzem *Caulerpa cylindracea* com a organisme model, ja que representa una de les espècies invasores marines actualment més estesa i perjudicials arreu del món. Concretament, pretenem: i) determinar si els mecanismes biòtics poden limitar l'èxit de l'espècie invasora i la seva dinàmica a llarg termini, i ii) avaluar com influeixen els factors abiòtics i les pertorbacions en la capacitat d'invasió de l'espècie, fet particularment rellevant tenint en compte l'actual escenari de canvi global.

En els primers tres capítols d'aquesta tesi (capítols 2, 3 i 4), hem avaluat la importància dels factors biòtics en l'èxit invasor utilitzant seguiments a llarg termini, crono-seqüencies, experiments manipulatius *in situ* i mostrejos de camp. Concretament, en el **Capítol 2**, demostrem que la dominància d'algues arborescents i d'algues erectes limita l'abundància de l'espècie invasora mitjançant mecanismes de competència interespecífica, i que l'herviborisme per part del peix *Sarpa salpa* redueix encara més l'abundància de l'invasor en hàbitats superficials, on la pressió herbívora és elevada. Així doncs, l'èxit de l'invasor a escala local està influenciat per la interacció entre ambdós mecanismes biòtics. Aquest fet, ha estat corroborat pel seguiment a llarg termini, demostrant que la distribució d'abundàncies de l'invasor al llarg de la franja batimètrica està modulada per la força d'aquests mecanismes biòtics. D'aquesta manera, els hàbitats que tenen una major intensitat de competència i herviborisme, són aquells que presenten les abundàncies més baixes de *C. cylindracea*. A més, a partir del seguiment a llarg termini de *C. cylindracea* vam observar una disminució en l'abundància de l'invasor, majoritàriament als hàbitats poc profunds, de manera que hipotetitzem que aquest fet podria ser degut a un increment en la intensitat de l'herviborisme al llarg de el temps. Així doncs, en el **Capítol 3** utilitzem mètodes de crono-seqüencia per avaluar si la interacció predador-presa entre *S. salpa* i *C. cylindracea* sorgeix des de l'inici de la invasió o si, contràriament, requereix cert temps per desenvolupar-se. Els nostres resultats demostren l'augment de la intensitat de l'herviborisme al llarg del temps, ja que *S. salpa* necessita cert temps per començar a consumir l'espècie invasora i així esdevenir un mecanisme de resistència biòtica efectiu. Concretament, *S. salpa* va necessitar uns 6 anys d'exposició a l'espècie invasora per incloure-la com a preferent a la dieta. De la mateixa manera, a localitats envaïdes des de fa molt de temps es van trobar els majors consums per càpita i les majors proporcions d'herbívors alimentant-se de l'invasor en comparació dels llocs envaïts recentment. De manera sorprenent, la selectivitat de *S. salpa* cap a l'invasor augmenta constantment al llarg del procés d'invasió, fins i tot quan l'abundància d'aquest disminueix. Aquest fet demostra que l'herbivorisme pot actuar com un mecanisme efectiu de resistència biòtica a llarg termini i que es manté durant la invasió. En els capítols anteriors, s'havia avaluat únicament l'efecte del peix herbívor *S. salpa*, però de manera ocasional a la Mediterrània s'ha observat que altres espècies de peixos també s'alimenten de *C. cylindracea*. En aquest context, en el **Capítol 4** investiguem si quatre dels peixos més comuns del Mediterràni occidental s'alimenten de l'invasor i determinem la seva preferència cap a *C. cylindracea*. L'anàlisi dels continguts estomacals va mostrar que tres espècies: *Diplodus annularis, Diplodus vulgaris* i *Spondyliosoma cantharus,* s'alimenten usualment de *C. cylindracea*, tot i que generalment la importància de l'alga invasora en la seva dieta va ser baixa, excepte per a *D. annularis*, ja que són espècies omnívores. De fet, els baixos valors obtinguts en l'índex de selectivitat suggereixen que aquestes espècies eviten consumir l'invasor i que el consum és accidental. No obstant, força individus van consumir grans quantitats de *C. cylindracea*, el que, juntament amb la importància numèrica d'aquestes espècies en els hàbitats rocosos de la Mar Mediterrània, suggereix que el consum de *C. cylindracea* per part dels peixos omnívors pot complementar el major impacte exercit per l'herbivoria de *S. salpa* i d'aquesta manera contribuir a limitar l'abundància de l'invasor en els hàbitats poc profunds.

A l'últim capítol d'aquesta tesi (**Capítol 5**), vam realitzar un experiment de termotolerància *ex situ* per i) determinar si els episodis de pertorbacions agudes, en aquest cas un episodi de temperatures extremes, afecten al rendiment de l'invasor, així com ii) avaluar la seva influència en la plasticitat de *C. cylindracea*. Períodes llargs d’exposició a elevades temperatures afecten a la supervivència de l'espècie, però, un cop es van revertir les condicions, *C. cylindracea* va mostrar una capacitat extraordinària per recuperar-se. De fet, sota l'exposició a les condicions d'estrès, l’invasor va desenvolupar una nova morfologia filamentosa que havia estat prèviament observada al camp després d'un episodi de pertorbació. Així doncs, aquests resultats demostren la tolerància de l'espècie invasora a les pertorbacions i la seva capacitat per adaptar-se a canvis ambientals mitjançant el desenvolupament d'una morfologia que millora la persistència, la resistència i la dispersió de l'espècie. Curiosament, aquesta nova morfologia també transforma l'invasor en una espècie críptica, fet que afecta a la gestió de l'espècie ja que dificulta una ràpida identificació al camp.

En resum, aquesta tesi proporciona una visió integrada d'alguns dels mecanismes i factors que influeixen en l'èxit i la dinàmica a llarg termini de *C. cylindracea* a la Mediterrània. D'aquesta manera, mostrem el paper que tenen els mecanismes de resistència biòtica sobre la història de la invasió, demostrant que en els hàbitats ben preservats, on les poblacions de peixos es mantenen sanes i equilibrades, l'hàbitat per si mateix presenta mecanismes per limitar l'èxit de l'invasor. A més, demostrem que *C. cylindracea* té capacitat per resistir pertorbacions agudes i que pot desenvolupar canvis morfològics dràstics que dificulten la seva identificació. Per aquests motius, per tal de reduir els impactes d'espècies invasores, proposem com a possible acció de gestió efectiva, garantir la conservació dels ecosistemes natius per fomentar la resistència i resiliència natural del sistema. Complementàriament, també proposem la implementació d’eines de seguiment que considerin la plasticitat morfològica dels invasors, amb l'objectiu de millorar la detecció precoç d'aquestes espècies. Concretament, tenint en compte que el canvi climàtic afavorirà l'establiment d’espècies invasores, assegurar el funcionament dels mecanismes que limiten l'èxit dels invasors serà cada vegada més necessari per limitar els impactes de les invasions presents i futures.

Resumen

Las invasiones biológicas son uno de los principales causantes del cambio antropogénico actual y se han convertido en uno de los impactos más generalizados para los ecosistemas de todo el mundo, ya que provocan la extinción de especies, perturban el funcionamiento de los ecosistemas, alteran el aprovisionamiento de importantes servicios ecosistémicos y causan grandes pérdidas económicas. Además, debido al incremento del comercio y del transporte, y al cambio climático, se espera que en el futuro el número de invasiones siga creciendo, algo que inevitablemente irá asociado con un incremento en los impactos para los ecosistemas nativos. Por estos motivos, encontrar maneras efectivas para manejar y gestionar a las especies invasoras se ha convertido en una de las prioridades globales. Desgraciadamente, cuando una especie invasora se establece en el nuevo hábitat, su control y erradicación es muy complicada, sobre todo en los ecosistemas marinos, donde la mayoría de los intentos de manejo han fracasado debido principalmente a la naturaleza abierta de estos sistemas. Sin embargo, durante el proceso invasivo, el éxito de las especies no nativas está influenciado por diversos mecanismos y características del hábitat receptor, que provocan que muchas invasiones fracasen y que pueden limitar la abundancia y los impactos nocivos de las especies invasoras ya establecidas. Por este motivo, existe un interés cada vez mayor en comprender como estos mecanismos influyen en el éxito de los invasores, con el fin de utilizar dicho conocimiento para encontrar opciones de gestión que fomenten la resiliencia de los hábitats nativos frente a las especies invasoras. Sorprendentemente, este conocimiento aún es muy limitado. Así pues, teniendo en cuenta esta falta de conocimiento y que las invasiones seguirán aumentando en número e impactos en el futuro, existe una necesidad urgente de estudios que aporten algo de luz sobre los mecanismos que afectan al éxito y a la dinámica de las especies invasoras bien establecidas, con el objetivo de encontrar formas para eliminar, o al menos reducir, los impactos de las invasiones.

En esta tesis, debido a que las algas marinas son unos de los invasores más exitosos y omnipresentes, nuestro objetivo consiste en descubrir algunos de los mecanismos que influyen en el éxito y la invasividad de estos organismos. Para ello, utilizamos *Caulerpa cylindracea* como organismo modelo, ya que es uno de las invasores marinos más extendidos y nocivos en todo el mundo. Concretamente, pretendemos: i) determinar si los mecanismos bióticos pueden limitar el éxito de la especie invasora e influenciar su dinámica a largo-plazo, y ii) evaluar como influyen los factores abióticos y las perturbaciones en la invasividad de la especie, algo que es particularmente relevante teniendo en cuenta el escenario actual de cambio global.

En los primeros tres capítulos de esta tesis (**Capítulos 2, 3 y 4**), hemos evaluado la influencia de los factores bióticos en el éxito invasor utilizando monitoreos a largo plazo, crono-secuencias, experimentos manipulativos *in situ* y muestreos de campo. En concreto, en el **Capítulo 2**, demostramos que la dominancia de algas formadoras de dosel y de algas erectas limita la abundancia de la especie invasora mediante mecanismos de competencia interespecífica, y que la herbivoría por el pez *Sarpa salpa* reduce aún más la abundancia del invasor en los hábitats someros, donde la presión de herbivoría es elevada. Así pues, el éxito del invasor a escala local está influenciado por la interacción entre los dos mecanismos bióticos. Esto ha sido además corroborado por el monitoreo a largo plazo, ya que la distribución de abundancias del invasor a lo largo del rango batimétrico está modulada por la fuerza de dichos mecanismos bióticos. De este modo, los hábitats que tienen una mayor intensidad de competencia y herbivoría, son aquellos que soportan las abundancias más bajas de *C. cylindracea*. Además, siguiendo la dinámica a largo plazo de *C. cylindracea* observamos una disminución en la abundancia del invasor, principalmente en los hábitats poco profundos, por lo que hipotetizamos que ésta podría deberse a un incremento en la intensidad de la herbivoría a lo largo del tiempo. Por lo tanto, en el **Capítulo 3** utilizamos una metodología de crono-secuencia para evaluar si la interacción predador-presa entre *S. salpa* y *C. cylindracea* surge desde el inicio de la invasión o si por el contrario, necesita tiempo para desarrollarse. Nuestros resultados demuestran nuestra hipótesis del aumento de la intensidad de herbivoría a lo largo del tiempo, ya que *S. salpa* necesita tiempo para empezar a consumir la especie invasora y convertirse en un mecanismo de resistencia biótica efectivo. Concretamente, *S. salpa* necesitó una exposición de al menos 6 años con la especie invasora para incluirla como una comida preferente, del mismo modo que en las localidades invadidas hace mucho tiempo se encontraron los mayores consumos *per capita* y las mayores proporciones de herbívoros alimentándose del invasor en comparación con los lugares invadidos recientemente. De forma sorprendente, la electividad de *S. salpa* hacia el invasor aumenta constantemente a lo largo de la invasión, incluso cuando la abundancia del invasor disminuye, lo que señala a la herbivoría como un mecanismo de resistencia biótica efectivo a largo plazo y que se mantiene durante la invasión. En los capítulos anteriores, se había evaluado únicamente el efecto de la herbivoría del pez herbívoro *S. salpa*, sin embargo en el Mar Mediterráneo se ha observado de forma ocasional, como otras especies de peces se alimentan de *C. cylindracea*. Por este motivo, en el **Capítulo 4** investigamos si cuatro de los peces más comunes en el Mar Mediterráneo occidental se alimentan del invasor y determinamos su electividad hacia *C. cylindracea*. El análisis de los contenidos estomacales reveló que tres especies: *Diplodus annularis*, *Diplodus vulgaris* y *Spondyliosoma cantharus*, se alimentan comúnmente de *C. cylindracea*, aunque en general la importancia del alga invasora en la dieta fue baja excepto para *D. annularis*, ya que son especies omnívoras. De hecho, los bajos valores obtenidos en el índice de electividad sugieren que estas especies evitan consumir al invasor y que el consumo es accidental. Sin embargo, bastantes individuos habían consumido grandes cantidades de *C. cylindracea*, lo que junto con la importancia numérica de estas especies en los hábitats rocosos del Mar Mediterráneo, sugiere que el consumo de *C. cylindracea* por parte de los peces omnívoros puede complementar el mayor impacto ejercido por la herbivoría de *S. salpa* y de este modo contribuir a limitar la abundancia del invasor en los hábitats poco profundos.

En el último capítulo de esta tesis (**Capítulo 5**), realizamos un experimento de termotolerancia *ex situ* para i) determinar si los eventos de perturbaciones agudos, en este caso en forma de ola de calor, afectan el rendimiento del invasor y ii) para evaluar su influencia en la plasticidad de *C. cylindracea*. Exposiciones largas a temperaturas elevadas afectan a la supervivencia de la especie, sin embargo, cuando las condiciones adversas remitieron, *C. cylindracea* demostró una capacidad extraordinaria para recuperarse. De hecho, la exposición a las condiciones de estrés propició la plasticidad morfológica del invasor y dio lugar a una nueva morfología filamentosa que había sido observada en el campo tras un evento de perturbación. Así pues, estos resultados demuestran la tolerancia de la especie invasora a las perturbaciones y su capacidad para adaptarse al cambio ambiental mediante el desarrollo de una morfología que mejora la persistencia, la resistencia y la dispersión de la especie. De forma interesante, esta nueva morfología también transforma al invasor en una especie críptica, lo que afecta a la gestión de la especie ya que compromete su rápida identificación en el campo.

En conjunto, esta tesis proporciona una visión integrada de algunos de los mecanismos y factores que influyen en el éxito y en la dinámica a largo plazo de *C. cylindracea* en el Mar Mediterráneo. De este modo, evidenciamos el papel que tienen los mecanismos de resistencia biótica sobre la historia de la invasión, demostrando que en los hábitats bien preservados, donde las poblaciones de peces estén sanas y bien equilibradas, el hábitat por si mismo posee mecanismos para limitar el éxito del invasor. Además, demostramos que *C. cylindracea* tiene la capacidad para resistir a las perturbaciones agudas y que puede sufrir cambios morfológicos drásticos que comprometen su identificación. Por estos motivos, podemos proponer que una acción de gestión efectiva para reducir los impactos de las especies invasoras consiste en garantizar la conservación de los ecosistemas nativos para fomentar la resistencia y resiliencia natural del sistema, mientras que de manera complementaria se implementan herramientas de monitoreo que tengan en cuenta la plasticidad morfológica de los invasores, con el objetivo de mejorar la detección temprana de dichas especies. En concreto, ya que el cambio climático favorecerá el establecimiento de las especies invasoras, promover el mantenimiento de los mecanismos que limitan el éxito de los invasores será cada vez más necesario para limitar los impactos de las invasiones presentes y futuras.

Chapter 1

General Introduction

Cover image: Detail of a *Caulerpa cylindracea* thallus as observed under the stereomicroscope.

Author: Jorge Santamaría Pérez.

This thesis is planned to improve our knowledge on the factors that influence the invasive success of species by focusing on the study of a successful marine invader. This knowledge is particularly relevant considering that invasions constitute a growing threat to native ecosystems worldwide and they need to be properly managed to lessen their negative impacts. However, when knowledge on the invasive process is scarce, it makes it difficult to find the appropriate management actions to prevent future invasions or at least to control the current ones.

Previous efforts have mainly considered the use of direct and active methods of eradication, but what if the answer relies in the ability of native communities to develop natural resistance mechanisms against invaders? To find out whether natural mechanisms can contribute to the control of invasions, we definitively need to expand our understanding of the factors that influence the long-term dynamics of invaders.

> "*They [biological invasions] are so frequent nowadays in every continent and island, and even in the oceans, that we need to understand what is causing them and try to arrive at some viewpoint about the whole business.*"
>
> "*Nowadays we live in a very explosive world, and while we may not know where and when the next outburst will be, we might hope to find ways of stopping it or at any rate damping down its force*"
>
> Charles S. Elton (1958)

1.1 Invasive species

> *"It is not just nuclear bombs and wars that threaten us, though these rank very high on the list at the moment: there are other sorts of explosions, and this book is about ecological explosions. An ecological explosion means the enormous increase in numbers of some kind of living organism – it may be an infectious virus like influenza (or coronavirus), or a bacterium like bubonic plague, or a fungus like that of the potato disease, a green plant like the prickly pear, or an animal like the gray squirrel. I use the word explosion deliberately because it means the bursting out from control of forces that were previously held in restraint by other forces."*
>
> Charles S. Elton (1958)

1.1.1 What is an invasive alien species?

Natural barriers have historically isolated animal and plant species, creating unique assemblages of species around the globe through adaptation, speciation, extinction and geological processes on a scale of millions of years (Futuyma, 2009). However, human activities are eroding those barriers at an unprecedented rate by allowing species to change their geographic distribution patterns, from local to continental scales (Vitousek et al., 1997; Mack et al., 2000; Simberloff et al., 2013). Due to this translocation of species outside their native home range, the term alien species was coined. Concretely, an **alien species** (also denoted as introduced species) refers to the organism that has been introduced, either deliberately or inadvertently, to an area outside its native range and that it could have not reached without human transportation (Richardson et al., 2000; Levine, 2008). This is clearly in contraposition with **native species**, that are defined as those that occur within their present or past natural range (IUCN, 2000). In this sense, although the definition of both alien and native species is quite clear, that of invasive alien species seems to be more problematic because it can be based on the spread (Richardson et al., 2000; Blackburn et al., 2011; Richardson and Pyšek, 2012), on the abundance (Colautti and MacIsaac, 2004; Valéry et al., 2008) or on the impacts of the alien species (IUCN, 2000; Mack et al., 2000; Lockwood et al., 2013). Actually, there is not a universally accepted definition of an invasive alien species, but for the matter of this thesis we will refer to **invasive alien species** (from now on **invasive species**) as introduced species that have negative impacts on native ecosystems (**1.1.2 Should we care about invasive species?**), that have the potential to spread over large areas and that sustain self-replacing populations in the introduced range.

1.1.2 Should we care about invasive species?

Invasive species are currently considered among the top 5 direct drivers of anthropogenic change, following land/sea use change, direct exploitation, climate change and pollution (Butchart et al., 2010; Brondizio et al., 2019; Pyšek et al., 2020)(**Figure 1.1**). That is so, because once established, invasive species can affect native species abundance and richness, threatening biodiversity worldwide (Vilà et al., 2011; Cameron et al., 2016; Gallardo et al., 2016). Actually, invasive species have been identified as the second cause of species extinctions since the 16th century (Bellard et al., 2016) and they have been the main driver of extinction for at least 261 animal species and 39 plant species worldwide (Blackburn et al., 2019). As an example of the pervasive impacts that a single invasive species can have for the biodiversity, the brown tree snake (*Boiga irregularis*), in less than 40 years, has caused the local extinction of more than half of Guam´s native bird, lizard and bat species and the global extinction of some of them (Fritts and Rodda, 1998; Simberloff and Rejmánek, 2011); while feral cats (*Felis catus*) are responsible for at least 14% of the modern global extinctions of birds, mammals and reptiles (Medina et al., 2011).

However, invasive species impacts expand beyond particular species and can also disrupt the functioning of the ecosystem and affect the provision of several ecosystem services (Pejchar and Mooney, 2009; Vilà et al., 2010; Simberloff et al., 2013; Vilà and Hulme, 2017). Among many others, invasive species have been reported to alter the nutrient cycling and the water flow (supporting services) (Castro-Díez and Alonso, 2017; Catford, 2017), to decrease the production of crop and timber and the fisheries yields (provisioning services) (Fried et al., 2017; Gozlan, 2017), and to disrupt pollination services and the quality of water (regulating services) (Costa et al., 2017; Morales et al., 2017). Additionally, invasive species can affect human well-being and health more directly as they can either be pathogens themselves, act as vectors of diseases or they can be poisonous to humans (Mazza et al., 2014; Nentwig et al., 2017; Rabitsch et al., 2017). Finally, for all these reasons, biological invasions have been associated to huge economic costs (Pimentel et al., 2001, 2005; Diagne et al., 2021), that just in Europe have been estimated to be over 20 billion € (Kettunen et al., 2008). Putting all this information together, it seems clear that there is an urgent need to consider the management of invasive species as a global priority, and this definitively goes associated to the need for better understanding the invasive process and the mechanisms that influence invader success.

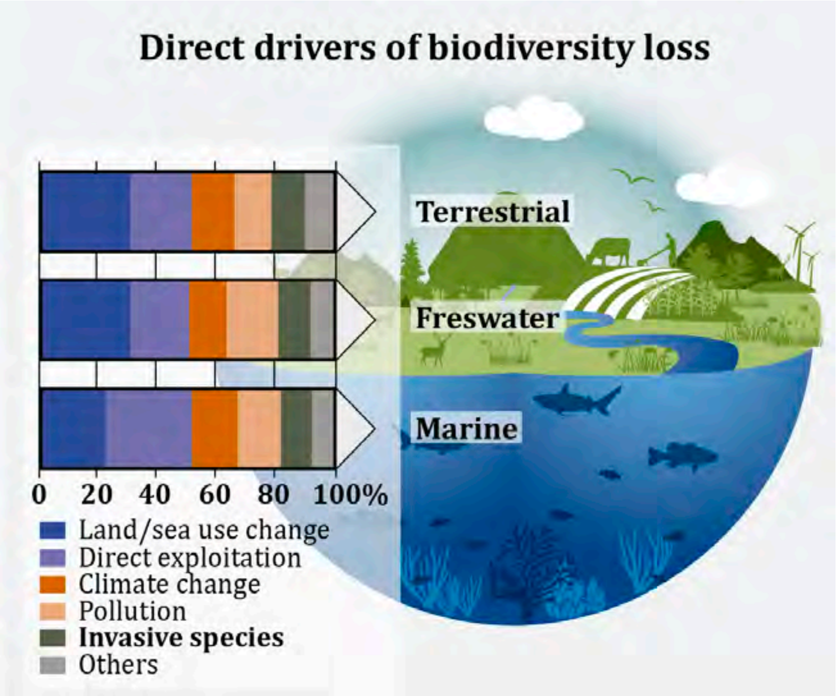

Figure 1.1. Most important direct drivers of biodiversity loss per ecosystem type based on the data published in the global assessment report on Biodiversity and Ecosystem Services (IPBES 2019). Figure modified from Bongaarts (2019).

1.1.3 How do alien species arrive to the new habitat?

The spread of non-native species is inherently associated to human activity and can be traced back at least to the 16th century (Mack, 2003; Genovesi et al., 2009; Pyšek et al., 2009; Roques et al., 2009). Since then, the transport of non-native species has increased steadily, following the increase through history in international trade and human movement (Hulme, 2009). Among the mechanisms by which alien species can be transported and introduced into a new region, six main pathways have been defined (Hulme et al., 2008): (1) intentional release (e.g., plants for erosion control or game animals), (2) escape from captivity (e.g., pets, ornamental plants), (3) contaminants (e.g., parasites or pests on traded goods), (4) stonaways on transport vectors (e.g., hull fouling or ballast water), (5) anthropogenic corridors (e.g., Panama and Suez Canals) or (6) unaided (e.g., natural dispersal from invaded regions). Different pathways predominate depending on the taxonomic group, with the unintentional pathways, mainly contaminants and stoneways, being most important for algae, invertebrates or fungi; whereas intentional pathways such as scape and release, are usually associated to the movement of alien plants and vertebrates (Pyšek et al., 2020). However, despite these pathways being well-known and several studies proposing risk-assessment frameworks for their management (Hulme et al., 2008; Banks et al., 2015; Hulme, 2015), rates in the accumulation of alien species have been growing steadily (Hulme, 2009), as the main vectors of introduction – global shipping, the expansion and opening of new canals, and the trade of exotic plants and animals – continue increasing (Hulme, 2015; Lockwood et al., 2019; Sardain et al., 2019). Actually, over the past 40 years, the estimated number of invasive alien species has increased by about 70% in the 21 countries that were assessed for the IPBES global assessment (Brondizio et al., 2019) and long-term temporal trends show a continuous increase in the cumulative number of established alien species over the last 200 years (Seebens et al., 2017). Unfortunately, the future looks far from promising, as climate change is predicted to increase the number, distribution and impact of invasive species (Vilà et al., 2007a; Walther et al., 2009; Hulme, 2017), with projections pointing to Europe and north eastern United States as the regions where the number of invasive species is expected to increase more (Bellard et al., 2013). Definitively, it seems clear that more effective measures of prevention, early detection and control are urgently needed to hinder the accumulation of invasive species (Seebens et al., 2017).

1.1.4 How do alien species establish in the new habitat?

Regardless of the pathway of introduction, every alien species, to become established and to be considered as an invasive species, has to go through a multi-stage process referred as the invasion process (Theoharides and Dukes, 2007; Blackburn et al., 2011; Lockwood et al., 2013). This process, despite being a continuum, has been divided into the following 4 stages: (1) transport, (2) introduction, (3) establishment and (4) spread and impact (**Figure 1.2**), to better assess the importance of the filters between the stages and to better consider the different spatio-temporal scales at which they operate (Theoharides and Dukes, 2007). These filters between the stages are ecological or environmental barriers (e.g. geographic, reproductive or dispersive) that prevent every non-native species to become established and dominant (Richardson et al., 2000; Theoharides and Dukes, 2007; Blackburn et al., 2011). Along the process, failure can happen at any stage, and even invasive species, when they have been well established and have successfully gone through every stage, can suddenly fail and suffer population crashes and local extinctions (Simberloff and Gibbons, 2004; Blackburn et al., 2011; Strayer et al., 2017).

Several factors such as (1) the propagule pressure, (2) the biotic mechanisms and (3) the abiotic characteristics of the invaded habitat, influence whether a species is able to go through any of the stages in the invasion process (**Figure 1.2**). Therefore, due to their importance for the success of invaders, these factors will be more deeply introduced in the following section: **1.1.5 Understanding the success of invaders**, and some of them will be further investigated in the main body of this thesis.

Finally, the filtering that occurs through the invasion process, only allows a small percentage of the introduced species to actually become invasive (Williamson and Fitter, 1996; Richardson et al., 2000; Jeschke and Strayer, 2005). Yet, despite their low number, they can have significant impacts for the native habitats and for the economy, as it was introduced in section: **1.1.2 Should we care about invasive species?**

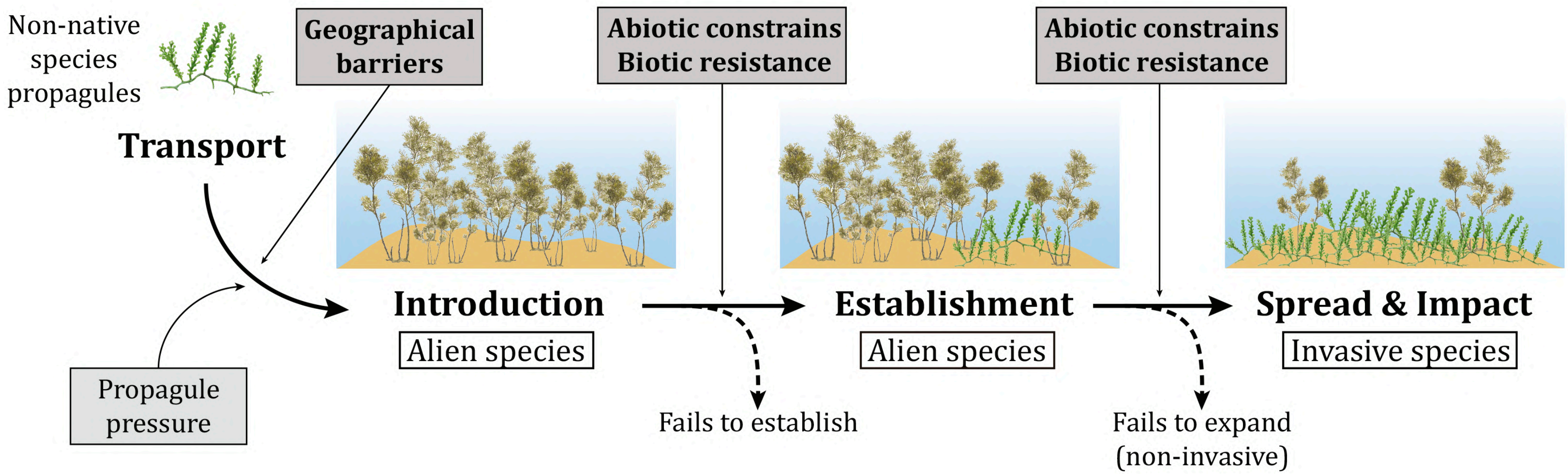

Figure 1.2. Schematic representation of the invasive process that every non-native species has to go through to become invasive. **Transport** – movement of the non-native species from the region of origin; **Introduction** – release and survival of the alien species in the introduced range; **Establishment** – populations of the alien species become self-sustaining; **Spread & Impact** – populations move across the landscape and alter the community. Along the process, geographical barriers, abiotic constrains and biotic resistance influence the success of the non-native species. Specifically, the abiotic constrains and the biotic resistance can: (1) act as a barrier before establishment and (2) regulate the spread and impacts of the alien species after its successful establishment. The latter situation will be the main focus of this thesis. Algae illustrations from the IUCN and Toni Llobet. Figure based on (Levine et al., 2004 and Theoharides and Dukes, 2007).

1.1.5 Understanding the success of invaders

The discipline of invasion ecology (invasion biology or invasion science) started to develop around 60 years ago with the seminal work by Elton (1958). Since then it has grown in relevance, probably associated to the increase in the perception of biological invasions as a global threat to biodiversity (**section 1.1.2**); but also because biological invasions can be used as natural experiments to understand the structure, function and dynamics of ecological communities (Levine, 2008). Lately, the use of this discipline has become essential to detect, understand and mitigate the impact of biological invasions (Simberloff et al., 2013).

In general, although the exact reasons behind the failure or success of most invasive species remain poorly understood (Hayes and Barry, 2008), a general framework of hypothesis has been developed to broadly address the factors influencing invasion success (Catford et al., 2009; Enders and Jeschke, 2018). Essentially, invasion success is influenced by the propagule pressure, the abiotic characteristics of the invaded habitat and the biotic characteristics of both, the recipient community and the invader (Richardson and Pyšek, 2006); and for an invasion to occur, all factors should be accommodating, if not favorable (Catford et al., 2009). Thus, the intensity and the extent of an invasion will be determined by a combination of the three factors (Fridley et al., 2007) and their fluctuation through time and space (Pyšek and Jarošík, 2005; Pauchard and Shea, 2006; Richardson and Pyšek, 2006). So far, the relative importance of each factor for the success of invaders is not clear, and while some studies suggest that the three factors strongly interact (Perelman et al., 2007; Catford et al., 2009), there are others that have found that single factors alone, such as the propagule pressure (Von Holle and Simberloff, 2005), the biotic factors (De Rivera et al., 2005; Davies et al., 2011) or the abiotic characteristics (Von Holle, 2005; Thomsen et al., 2006), can predominantly control the fate of an invasion.

In this sense, we will just focus in the biotic and abiotic factors of the invasion, to assess whether several characteristics of the habitat can limit the spread and the success of already established species.

I. Biotic resistance

When an alien species establishes in a new range, it necessarily interacts with the biotic component of the habitat and gains several interactions with the native community in the form of competition, predation, disease or mutualism. These interactions, depending on their direction and strength, can either facilitate the alien species (positive interactions) or limit its establishment and spread (negative interactions) (Lockwood et al., 2013), being the latter the ones that contribute to the resistance of the community against invaders (Levine et al., 2004; Lockwood et al., 2013). Actually, although this biotic resistance is generally not enough to completely prevent an invasion, it can strongly reduce the abundance and spread of the invader once it has been established (Levine et al., 2004; Theoharides and Dukes, 2007). More specifically, we will focus on two of the main mechanisms of biotic resistance to alien species: (1) competition by the native species and (2) herbivory.

I.I. Competition

The classical biotic resistance hypothesis, also known as the diversity-invasibility hypothesis, suggests that high diverse communities would resist invasions more effectively because less niches would be available for the invader to occupy (Elton, 1958; Levine and D'Antonio, 1999) and there would be a more efficient use of resources by the community members (Hooper, 1998). However, contrasting support for this hypothesis has been found depending on the spatial and temporal scales of the study (Levine, 2000; Shea and Chesson, 2002; Stohlgren et al., 2006; Fridley et al., 2007; Clark et al., 2013), on the habitat considered (Vilà et al., 2007b) and on the nature of the experiment – observational vs. experimental (Naeem et al., 2000; Stohlgren et al., 2003; Frankow-Lindberg, 2012; Jeschke et al., 2018; Peng et al., 2019). Actually, in general, this hypothesis has received low support (Jeschke et al., 2012b, 2018).

Nowadays, it is more widely suggested that community resistance is more influenced by the properties of the community members than by the species diversity *per se*. Thus, there is a growing interest in determining how the composition of the native assemblage and the specific functional traits of the native species can influence the interspecific competition against the invader (Perelman et al., 2007; Byun et al., 2013; Lockwood et al., 2013). So far, increases in the functional diversity of the communities have been associated to decreases in the invasion success through the preemption of available resources (Pokorny et al., 2005; Perelman et al., 2007; Byun et al., 2013), while the presence of certain functional groups and traits among the community can also significantly affect the community resistance towards the invader (Lindig-Cisneros and Zedler, 2002; Sheley and James, 2010; Frankow-Lindberg, 2012; Byun et al., 2013; Zhang et al., 2018). The particular functional group that better resists the invasion is not always consistent however, and in some cases it is the functional group more similar to the invader (limiting similarity) (Pokorny et al., 2005; Mwangi et al., 2007; Hooper and Dukes, 2010; Petruzzella et al., 2018), whereas in other cases there are functional groups different to the invader the ones that increase resistance (fitness inequality) (Lulow, 2006; Sheley and James, 2010). Despite the lack of a general mechanism, what seems clear is that the interspecific competition can negatively affect invader success as has been previously reported from different ecosystems (Amsberry et al., 2000; Fine, 2002; Morris et al., 2015; Petruzzella et al., 2018; te Beest et al., 2018). However, on its own, this effect might not be enough to strongly control well-established invaders (Levine et al., 2004; Vilà and Weiner, 2004).

I.II. Herbivory

Plant performance and distribution can be significantly reduced by herbivores (Maron and Crone, 2006; Wood et al., 2017), so we can expect herbivory to affect the performance and success of invasive species. However, the effect of herbivory on invader success is not fully understood yet as it can take two directions (Maron and Vilà, 2001). On the one hand, herbivory has been reported to have no effect on the performance of invaders (Stohlgren et al., 1999) and even to promote them (Eschtruth and Battles, 2009a; Kalisz et al., 2014), in part because invasive plants are usually released from their coevolved specialist herbivores in the invaded habitat (Keane and Crawley, 2002; Colautti et al., 2004; Liu and Stiling, 2006). On the other hand, however, several studies have reported the ability of herbivores to reduce the recruitment, growth and survival of invaders (Parker et al., 2006; Cushman et al., 2011; Pearson et al., 2012; Li et al., 2014; Zhang et al., 2018).

This lack of consensus clearly highlights the need for more studies that assess the impact of herbivory in different systems and invaders to try to understand the conditions under which herbivory can be effective for the control of an invader. In fact, despite previous studies reporting negative impacts of herbivores on invader plant performance, the lack of exclusion experiments and long-term assessments makes it difficult to assess whether these herbivory impacts actually render a limiting effect on the population dynamics of the invader (Liu and Stiling, 2006; Pearson et al., 2012). These types of assessments might be particularly relevant to understand the effectiveness of herbivory as a resistance mechanism considering that invasive species can have strong compensatory mechanisms to overcome the herbivory impacts (Garren and Strauss, 2009; Ortega et al., 2012).

I.III. Interaction between mechanisms

Previous evidence suggests that both competition and herbivory can limit the success of invaders to a certain extent (Levine et al., 2004). However, whether the interaction between them can further influence the overall resistance of native habitats towards invaders has been rarely assessed (but see: Suwa and Louda, 2012; Li et al., 2014; Zhang et al., 2018). In this sense, considering that competition and herbivory are mechanisms known to strongly interact between each other (Gurevitch et al., 2000; Hambäck and Beckerman, 2003), it might be necessary to consider and assess the interaction between these mechanisms if we want to better assess the strength of biotic resistance against invaders (Levine et al., 2004; Mitchell et al., 2006; Kimbro et al., 2013)(**Figure 1.3**).

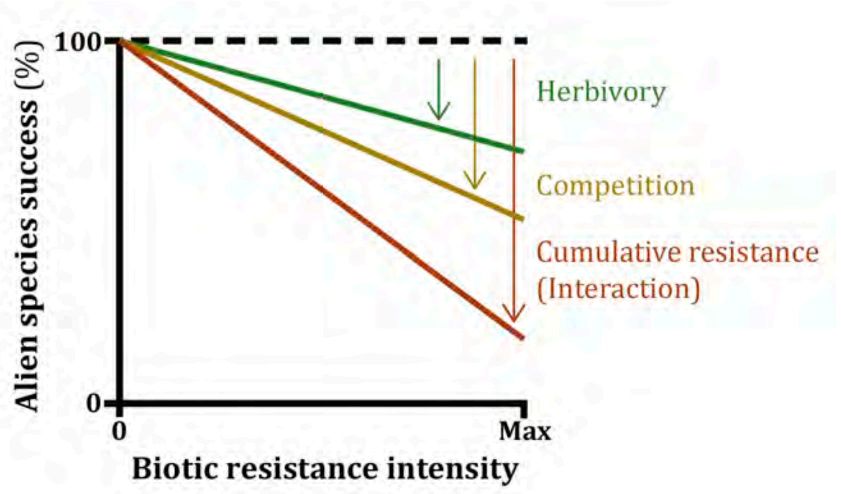

Figure 1.3. Conceptual figure showing how the biotic mechanisms assessed in this thesis might influence the success of an invader. Both, competition and herbivory can affect invader success to a certain extent, but it might be only when the interaction between them is considered that a more realistic assessment of the biotic resistance strength might be obtained. Figure modified from Theoharides and Dukes (2007).

II. Abiotic characteristics

The physical environment is considered to be the main factor repelling non-native species and preventing them for becoming established (Levine et al., 2004; Theoharides and Dukes, 2007; Levine, 2008). However, under certain circumstances, it can also influence the success of already established invaders, particularly when there are sudden changes to the abiotic conditions such as under extreme climatic events (Collinge et al., 2011; Goldstein and Suding, 2014). In such situations, the disturbance created can open windows of opportunity for invaders

(Shea and Chesson, 2002; Lockwood et al., 2013; Jauni et al., 2015), because, in general, invasive species have wider ecological tolerances, higher fitness and are phenotypically more plastic than the natives (Pyšek and Richardson, 2007; Van Kleunen et al., 2010; Davidson et al., 2011). This might be particularly relevant in a scenario where climatic variability and the recurrence of extreme climatic events are expected to increase due to climate change (Meehl and Tebaldi, 2004; Karl et al., 2008; Füssel, 2009), highlighting the potential for invasions to increase in the future (Vilà et al., 2007a; Hellmann et al., 2008; Diez et al., 2012; Bellard et al., 2013). However, our knowledge on how invaders specifically respond to extreme climatic events is still quite limited (but see: Song et al., 2010; Sorte et al., 2010), but this information might be of paramount importance to understand the success of invaders under future climatic conditions.

Taking into account the importance of alien species as drivers of biodiversity loss and the lack of knowledge regarding the mechanisms and conditions that influence their success, in this thesis we will try to unravel the importance of some of those mechanisms for the success of a well established invader, to later use this information to propose effective management measures that could reduce the impacts of invaders. In particular, we will focus in marine systems as we will introduce in the following sections, but we believe that our findings could also be applied to other systems.

1.2 Marine invaders

Marine invaders are considered to be one of the main threats to marine ecosystems worldwide due to the significant impacts they cause in native communities and in ecosystems services (Bax et al., 2003; Halpern et al., 2008; Molnar et al., 2008; Katsanevakis et al., 2014b). Similarly to the pattern observed in terrestrial ecosystems, their number is on the rise following the increase in their main vectors of introduction – the aquarium trade, the opening and widening of canals and shipping traffic (Katsanevakis et al., 2013; Seebens et al., 2013; Galil et al., 2017; Castellanos-Galindo et al., 2020). Additionally, global warming can also favor the spread of alien species both, directly by increasing the chance of establishment and indirectly by weakening native species (Walther et al., 2009; Diez et al., 2012), which will further intensify the negative impacts of invaders. Currently there are 1,111 marine alien species listed in the Global Register of Introduced and Alien Species (GRISS; http://www.griis.org/) (Pagad et al., 2018), being the Mediterranean Sea (**Box 1**) one of the regions where more alien species have been recorded.

Regarding the influence of negative interactions on the success of marine invaders, previous evidence suggests that competition and consumer pressure are weaker on invasive producers than on invasive consumers (Kimbro et al., 2013; Papacostas et al., 2017), which highlights that, in general, invasive marine producers are released more effectively from biotic control than their terrestrial counterparts. Still, it is important to consider that research on marine invaders falls well behind that on terrestrial invaders (**Figure 1.4, A**) (Lowry et al., 2013) and that knowledge on the mechanisms that drive the success of marine invaders is still quite limited (Papacostas et al., 2017), particularly considering that manipulative experiments, long-term assessments and experiments assessing multiple mechanisms are seldom done. Given that marine algae are the trophic group that has the highest impacts on marine communities (Anton et al., 2019) and that they are one of the less studied taxonomic groups worldwide (**Figure 1.4, B**) (Pyšek et al., 2008; Jeschke et al., 2012a), they will be the main focus of this thesis (**1.2.1 Invasive algae**).

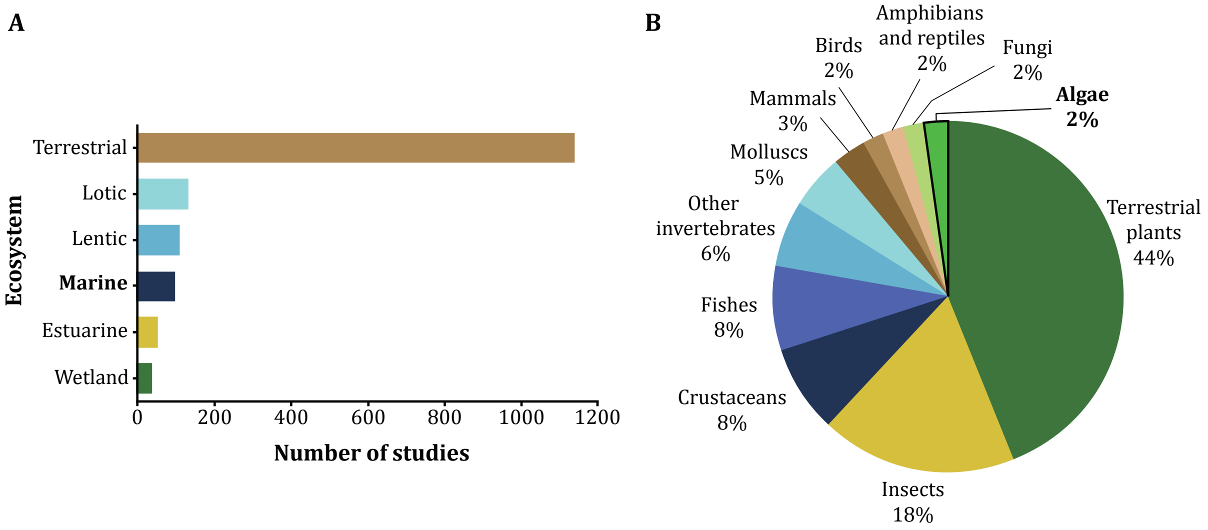

Figure 1.4. Summary of invasion ecology studies done: **A)** per ecosystem type and **B)** per taxonomic group. Figures modified from Lowry et al. (2013) and from Pyšek et al. (2008).

1.2.1 Invasive algae

Algae are one of the most conspicuous and successful groups of marine invaders worldwide (Schaffelke et al., 2006), with at least 346 alien taxa identified (Thomsen et al., 2016). These organisms, once established, have direct negative effects on single species abundance, community structure and biodiversity (Williams and Smith, 2007; Guy-Haim et al., 2018), mainly affecting native algae species (Thomsen et al., 2009, 2016; Maggi et al., 2015). Specifically, they contribute to the homogenization of marine habitats through the monopolization of space, over-simplifying the communities and causing reductions in the abundance of fauna (Schaffelke and Hewitt, 2008). Additionally, these species can act as foundation species and ecosystems engineers, and modify both, the habitat and the availability of resources, which is associated to greater negative consequences for the native communities (Wallentinus and Nyberg, 2007). However, our knowledge on invasive algae is still quite limited as they have earned little attention in invasion ecology (**Figure 1.4, B**) (Pyšek et al., 2008; Jeschke et al., 2012a).

Nowadays, knowledge on the mechanisms that determine the long-term establishment and persistence of invasive algae is still negligible, although several mechanisms such as competition and herbivory have been suggested to influence the success of marine algae (Inderjit et al., 2006; Williams and Smith, 2007; Kimbro et al., 2013; Papacostas et al., 2017). On the one hand, some studies have reported the ability of native algae, mostly canopy-forming and erect species, to reduce, to some extent, the success of invasive algae through competition mechanisms (Ceccherelli et al., 2002; Arenas et al., 2006; Britton-Simmons, 2006; Inderjit et al., 2006; Williams and Smith, 2007; Piazzi and Balata, 2009; Vaz-Pinto et al., 2012). On the other hand, despite marine communities being dominated by generalist herbivores (Hay and Steinberg, 1992; Hay et al., 1992; Enge et al., 2017), herbivory seems to be ineffective for the control of invasive algae (e.g., Wikström et al., 2006; Williams and Smith, 2007; Forslund et al., 2010; Cebrian et al., 2011; Tomas et al., 2011b; Nejrup et al., 2012; Enge et al., 2017). This clearly contrasts with the significant role that herbivores have on marine systems, where herbivory has been reported to exert stronger control impacts on algae compared to terrestrial plants (Cyr and Face, 1993; Shurin et al., 2002, 2006; Poore et al., 2012). However, it should not be disregarded that most of the previous assessments relied only on preference and laboratory experiments, they just considered a particular time frame in the invasion and they mainly assessed the role of invertebrates (Williams and Smith, 2007; Kimbro et al., 2013; Enge et al., 2017; Papacostas et al., 2017). Actually, this could have definitively influenced the patterns reported because: (1) preference and laboratory assessments provide feeble information on the real effect of herbivory at the community level and in natural environments (Liu and Stiling, 2006; Pearson et al., 2012), (2) relying on a specific time frame neglects the ability of native herbivores to adapt to the invader (Carroll et al., 1998; Siemann et al., 2006; Dostál et al., 2013), (3) and fishes could significantly influence invader success according to their capacity to regulate algae abundance and distribution (Vergés et al., 2009; Poore et al., 2012). Additionally, despite omnivory being more prevalent in marine food webs than in the terrestrial ones (Thompson et al., 2007), previous assessments have only considered the strict herbivores, disregarding the potential role that omnivorous organisms could have for the success of invasive algae by directly consuming the invaders. Finally, no study before has considered the interaction between competition and herbivory, which could prevent us from assessing the true strength of biotic resistance mechanisms in natural marine communities.

Complementary, algae success has also been suggested to be influenced by the abiotic conditions of the receiving habitat (Inderjit et al., 2006; Schaffelke et al., 2006; Thomsen et al., 2016), which highlights the importance of understanding how algae respond to environmental change, particularly considering that marine climatic conditions are shifting abruptly worldwide (Belkin, 2009; Oliver et al., 2018, 2019). Unfortunately, knowledge on how invasive algae might respond to changes in the abiotic conditions, particularly to climate change and to extreme climatic events, is still in its infancy (but see: Roth-Schulze et al., 2018; Atkinson et al., 2020), but it definitively needs to gain relevance if we are to better predict how climate change might affect current and future invasions.

Finally, among algae species, *Caulerpa cylindracea* has been recently ranked as one of the marine invaders with the largest negative impacts (Anton et al., 2019) and it is also the most wide-spread invader within the Mediterranean Sea (Klein and Verlaque, 2008), making it the perfect target species for this thesis (**Box 2**).

Box 1| Study site: the Mediterranean Sea, a biodiversity hotspot under threat

The Mediterranean Sea is the deepest and largest semi-enclosed sea on Earth and it occupies just 0.82% of the world´s oceans surface (Coll et al., 2010; Boudouresque et al., 2017). Still, despite its small size, it is home for an unusually rich and diverse biota that represents around 7% of the world´s marine biodiversity, including a high percentage of endemic species (Bianchi and Morri, 2000; Coll et al., 2010; Lejeusne et al., 2010). As a result, it is considered as one of the main biodiversity hotspots for marine species (Boudouresque, 2004; Coll et al., 2010). However, it is also one of the marine regions more impacted by climate change and anthropogenic pressures (Giorgi, 2006; Halpern et al., 2008; Belkin, 2009; Coll et al., 2010; Lejeusne et al., 2010), and for the past decades it has also suffered an increase in the establishment and spread of alien species, becoming a hotspot for marine invasions (Rilov and Galil, 2009). Actually, since the beginning of the 20th century, the number of alien species in the basin has doubled every 20 years (Galil, 2008), with more than 800 species introductions since 1950 (Zenetos et al., 2012).

Three main pathways of introduction: the Suez Canal, aquaculture and shipping traffic through fouling and ballast water (Zenetos et al., 2012; Katsanevakis et al., 2013, 2014a), are responsible for most of the close to 1,000 species introductions that have been reported in the basin, being more than half of these species already established and spreading (Zenetos et al., 2010, 2012). Interestingly, due to most of the alien species entering to the Mediterranean Sea through the Suez Canal (Galil et al., 2014, 2015; Katsanevakis et al., 2014a), the biodiversity spatial pattern of alien species differs to that of native species, with native biodiversity decreasing from the northwest to the southeast (Coll et al., 2010) while alien biodiversity decreases from the southeast to the northwest (Katsanevakis et al., 2014a). Actually, due to this increase in the establishment of species native to warmer regions and to the rise in the seawater temperature, several authors propose that the Mediterranean Sea is going through a process of "tropicalization" (Bianchi, Carlo and Morri, 2003), that will be further exacerbated in the future with the increase in the warming of the Mediterranean Sea and the likely increase in the establishment and spread of more thermophilic species (Bianchi, 2007; Bianchi et al., 2013).

Nowadays, there is a contrast in the taxonomic groups that predominate in each region of the Mediterranean Sea, with alien macrophytes predominating in the Western Mediterranean, whereas alien fishes and invertebrates predominate in the eastern and central part (Zenetos et al., 2012). What is common, however, is that the arrival and establishment of these species is altering the native diversity and the functioning of the system in really profound ways (Streftaris and Zenetos, 2006; Galil, 2007; Katsanevakis et al., 2014b; Boudouresque et al., 2017; Mannino et al., 2017). Still there is a lot of debate on whether all alien species are actually bad, as negative effects have only been reported for a small proportion of them (Katsanevakis et al., 2014b; Giangrande et al., 2020). However, care should be taken when making such considerations, as our understanding of the impacts of marine invasions is still quite limited.

A rich diversity of established alien species can be currently found in the Mediterranean Sea (**Figure 1.5**), being *Caulerpa cylindracea* one of the most wide-spread species in the basin (Klein and Verlaque, 2008). This species, being the target species in this thesis, will be introduced in detail in **Box 2**.

Figure 1.5. Some of the alien species that have established in the Mediterranean Sea with the arrows indicating the main dispersion routes they use. Credit: Glynn Gorick/CLAMER.

Box 2| Target species: *Caulerpa cylindracea*

Caulerpa cylindracea is a siphonaceous green macroalga native to the Southwestern Coast of Australia (Verlaque et al., 2003), which was reported for the first time in the Mediterranean Sea in 1990, off the coasts of Libya (Nizamuddin, 1991). In less than 20 years, *C. cylindracea* colonized marine communities throughout the entire basin and even reached the North Atlantic (Verlaque et al., 2004), becoming the most wide-spread invasive species in the Mediterranean Sea (Klein and Verlaque, 2008; Katsanevakis et al., 2016). Currently, this species can be found thriving on a wide array of substrata in depths from 0 to 70 m, where it can form compact mono-specific mats that can grow on top of the benthic assemblages (Klein and Verlaque, 2008). This causes the homogenization of native communities and it has strong negative impacts on the native communities, through the limitation of other macrophyte and invertebrate species (Piazzi et al., 2001; Piazzi and Ceccherelli, 2006; Klein and Verlaque, 2008, 2011; Kružić et al., 2008; Žuljević et al., 2011) and through the creation of an anoxic layer underneath the mat (Piazzi et al., 2007; Klein and Verlaque, 2008). Consequently, *C. cylindracea* is considered to be the most-harmful invasive species in the Mediterranean Sea (Klein and Verlaque, 2008; Katsanevakis et al., 2016) and one of the marine invaders with the strongest impacts worldwide (Anton et al., 2019). Lately, despite its rampant success, several meadows have suffered steep abundance declines (Klein and Verlaque, 2008; García et al., 2016), which suggests that after a period of successful establishment, there might be some resistance mechanisms that can be effective for the control of the invader and could limit its invasive success. So far, however, the identity of those mechanisms remains unknown.

Morphologically, *C. cylindracea* is characterized by a simple morphology that is formed by erect shoots with grape-like ramuli that can be arranged radially or distichously and creeping stolons that attach to the substrate by thin rhizoids (Klein and Verlaque, 2008). This characteristic morphology, together with its vivid green color, makes species identification an easy task, both underwater and on land (**Figure 1.6**).

Figure 1.6. *Caulerpa cylindracea* growing in close proximity to the tunicate *Aplidium proliferum* and to a small colony of the bryozoan *Myriapora truncata*. Photo credit: Eneko Aspillaga.

1.3 Objectives

This thesis is encapsulated in the discipline of invasion ecology and has the general aim of improving our understanding of the factors that influence the success of invasive species by using *Caulerpa cylindracea* as a model organism. Particularly, on the first 3 chapters (**2, 3** and **4**) we focus on assessing the role that biotic factors might have on the invasion dynamics of the studied species (**Figure 1.7**), while in **Chapter 5** we focus in assessing how the abiotic factors, concretely temperature, can influence the invasive process of the studied species (**Figure 1.7**).

For this thesis we have chosen *Caulerpa cylindracea* as the target species for several reasons. First, this macroalga species is currently considered the most widespread and harmful invader in the Mediterranean Sea (Klein and Verlaque, 2008; Katsanevakis et al., 2016) and has even been considered as one of the most damaging marine invaders worldwide (Anton et al., 2019). However, despite this species probably being amongst the most studied invasive algae, the role of several factors on its invasion dynamics, particularly herbivory and temperature, are still poorly understood (**Figure 1.7**), although they could potentially influence its invasion success considering the importance that biotic and abiotic factors have for the invasive process in general (**1.1.5 Understanding the success of invaders**). Actually, several regressions of *C. cylindracea* meadows have been recently reported after the onset of the invasion, and although their causes remain elusive (Klein and Verlaque, 2008; García et al., 2016), they could be related to the existence of resistance mechanisms against the invader. Still, which are those mechanisms and whether they develop with time remains poorly understood, but considering that reliable long-term data on the presence and abundance of the species is available from the research team since the beginning of the invasion (Piazzi et al., 2005; Ballesteros et al., 2008; Cebrian and Ballesteros, 2009; Cebrian et al., 2011; García et al., 2016), we combined it with *in situ* and *ex situ* experimentation to further investigate on the mechanisms that influence the long-term dynamics of the invader. Particularly, given that long-term data on invaders is scarce and that manipulative experimentation in the field is seldom done, the findings reported in this thesis can be relevant for biological invasions in general, as they might allow us to understand the mechanisms that influence invader success and the temporal dynamics of invasive species dwelling in different systems.

The chapters here presented combine field surveys (**Chapter 2, 3, 4** and **5**), manipulative experiments in the field (**Chapter 2** and **3**), controlled experiments in the laboratory (**Chapter 5**) and molecular analysis techniques (**Chapter 5**). The specific objectives of each chapter are detailed below:

- **Chapter 2**. *The role of competition and herbivory in biotic resistance against invaders: A synergistic effect.* Using benthic assemblage sampling and *in situ* field experiments, we assess whether the competition mediated by the complexity of the assemblage and the herbivory by the fish *Sarpa salpa*, can contribute to the control of the abundance of *C. cylindracea*. Additionally, by using long-term data on the abundance and distribution of the invader at the study site we further elucidate on the influence that the interaction between both biotic mechanisms, competition and herbivory, has for the invasion dynamics of *C. cylindracea*.

- **Chapter 3**. *Learning takes time. Biotic resistance by native herbivores increases throughout the invasion process.* In this chapter, taking into account the decrease in *C. cylindracea* abundance that we observed some time after the onset of the invasion in chapter 2, we investigate how time since the invasion and abundance of the invader shape the predator-prey interaction between *S. salpa* and *C. cylindracea*. Particularly, we experimentally assess whether the preference, the consumption and the electivity towards the invader change throughout the invasion, in relation to both, exposure time and invader availability.

- **Chapter 4**. *Fish herbivory on Caulerpa cylindracea: The role of omnivorous sparid fishes.* Given the important effect that consumption by *S. salpa* has on *C. cylindracea* abundance, observed in chapters 2 and 3, in this chapter we assess whether non-strictly herbivorous fishes can also contribute to the biotic resistance towards the invader. Specifically, we study the diets of four of the commonest omnivorous sparid fishes in the Mediterranean Sea to elucidate whether they consume *C. cylindracea*, whether the invader is an important part of their diet and whether the consumption is intentional or accidental.

- **Chapter 5**. *Stressful conditions give rise to a novel and filamentous form of Caulerpa cylindracea.* In this chapter, after detecting a previously unreported morphology of *C. cylindracea* in the field, we explore the influence of extreme temperatures on the morphological plasticity of the invader to disentangle the causes behind the observed morphology and to assess the tolerance of the invader to disturbance. Furthermore, given the novelty of the new morphology, we characterize it morphologically, both macroscopically and microscopically; and we use molecular analyses to confirm the identity of the morphology due to its lack of resemblance with previous reported morphologies for the genus.

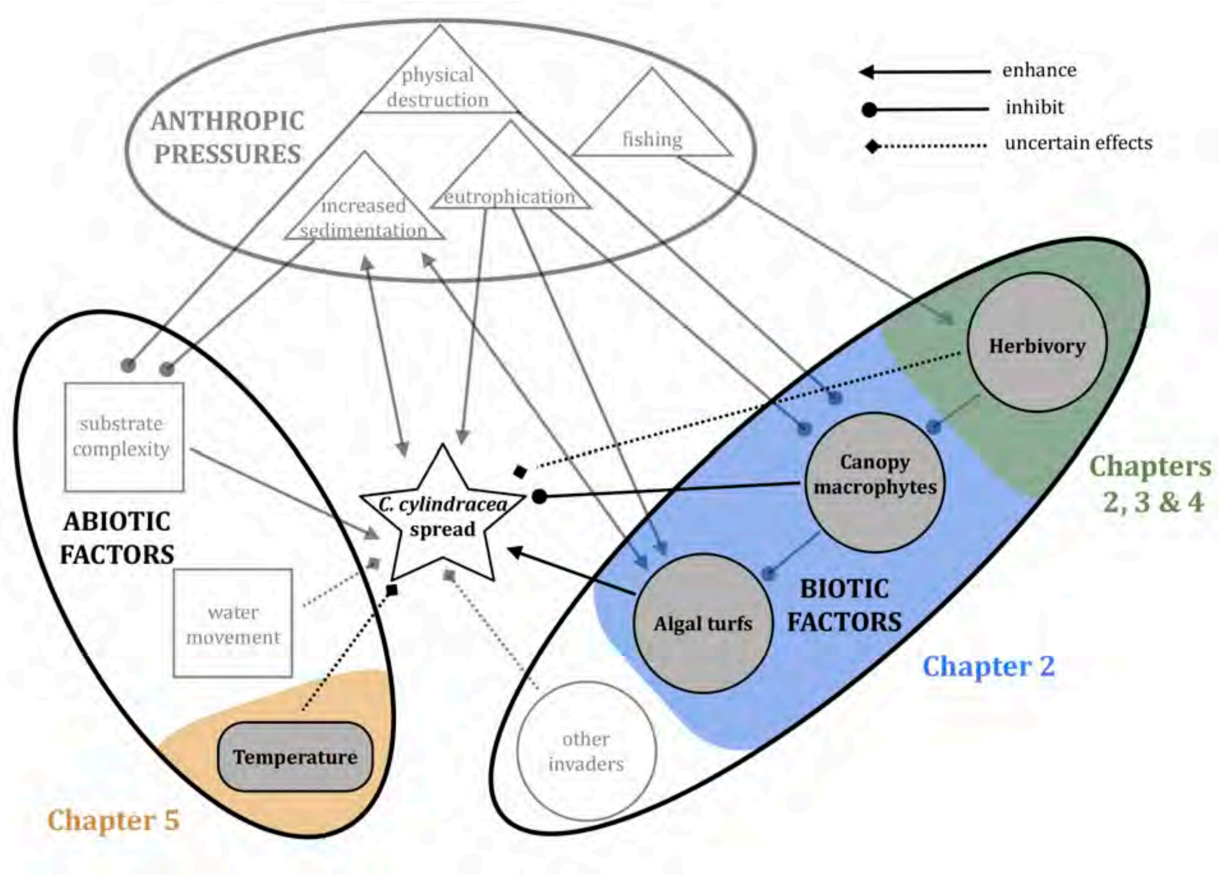

Figure 1.7. Conceptual model showing the factors influencing *Caulerpa cylindracea* spread. The factors assessed in this thesis are highlighted in bold, with the different colors showing the specific chapters in which they are assessed. Conceptual model modified from Piazzi et al. (2016).

Chapter 2

The role of competition and herbivory in biotic resistance against invaders: A synergistic effect.

Jorge Santamaría, Fiona Tomas, Enric Ballesteros, Juan M. Ruiz,
Jaime Bernardeau-Esteller, Jorge Terrados, Emma Cebrian

***Ecology* (2021)** *Accepted*

Cover image: *Caulerpa cylindracea* thriving in a community dominated by turf species like the invasive alga *Womersleyella setacea*.

Author: Jana Verdura Brugarola.

2.1 Abstract

Invasive species pose a major threat to global diversity and once they are well established their eradication typically becomes unfeasible. However, certain natural mechanisms can increase the resistance of native communities to invaders and can be used to guide effective management policies. Both competition and herbivory have been identified as potential biotic resistance mechanisms that can limit plant invasiveness but it is still under debate to what extent they might be effective against well-established invaders. Surprisingly, whereas biotic mechanisms are known to strongly interact, most studies up to date have examined single biotic mechanisms separately, which likely influences our understanding of the strength and effectiveness of biotic resistance against invaders. Here we use long-term field data, benthic assemblage sampling and exclusion experiments to assess the effect of native assemblage complexity and herbivory on the invasion dynamics of a successful invasive species, the alga *Caulerpa cylindracea*. A higher complexity of the native algal assemblage limited *C. cylindracea* invasion, probably through competition by canopy-forming and erect algae. Additionally, high herbivory pressure by the fish *Sarpa salpa* reduced *C. cylindracea* abundance by more than 4 times. However, long-term data of the invasion reflects that biotic resistance strength can vary across the invasion process and it is only where high assemblage complexity is concomitant with high herbivory pressure, that the most significant limitation is observed (synergistic effect). Overall, the findings reported in this study highlight that neglecting the interactions between biotic mechanisms during invasive processes and restricting the studied time scales may lead to underestimations of the true capacity of native assemblages to develop resistance to invaders.

2.2 Introduction

Biological invasions are one of the main threats to biodiversity and ecosystem function worldwide, being the second most prominent cause of species extinctions and playing an important role in diversity reduction (Vilà et al., 2011; Bellard et al., 2016). Furthermore, bioinvasions can produce alterations in a number of ecosystem services and basic ecosystems processes (Pejchar and Mooney, 2009; Vilà et al., 2010; Simberloff et al., 2013), often at great economic cost (Pimentel et al., 2005). Still, our understanding of the factors that influence invasion success remains limited (Simberloff et al., 2013), complicating the development of effective management strategies to prevent and mitigate the negative effects of invasive species.

The success of an invasion is dependent on multiple processes across a wide range of temporal and spatial scales (Perelman et al., 2007; Theoharides and Dukes, 2007; Eschtruth and Battles, 2009b; Byun et al., 2015). Among these processes, most of the attention has fallen on biological processes, in the context of the Biotic Resistance Hypothesis (Elton, 1958; Keane and Crawley, 2002; Levine et al., 2004). The strength of biotic resistance against an invader is strongly influenced by the native assemblage and by the functional traits of the native species (Pokorny et al., 2005; Perelman et al., 2007; Byun et al., 2013), which modulate the interspecific competition; but also by the consumer pressure on both, the invasive and the native species (Levine et al., 2004; Parker and Hay, 2005; Mitchell et al., 2006). In this sense, negative effects of competition on several invasive plants, mainly caused by the limitation of essential resources such as water, nutrients or light, have been previously reported in tropical forests (Fine, 2002), saltmarshes (Amsberry et al., 2000), grasslands (Corbin and D'Antonio, 2004; te Beest et al., 2018), mangroves (Li et al., 2014; Zhang et al., 2018), shrublands (Morris et al., 2015) and freshwater ecosystems (Petruzzella et al., 2018). However, competition alone might not be enough to exert a strong biotic control against a well-established invader (Levine et al., 2004; Vilà and Weiner, 2004). On the other hand, herbivory has been also acknowledged as an important biotic resistance mechanism for native ecosystems (Levine et al., 2004; Parker and Hay, 2005; Parker et al., 2006), although its effectiveness is controversial (Maron and Vilà, 2001; Keane and Crawley, 2002; Liu and Stiling, 2006). In fact, herbivores can promote (Eschtruth and Battles, 2009a; Relva et al., 2010; Kalisz et al., 2014), deter (Cushman et al., 2011; Pearson et al., 2012; Zhang et al., 2018), or have no effect on the dominance of invasive plant species (Stohlgren et al., 1999), which makes it difficult to understand the conditions under which herbivory can be an effective biotic resistance mechanism against an invasion. Taking into account that herbivory can be highly influenced by other factors such as native plant traits (Grutters et al., 2017) or habitat features (Alofs and Jackson, 2014; Li et al., 2014; Ender et al., 2017; Zhang et al., 2018), contrasting observations on the role of biotic mechanisms in controlling invasive species may be partially explained by the fact that they are often assessed neglecting the relative importance of the interactions between mechanisms (Levine et al., 2004; Mitchell et al., 2006; Alofs and Jackson, 2014; Zhang et al., 2018; Petruzzella et al., 2020). Indeed, the interaction between biotic mechanisms has been suggested to be responsible for an enhancement in the biotic resistance capacity of the invaded community (Suwa and Louda, 2012; Li et al., 2014; Zhang et al., 2018).

In marine ecosystems, macroalgae are one of the most conspicuous and successful invaders, as well as one of the most harmful, comprising 20% of marine invasive species worldwide (Schaffelke et al., 2006) and causing potentially important ecological and economic damage (Williams and Smith, 2007). Despite this, the factors that drive their invasive success remain largely unknown (Inderjit et al., 2006), although similarly to what has been observed for plant invasions in terrestrial ecosystems, both competition and herbivory are suspected to determine their invasion success (Kimbro et al., 2013; Papacostas et al., 2017). In this regard, functional traits of the native species can influence habitat resistance to algae invasion through their contribution to interspecific competition (Arenas et al., 2006; Britton-Simmons, 2006; Vaz-Pinto et al., 2012). Surprisingly, however, most studies seem to confirm the inability of herbivores to limit the spread of well-established invasive macroalgae (e.g., Wikström et al., 2006; Forslund et al., 2010; Cebrian et al., 2011; Tomas et al., 2011b; Nejrup et al., 2012), despite the important role of herbivory regulating algal abundance and distribution (Vergés et al., 2009; Poore et al., 2012). Overall, previous research on biotic resistance against invasive macroalgae seems to suggest that, in most cases, the effect of single biotic mechanisms might not be enough to significantly affect invader performance (Kimbro et al., 2013; Papacostas et al., 2017). Probably, as has been suggested above, only by considering the interaction between biotic mechanisms (both competition and herbivory), more robust conclusions on the true resistance of an assemblage towards a particular invader can be obtained.

In this study, we aim to test whether herbivory interacts with competition to modulate the resistance of a marine habitat towards a particular invader. We use *Caulerpa cylindracea*, one of the most invasive macroalgae worldwide, to assess the simultaneous role that both assemblage structure and herbivory pressure have on *C. cylindracea* invasion by using *in situ* field experiments that assess herbivory and assemblage characteristics. Additionally, we monitor the abundance of the invader over time to further elucidate whether the studied biotic mechanisms and their interaction influence the long-term dynamics of the invader.

2.3 Materials and methods

2.3.1 Study species

Caulerpa cylindracea is a green alga, native to the Southwestern coast of Australia (Verlaque et al., 2003), which is currently considered one of the most invasive species within the Mediterranean Sea (Klein and Verlaque, 2008; Katsanevakis et al., 2016), having also invaded areas in the North Atlantic (Verlaque et al., 2004).

Actually, it has recently been ranked as one of the marine invaders with the highest negative ecological impacts worldwide (Anton et al., 2019). However, despite its formidable ability to spread and grow, the abundance of *C. cylindracea* appears to differ markedly among invaded assemblages (Klein and Verlaque, 2008; Cebrian and Ballesteros, 2009), suggesting that there might be, in some cases, some natural mechanisms controlling *C. cylindracea* abundance. Among such mechanisms, competition may play an important role, since canopy-forming and erect algae (typical of high-complexity assemblages) can outcompete *C. cylindracea* (Ceccherelli et al., 2002; Bulleri and Benedetti-Cecchi, 2008; Piazzi and Balata, 2009; Bulleri et al., 2010), whereas herbivory seems to fail as a control mechanism for *C. cylindracea* when it is well established (Bulleri et al., 2009; Cebrian et al., 2011), even though several species are known to commonly feed on it (Ruitton et al., 2006; Box et al., 2009; Cebrian et al., 2011; Tomas et al., 2011b). However, it is important to consider that previous studies have focused on only one of these mechanisms (either competition or herbivory) while, in nature, both mechanisms might act together to influence the abundance of the invasive species.

2.3.2 Study system

The Mediterranean Sea is the largest and deepest semi-enclosed sea on Earth and it is considered a hotspot for marine biodiversity as it harbors around 17,000 marine species, 20% of them being endemic to the region (Coll et al., 2010). Due to its temperate climatic conditions, Mediterranean benthic shallow habitats are dominated by macroalgae. Unfortunately, the Mediterranean Sea is one of the areas most susceptible to the introduction of non-native species worldwide (Galil, 2007) and it is considered to be a hotspot for invasive algae (Williams and Smith, 2007; Thomsen et al., 2016).

The study was conducted in the Cabrera Archipelago National Park, in the Balearic Islands (western Mediterranean; 39° 12’ 21” N, 2° 58´44” E) (**Figure 2.1**).

This maritime-terrestrial national park was established in 1991 and currently harbors some of the best-preserved benthic and fish assemblages in the Mediterranean Sea (Sala et al., 2012; Guidetti et al., 2014).

The invasive alga, *Caulerpa cylindracea*, was first detected in the area in 2003 at a depth of 30 m (Cebrian and Ballesteros, 2009) and has, since then, colonized most of the park´s benthic habitats at depths from 0 to 65 m. Indeed, in some of these habitats, it has become the dominant species (Cebrian et al., 2011).

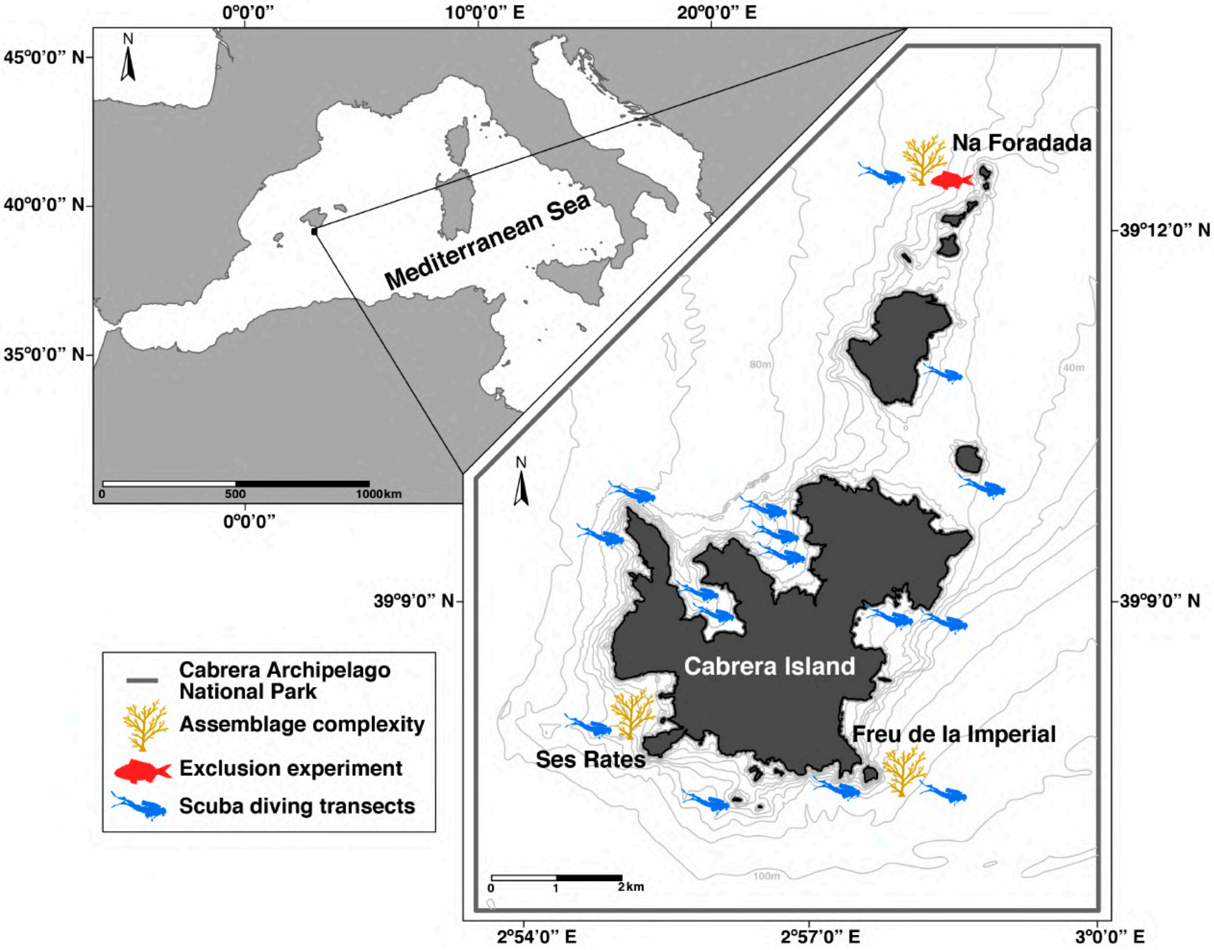

Figure 2.1. Location of the Cabrera Archipelago National Park. The points show the locations of the sampling sites where assemblage complexity was assessed (3 sites), the exclusion experiment was performed (1 site) and the scuba diving transects were done (16 sites). Shapefile for the Mediterranean Sea downloaded from www.naturalearthdata.com and for the Cabrera Archipelago National Park from www.miteco.gob.es.

2.3.3 Benthic habitat sampling and assemblage complexity

To assess the role that benthic assemblage complexity might have on *C. cylindracea* coverage, different assemblages were surveyed in three sites around the Cabrera Archipelago: Ses Rates, Na Foradada and Freu de la Imperial (**Figure 2.1**). A shallow assemblage (10 m) and a deep assemblage (30 m) were surveyed at each site to take into account the wide range of benthic assemblage complexities (Ballesteros et al., 1993) and contrasting herbivory pressures (Vergés et al., 2009; Tomas et al., 2011b) in relation to depth. Assemblages were sampled in 2005, 2006 and 2007. At each site and depth, three random samples measuring 20 x 20 cm^2 were collected, with the whole benthic cover removed using a hammer and a chisel (Boudouresque, 1971; Sant et al., 2017). After removing the erect algae, and before scraping each quadrat, the cover of each encrusting species was visually estimated to obtain a more reliable value of their abundance in the encrusting layer. After collection, samples were preserved in 4% formalin in seawater, and once in the laboratory, they were sorted and all algae were identified to species level. Species coverage was calculated by placing the species specimens horizontally over a laboratory tray and measuring the area they covered (Ballesteros, 1986). Then, each algal species was assigned to a different category ("Canopy-forming", "Erect", "Turf" and "Encrusting") based on their morphological traits (size and morphology) (**Appendix A: Table A.1**). In order to avoid the effect of assemblage complexity being subject to a confounding effect of the presence of *Caulerpa cylindracea*, this species was not included in any of the previous categories. Finally, the percent cover of each category in the sample was calculated.

Capacity of canopy-forming and erect algae to outcompete *C. cylindracea* in contrast to turf and encrusting species has been experimentally proven for coastal shallow rocky bottoms of the Mediterranean Sea (Ceccherelli et al., 2002; Bulleri and Benedetti-Cecchi, 2008; Piazzi and Balata, 2009; Bulleri et al., 2010). Consequently, complexity of each sample was defined based on the percentage abundance of the "Canopy-forming" and "Erect" categories. Three levels of complexity were defined for the samples based on the percentage of coverage that comprised canopy-forming and/or erect species: "high complexity" (more than 50%); "medium complexity" (between 15% and 50%) and "low complexity" (lower than 15%).

2.3.4 Exclusion experiment

An exclusion experiment was performed to assess whether fish herbivory could act as a biotic resistance mechanism against *Caulerpa cylindracea* invasion by reducing the abundance of the invasive alga. To this end, in order to obtain a proxy of contrasting herbivory intensities, and bearing in mind that herbivory pressure decreases strongly through the water column (Vergés et al., 2009, 2012; Steneck et al., 2017), the exclusion experiment was performed at two different depths: 10 m, where herbivory pressure is high, and 30 m, where it is low (Reñones et al., 1997; Tomas et al., 2011b; Vergés et al., 2012).

This experiment mainly targeted *Sarpa salpa*, because it is the only truly herbivorous fish in the western Mediterranean Sea (Verlaque, 1990; Gianni et al., 2017), it plays an important role structuring algal communities (Vergés et al., 2009) and it regularly consumes *C. cylindracea* (Ruitton et al., 2006; Tomas et al., 2011b). This fish species is quite abundant throughout the Cabrera Archipelago, where it can reach densities up to 14 individuals per 250 m^2 above depths of 20 m (Reñones et al., 1997), spending around 90% of the time above 20 m (Tomas et al., 2011b). S*arpa salpa* is known to have a quite heterogeneous diet (Havelange et al., 1997), but it can also be very selective (Verlaque, 1990; Marco-Méndez et al., 2017) and even shows a preference for *C. cylindracea* over many native species (Tomas et al., 2011b), which makes it a potential candidate for the control of *C. cylindracea.*

The exclusion experiment was set up at the end of June 2011 in Na Foradada (**Figure 2.1**), an area where fish communities are well established, sea urchin (*Paracentrotus lividus* and *Arbacia lixula*) densities are very low (<0.1 per m^2) (Cebrian et al., 2011) and the highest densities of the fish *S. salpa* are found within the National Park, with more than 20 individuals per 250 m^2 (Reñones et al., 1997). Furthermore, we chose this area because both the shallow and the deep benthic habitats displayed a similar medium complexity (with coverage of erect and canopy-forming species at between 25-35%) and similar abundances of native species. This meant there was sufficient abundance of *C. cylindracea* to experimentally assess, in the field, the effect of herbivory pressure on it.

At each depth, 3 treatments were used: "Exclusion", which consisted of cages of 50 x 50 x 50 cm^3 made of plastic netting with a mesh size of 2.5 cm; "Control-Exclusion", consisting of cages with open sides; and "Control", consisting of 50 x 50 cm^2 quadrats marked permanently on the corners and without a cage. A total of 5 interspersed replicates per treatment were set (15 plots per depth) within an area of <100 m^2 to avoid different abiotic conditions between plots. At the beginning (July) and at the end (August) of the experiment, pictures were taken at each plot to subsequently assess *C. cylindracea* abundance, which was calculated with the computer program *photoQuad* version 1.4 (Trygonis and Sini, 2012). In each photograph, 50 random points were placed and then, each of these points was assigned to the category of either "*Caulerpa cylindracea*" or "other algae". The proportion of points in each category was then used as a proxy of the percentage abundance for each of those two categories.

2.3.5 Abundance of *Caulerpa cylindracea*

The abundance of *C. cylindracea* at the Cabrera Archipelago was assessed in 2008 and then again in 2017. To do so, 16 representative sites around the archipelago were chosen (**Figure 2.1**) and a perpendicular transect to shore was performed at each site by means of scuba diving. The depth of the transects ranged from 5 to 45 m to cover the main bathymetric range at which *C. cylindracea* can be found

(Cebrian et al., 2011). The abundance of *C. cylindracea* was estimated by means of 25 x 25 cm^2 quadrats, divided into 25 subquadrats of 5 x 5 cm^2 (Sala and Ballesteros, 1997; Sant et al., 2017) and the number of subquadrats where *Caulerpa* was detected, was used as a unit of abundance. A total of thirty quadrats were randomly positioned within each 5 m-depth range and then the mean *C. cylindracea* abundance per each 5 m-depth stratum was calculated. Also, to take into account the effect that assemblage complexity might have on the bathymetric abundance of *C. cylindracea*, each 5 m-depth range at each site was classified as having either high or low complexity based on the dominant morphological categories in the assemblage ("canopy-forming", "erect", "turf" and "encrusting"), while ignoring the presence of *C. cylindracea* to avoid confounding effects.

2.3.6 Statistical analysis

The effect of benthic assemblage complexity on the cover of *Caulerpa cylindracea* was assessed with binomial generalized linear models (GLM), because the response variable was measured as a percentage (% of *C. cylindracea* coverage in each sample) and it could be approximated to a logistic distribution (e.g.: success = % coverage of *C. cylindracea*, failure = % coverage of species other than *C. cylindracea*). Two models were fitted, one to assess the role of "assemblage complexity" and another to assess the role of both "depth" and "assemblage complexity" on *C. cylindracea* coverage. In the latter, both factors were included as fixed effects and if the interaction between them was significant, it was also included in the model. To fit both models, the statistical environment R was used (R version 3.6.3) (R Core Team, 2018); and to compare the effects between levels in the assemblage complexity factor itself ("high complexity", "medium complexity" and "low complexity"), and in the assemblage complexity factor at each depth ("shallow" and "deep"), Tukey *post hoc* tests were performed using the functions "pairs" and "emmeans" from the package *emmeans* (Lenth, 2018).

To evaluate the effect of the exclusion treatment on the abundance of *C. cylindracea* at the end of the experiment, binomial mixed effects GLMs were used because the response variables were measured as proportions and could be approximated to a logistic distribution (e.g., success = points that corresponded to *C. cylindracea*; failure = points that did not correspond to *C. cylindracea*), while the random terms were used to take into account the repeated measures. In the models, the factors "treatment" and "time" were included as fixed effects, whereas "plot" was included as a random effect. If the interaction between "treatment" and "time" was significant, it was also included in the fixed part of the model. Two models were fitted, one per depth (10 m or 30 m) by means of the package *lme4* (Bates et al., 2015) for R. Tukey *post hoc* tests, which were performed using the functions "pairs" and "emmeans" in the package *emmeans*, were used to compare effects between levels in the treatment factor ("Exclusion", "Control-Exclusion", "Control") at each time observation ("beginning" and "end").

The R code used to perform all the statistical analyses can be found on Zenodo: https://doi.org/10.5281/zenodo.4664432 (Santamaría, 2021).

2.4 Results

2.4.1 Effect of benthic assemblage complexity on *Caulerpa cylindracea* coverage

Benthic assemblage complexity had a significant effect on *Caulerpa cylindracea* coverage (p<0.001, **Appendix A: Table A.2**), with high complexity assemblages supporting low *C. cylindracea* covers (**Figure 2.2, A**). In fact, there were significant differences in *C. cylindracea* cover among the different levels of assemblage complexity (p<0.001, **Appendix A: Table A.3**), with *C. cylindracea* coverage lower than 5% in very complex assemblages, whereas in low complexity assemblages, coverage of the invasive species reached values of almost 30% (**Figure 2.2, A**).

When the depth of the sampled assemblages was included in the model, *C. cylindracea* coverage varied significantly by depth, by assemblage complexity and by the interaction term (**Appendix A: Table A.4**). In fact, although the previous pattern of lower *C. cylindracea* cover in high complexity assemblages is maintained, the coverage of the invasive alga is significantly higher at deeper habitats, independently of assemblage complexity (**Appendix A: Table A.5, A**; **Figure 2.2, B**). In particular, in shallow habitats, high complexity assemblages had 20 times less *C. cylindracea* coverage than low complexity assemblages, but only 10 times less coverage at deeper habitats (**Figure 2.2, B**). At each depth, there were significant differences between all levels of assemblage complexity, except between high complexity and medium complexity assemblages in shallow areas (**Appendix A: Table A.5, B**).

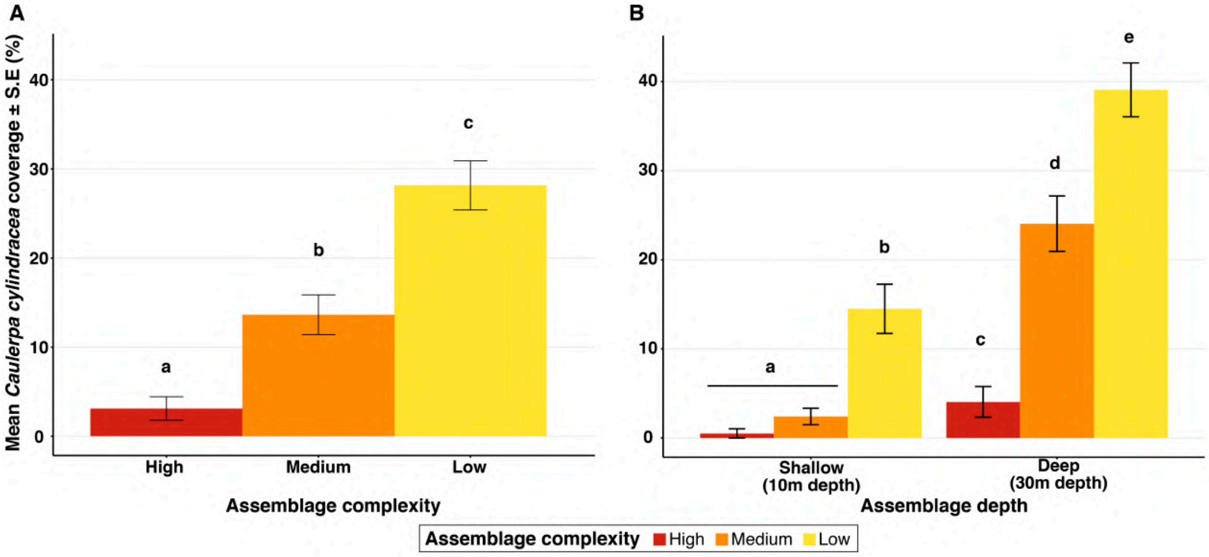

Figure 2.2. Mean *Caulerpa cylindracea* coverage ± S.E for **A)** each level of assemblage complexity, and for **B)** each level of assemblage complexity at the two different depths. Significant differences between assemblage complexity levels (p-values from Tukey´s test with 95% confidence intervals) are indicated with letters.

2.4.2 Exclusion experiment

Significant effects of herbivory on *Caulerpa cylindracea* abundance were only detected at shallow depths (10 m), where *C. cylindracea* abundance varied significantly by treatment, by time and by the interaction between the two (**Appendix A: Table A.6**). While at the beginning of the experiment, all treatment areas displayed similar *C. cylindracea* abundance, both the "Control-Exclusion" and the "Control" treatments exhibited lower *C. cylindracea* abundances at the end of the experiment (p-value < 0.001; **Appendix A: Table A.7, A**), whereas *C. cylindracea* abundance in the "Exclusion" treatment remained constant (**Appendix A: Table A.7, B**). Indeed, at the end of the experiment, *C. cylindracea* abundance was 4.33 and 2.36 times higher in the full exclusion cages compared with the uncaged control plots and the side-open cages, respectively (**Figure 2.3, A**).

On the other hand, at 30 m, the abundance of *C. cylindracea* varied significantly across time but not between treatments (**Appendix A: Table A.6**). In fact, for all three treatment levels, abundance was significantly higher (1.88 times on average) at the end of the experiment, than at the beginning (**Figure 2.3, B**).

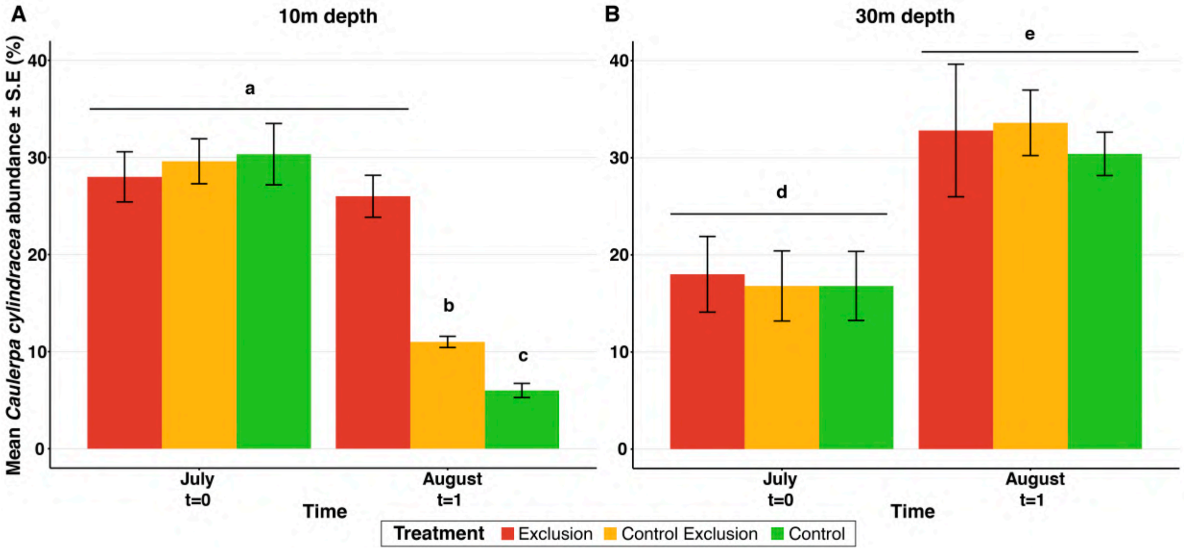

Figure 2.3. Mean *Caulerpa cylindracea* abundance ± S.E., at each time observation and for each treatment in the exclusion experiment. **A)** Exclusion experiment at 10 m depth and **B)** exclusion experiment at 30 m depth. Significant differences between exclusion

treatments (p-values from Tukey´s test with 95% confidence intervals) are indicated with letters in each graph.

2.4.3 Abundance of *Caulerpa cylindracea*

The abundance of *Caulerpa cylindracea* in the Cabrera Archipelago decreased between 2008 and 2017 at depths of between 5 and 35 m, but remained more or less constant below 40 m (**Figure 2.4**). In the entire bathymetric distribution, assemblage complexity showed a considerable effect on *C. cylindracea* abundance, with high complexity assemblages exhibiting lower abundances of the invasive alga than low complexity assemblages. Furthermore, it was in shallow and highly complex assemblages, from 5 to 25 m deep, where *C. cylindracea* reached its lowest abundance, with values ranging between 5 and 10% (**Figure 2.4, B**).

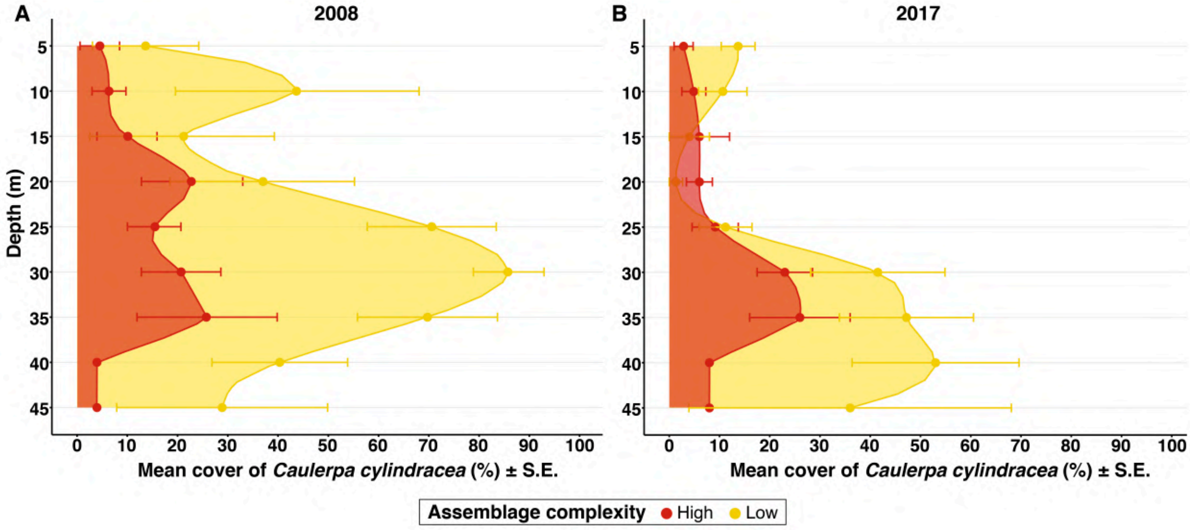

Figure 2.4. Bathymetric abundance of *Caulerpa cylindracea* (mean ± S.E.) at the Cabrera Archipelago National Park: **(A)** in 2008 and **(B)** in 2017, on assemblages with different complexities.

2.5 Discussion

Our results indicate that while both, competition and herbivory, can provide biotic resistance to a certain extent, strong synergistic effects are observed when the two mechanisms act together. Indeed, competition (by canopy-forming and erect algae) and herbivory (by *Sarpa salpa*), significantly affect the invasion outcomes of *C. cylindracea*, particularly limiting its abundance in complex algal assemblages in which herbivory pressure is high.

Benthic assemblage complexity had a strong influence on preventing *C. cylindracea* invasion. The dominance of canopy-forming and erect species resulted in reduced cover of *C. cylindracea*, probably determining resistance to *C. cylindracea* invasion through competition mechanisms (Ceccherelli et al., 2002; Piazzi and Balata, 2009), such as the reduction in light availability, which can limit the photosynthetic performance of *C. cylindracea* (Bernardeau-Esteller et al., 2015; Marín-Guirao et al., 2015; Bernardeau-esteller et al., 2020) and the prevention of reattachment of fragments through the branch-sweeping of the substratum (Bulleri and Benedetti-Cecchi, 2008; Piazzi et al., 2016). In contrast, when the abundance of canopy-forming algae was low and the communities were dominated by turf and encrusting species, *C. cylindracea* cover was much higher. In such habitats, the colonization and spread of *C. cylindracea* seems to be facilitated because turf assemblages provide an optimal 3-D matrix which favors the anchoring of the stolons and trapping of fragments (Ceccherelli et al., 2002; Bulleri and Benedetti-Cecchi, 2008; Bulleri et al., 2009). In this sense, these findings support previous evidence from marine and terrestrial ecosystems, where the presence and dominance of species with certain functional traits (e.g., growth form, size and height) among the assemblage, strongly influences the strength of the biotic resistance against invasive primary producers (Lindig-Cisneros and Zedler, 2002; Arenas et al., 2006; Britton-Simmons, 2006; Byun et al., 2013; Bernardeau-esteller et al., 2020) by limiting one or several essential resources for the invader.

However, the significant differences we observed in the abundance of *C. cylindracea* between shallow and deep communities, regardless of assemblage complexity, suggest that mechanisms other than assemblage complexity are playing an important role on the invasion of *C. cylindracea*, particularly given that *C. cylindracea* can readily colonize habitats from 0 to 50 m depth (Klein and Verlaque, 2008; Cebrian and Ballesteros, 2009). In this sense, although several factors (e.g. propagule pressure, disturbance or abiotic conditions) cannot be disregarded, we suggest that the observed differences in invader abundance mainly reflect the contrasting consumer pressures found between deep and shallow communities. Concretely, given similar assemblage complexity, when herbivory pressure was high (here shallow habitats) (Reñones et al., 1997; Vergés et al., 2009, 2012; Tomas et al., 2011b), the abundance of *C. cylindracea* was significantly lower compared to areas where herbivory pressure was low or non-existent (deeper habitats). Actually both, the exclusion experiment and the pattern of *C. cylindracea* abundance and distribution across the Cabrera Archipelago, further support that herbivory is also contributing to the biotic resistance of native assemblages to the *C. cylindracea* invasion. In this sense, where herbivory is weak, such as in deep habitats or if herbivores have been depleted, *C. cylindracea* is subject to limited control and its abundance depends largely on assemblage complexity, which results in higher abundances of the invader among the native assemblage (**Figure 2.5, A and B**). Similarly, when herbivory pressure is high but assemblage complexity is low, the invader will also suffer limited control (**Figure 2.5, C**). However, when high herbivory pressure is concomitant with high assemblage complexity, both mechanisms (competition and herbivory) strongly limit the abundance of *C. cylindracea* (**Figure 2.5, D**). Taking into account that sea urchin densities were very low in the study area (<0.1 per m^2), the herbivory pressure observed can be mainly attributed to the effect of *Sarpa salpa*, a fish which is mostly distributed at shallow depths and which has a certain preference for *C. cylindracea* (Tomas et al., 2011b). Nevertheless, considering that sea urchins have been previously reported feeding on *C. cylindracea* (Ruitton et al., 2006; Bulleri et al., 2009; Cebrian et al., 2011; Tomas et al., 2011a), additional negative effects on *C. cylindracea* abundance may occur in areas with high sea urchin abundances. These results where the interaction between biotic mechanisms yielded a stronger biotic resistance against the invader than the single mechanisms alone, agree with previous studies where habitat characteristics interacted with herbivory pressure to influence the overall biotic resistance of certain terrestrial ecosystems (Suwa and Louda, 2012; Li et al., 2014; Zhang et al., 2018).

Also, and importantly, by following the long-term dynamics of *C. cylindracea* at the study area, we observe that the overall strength of the assemblage´s biotic resistance has increased over time as the abundance of the invasive alga in assemblages subjected to higher biotic resistance (i.e., communities at depths of between 0 to 25 m), has decreased over a 10-year period (**Figure 2.4**). This regression, restricted to areas with high herbivore pressure, may be the result of either an increase in the abundance of the herbivores or to some herbivores becoming more efficient in consuming the invasive species as the invasion progresses (Strayer et al., 2006; Carlsson et al., 2009). However, given that the abundance of *S. salpa* has remained more or less stable during the assessed period (Coll, 2020), we suspect that this herbivore has become more efficient at targeting the invader and has increased its *per capita* consumption rates over time (Santamaría J., unpublished manuscript, **Chapter 3**). Nevertheless, other mechanisms that can increase biotic resistance, such as, for instance, allelochemical defenses deployed by native species and assemblages against the invader (Strayer et al., 2006), cannot be ruled out.

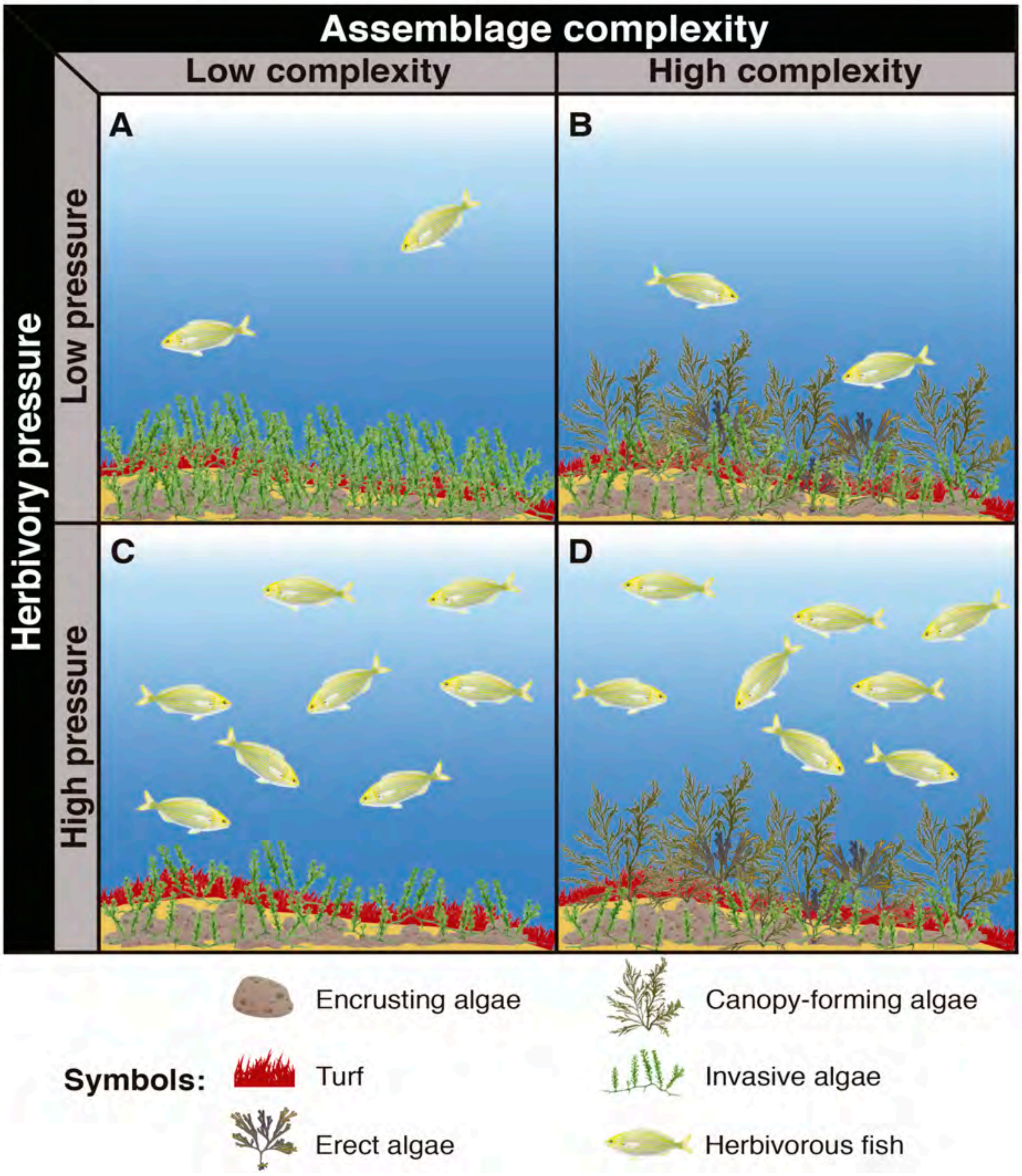

Figure 2.5. Depiction of how different combinations of assemblage complexity and herbivory pressure determine biotic resistance against a marine invasive alga (e.g. *Caulerpa cylindracea*), representing four scenarios: **A)** low assemblage complexity and low herbivory pressure; **B)** high assemblage complexity and low herbivory pressure; **C)** low assemblage complexity and high herbivory pressure and **D)** high assemblage complexity and high herbivory pressure. (Algae illustrations obtained and modified from the Integration & Application Network (IAN) Image Library (Tracey Saxby and Joanna Woerner), the IUCN and freepik (https://www.freepik.com/macrovector); the fish illustration, by João T. Tavares, was obtained from www.deviantart.com).

Unfortunately, in general, knowledge on whether the effects of biotic mechanisms are maintained throughout an invasion process or whether they change in relation to time since invasion is still quite limited, because most studies just assess a particular time frame in the invasion (Maron and Vilà, 2001; Levine et al., 2004; Mitchell et al., 2006; Alofs and Jackson, 2014; Papacostas et al., 2017). To our knowledge, our study is the first that reports the importance of exposure time for the development of resistance against invaders in natural ecosystems (but see: Diez et al., 2010 and Dostál et al., 2013 for assessments in common garden experiments) and highlights that increases in biotic resistance observed over time (due to increased consumer pressure, competitive impacts or a combination of the two), could explain why invaders that have previously been considered as hyper-successful (e.g., *Myriophyllum spicatum*, *Elodea canadiensis*, *Dreissena polymorpha*, *Carcinus maenas*, *Caulerpa* species) can suffer marked reductions in population size some years after the onset of the invasion (Simberloff and Gibbons, 2004; De Rivera et al., 2005; Ivešа et al., 2006; Carlsson and Strayer, 2009; Bernardeau-esteller et al., 2020). Therefore, by focusing on only a small time frame or just the beginning of an invasion, we may be underestimating the true capacity of native assemblages to develop resistance to invaders (Strayer et al., 2006, 2017; Rius et al., 2014; Papacostas et al., 2017). For this reason, the use of long-term data, despite being scarce, should be prioritized whenever possible, to assess the true effect that biotic resistance mechanisms might have on the overall invasion process.

Generally, our findings highlight the importance of considering several factors and their interaction when assessing the strength of biotic resistance mechanisms against a particular invader, especially considering that herbivory and competition are universal processes that operate across ecosystems and that naturally interact with each other (Gurevitch et al., 2000; Meiners and Handel, 2000; Hambäck and Beckerman, 2003). In fact, it has been proposed that herbivory reinforces competition and in turn releases the chance for coexistence, favoring those species that are better competitors (Gurevitch et al., 2000). However, despite that, in invasion ecology, the interaction between these mechanisms has been rarely assessed (but see: Suwa and Louda, 2012; Li et al., 2014; Zhang et al., 2018) and most studies rely on the assessment of single biotic mechanisms (Maron and Vilà, 2001; Levine et al., 2004; Vilà and Weiner, 2004; Kimbro et al., 2013; Papacostas et al., 2017, and references therein). This can definitively underestimate the true role of biotic processes (e.g., competition and herbivory) against invasive species and may explain why our findings, reporting a strong effect of biotic mechanisms against a successful invader, contrast with many previous studies. Actually, our results, together with those recently reported for mangrove ecosystems (Li et al., 2014; Zhang et al., 2018), where a successful invader (*Spartina alterniflora*) was limited and excluded due to the interaction between competition and herbivory, suggest that synergisms between biotic resistance mechanisms may be an important but overlooked process driving the invasion success of plant invaders (**Figure 6**). In this sense, *in situ* field experiments, in which competition (e.g., assemblage complexity), herbivory, and the interaction between the two can be simultaneously assessed, can provide a better understanding of the true extent of biotic resistance against an invader (Levine et al., 2004; Mitchell et al., 2006; Kimbro et al., 2013; Li et al., 2014; Enge et al., 2017; Zhang et al., 2018; Petruzzella et al., 2020) and will definitively help in the understanding of the invasion success and the dynamics of different invaders.

Finally, the findings reported in this study highlight the importance of improving our knowledge regarding the factors that influence invasive species success in order to fully understand the invasion process of different species and adopt successful mitigation and management measures. As a practical example, while the removal of invasive algae has proven to be, in most cases, ineffective or infeasible (Epstein and Smale, 2017; Giakoumi et al., 2019a), results obtained in this and other studies (Caselle et al., 2018; Bernardeau-esteller et al., 2020) suggest that by promoting the conservation of marine habitats and herbivorous populations, we can foster biotic resistance within an ecosystem-based approach to marine environment management and contribute to the long-term control of marine invasions.

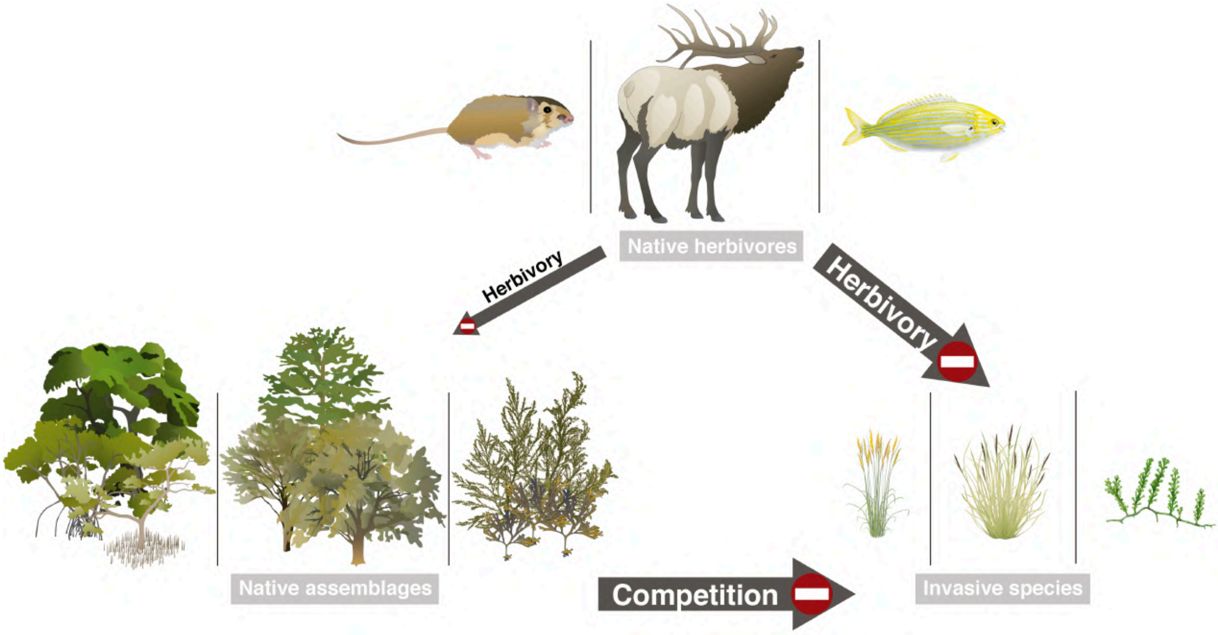

Figure 2.6. Schematic representation of how the interaction between competition and herbivory might determine the overall biotic resistance against an invader. Depicted illustrations correspond to *Ratus losea*, *Cervus elaphus* and *Sarpa salpa* as native herbivores; a mangrove forest, a temperate forest and a marine algal forest as native assemblages; and two grasses and an alga species (here *Caulerpa cylindracea*) as invasive species. (Illustrations obtained from the Integration & Application Network (IAN) Image Library (Tracey Saxby, Kim Kraeer and Lucy Van Essen-Fishman), the IUCN and the fish illustration, by João T. Tavares, was obtained from www.deviantart.com).

2.6 Acknowledgments

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Chapter 3

Learning takes time. Biotic resistance by native herbivores increases through the invasion process.

In preparation

Cover image: *Sarpa salpa* targeting the native alga *Cystoseira compressa* in one of the paired-choice feeding experiments.

Author: Jorge Santamaría Pérez.

3.1 Abstract

The arrival of non-native species into a new habitat normally triggers the development of novel interactions that can influence the success of invaders. Among those, predator-prey interactions have been reported to reduce the invasion success of several species, but it is also common to observe invaders being released from the effects of native enemies, thus escaping biotic control. Therefore, several mechanisms have been proposed to affect the strength and direction of the predator-prey interactions. Surprisingly, despite ecological interactions being dynamic processes, native predator-invasive prey interactions are normally assessed considering a fixed time frame, thus making it difficult to infer whether they can shift through the invasion process. Actually, both the exposure time and the abundance of the invader in the native community can potentially influence the strength and the development of these novel interactions. In this study we use a Space-for-Time substitution methodology to assess whether the strength of the interaction between a Mediterranean keystone native herbivore (*Sarpa salpa*) and a hyper-successful invasive alga (*Caulerpa cylindracea*) is mediated by the exposure time to the invader and/or by its availability in the community. Our results show that preference for *Caulerpa* develops after at least 5 years since the introduction and first contact of the invader with the herbivore, regardless on the abundance of the invader; whereas the percentage of herbivores feeding on *Caulerpa* and the *per capita* consumption rates were influenced by both, the exposure time and the availability of the invasive alga. In this sense, sites historically invaded and with higher invader abundances, where the ones that supported the strongest predator-prey interaction compared to sites recently invaded. Remarkably, even when the abundance of the invader decreased with time, the electivity towards the invasive alga continued increasing through the invasion, suggesting that the learned herbivory preference is continuously transmitted within the grazer population, even when *Caulerpa* abundance is anecdotic. Overall, our findings suggest that novel predator-prey interactions need time to develop and that their strength can increase through the invasion process, thus becoming an effective biotic resistance mechanism.

3.2 Introduction

Biological invasions, together with climate change, land use and pollution, are currently considered one of the leading drivers of the current biodiversity crisis due to the profound impacts they cause in native ecosystems (Butchart et al., 2010; Brondizio et al., 2019; Pyšek et al., 2020). These negative impacts also cascade through the ecosystem, affecting the normal functioning of the system, disrupting the provision of important ecosystem services (Simberloff et al., 2013; Vilà and Hulme, 2017) and even impacting the human well-being and health (Pejchar and Mooney, 2009; Pyšek and Richardson, 2010). For these reasons, the management of invasive species has been positioned as an urgent priority at the global scale (Pyšek et al., 2020), but still, rates in the accumulation of invasive species have been growing for the past decades (Hulme, 2009; Seebens et al., 2017). Therefore, in order to find effective management measures for the control of the current invasions and those to come, we need to improve our knowledge on the long-term dynamics of successful invasive species and on how they interact with the native communities (Seebens et al., 2017).

It is well known that the arrival of an invader will develop several novel interactions in the receiving community – either in the form of competition or predation – and their strength and direction will be key for the success of non-native species (Colautti et al., 2004; Levine et al., 2004; Sih et al., 2010; Pintor and Byers, 2015). Among them, predator-prey interactions have been reported to reduce the invasion success of several species by predators directly consuming the invader (Maron and Vilà, 2001; Levine et al., 2004; Carpenter and Cappuccino, 2005; De Rivera et al., 2005; Carlsson and Strayer, 2009; Carlsson et al., 2011; Robbins et al., 2013; Kremer and da Rocha, 2016; Caselle et al., 2018; Zhang et al., 2018). However, many studies have also reported that invasive species can be commonly released from the effects of their natural enemies (Maron and Vilà, 2001; Keane and Crawley, 2002; Colautti et al., 2004; Liu and Stiling, 2006), allowing the invader to escape biotic control. In this sense, several characteristics have been proposed to influence the strength and direction of consumer pressure, such as the defense capabilities of invaders and their ability to release allelophatic chemicals (Mollo et al., 2008; Inderjit et al., 2011; Mennen and Laskowski, 2018), the existence of predator avoidance mechanisms (Strauss et al., 2006; Berthon, 2015; Ruland and Jeschke, 2020) or the inability of predators to identify an invader as prey – native predator "naïveté" (Verhoeven et al., 2009; Sih et al., 2010; Kimbro et al., 2013).

However, it is important to consider that most studies assessing predator-prey interactions against invasive species do so disregarding the temporal component of the invasions, just assessing a particular time frame in the invasion process. Assuming that invasions are static in contraposition with their real dynamic nature can definitively influence the perceived strength of the novel predator-prey interactions depending on the time considered (e.g., recent vs. old invasions). Actually, predators, through the exposure time to the invader, can develop adaptations that allow them to increase their feeding efficiency on the novel prey (e.g., Carroll et al., 1998; Phillips and Shine, 2004; Carlsson et al., 2009). However, the role that exposure time might have for the development of effective novel predator-prey interactions is still under debate, because while several studies have reported increased consumptions and damages on invaders as the invasion progresses (Siemann et al., 2006; Carlsson and Strayer, 2009; Diez et al., 2010; Carlsson et al., 2011; Dostál et al., 2013; Stricker et al., 2016; Kurr and Davies, 2017); there are others that have not found a positive relationship between exposure time and the damage or the consumption of invaders (Carpenter and Cappuccino, 2005; Harvey et al., 2013; Pintor and Byers, 2015; Schultheis et al., 2015).

Conflicting evidence on the importance of time since invasion for the success of invasive species suggests that, although time might play a crucial role in the development of novel predator-prey interactions, other factors and the interactions between them might also have an influence for the development of such novel interactions (Mitchell et al., 2006). For instance, despite the importance that prey abundance has for the strength of predator-prey relationships (Wellenreuther and Connell, 2002; Carbone et al., 2011; Davies et al., 2012) and considering that the experience with novel prey follows a density-dependent increase (Saul and Jeschke, 2015), the abundance of the invader in the community has been rarely taken into account when assessing the development of the novel predator-prey interactions (but see: Nelson et al., 2011). Therefore, contributions on whether the abundance of the invader might mediate the increase in novel prey consumption through time and whether both factors – abundance and exposure time – interact to modulate the development of the novel predator-prey interactions, are urgently needed to understand the long-term dynamics of invaders. Unfortunately, as long-term data on most invaders is rare (Strayer et al., 2006), it is difficult to draw stronger conclusions on how predator-prey interactions might develop through time (Strayer et al., 2017) and whether other factors, such as the abundance of the invader, contribute to their development. Nevertheless, the use of Space-for-Time methodologies or chronosequences, where populations with different invasion times are studied, provides a cost-effective, viable and valid alternative to Time-for-Time methodologies (long-term studies) (Thomaz et al., 2012; Dostál et al., 2013; Gruntman et al., 2017). Actually, this methodology might allow to effectively assess whether the strength of the novel consumer-prey interactions shifts throughout the invasion process in relation to exposure time alone or in combination with other factors such as the invasion intensity. Finally, the use of these types of approaches could definitively enhance our understanding of the long-term dynamics of certain invasive species and could provide us with important information for improving the management of invasions.

In this study, by using a Space-for-Time substitution methodology, we assess whether the strength of the predator-prey interaction between a keystone herbivore and a hyper-successful invader is mediated by the time since the invasion and/or by the abundance of the invader in the community. Preference and consumption assessments were carried in populations with contrasting exposure times and abundances of the invader, to assess whether the preference feeding, the number of herbivores feeding on the invader and the consumption *per capita* rates of the herbivore are influenced by the temporal and numerical characteristics of the invasion. Additionally, to further disentangle the influence of time since invasion on the strength of the predator-prey interaction, electivity for the invader was assessed at three succeeding sampling times in two populations characterized for their differential exposure time to the invader.

3.3 Materials and methods

3.3.1 Target species

Caulerpa cylindracea is a siphonaceous green alga native to the Southwestern coast of Australia that is invasive in the Mediterranean Sea and in some regions in the Atlantic Ocean and in the Indian Ocean (Klein and Verlaque, 2008). In the Mediterranean Sea, *C. cylindracea* has experienced a rampant success, where it has colonized marine communities throughout the entire basin in less than 20 years since its first detection (Piazzi et al., 2005; Klein and Verlaque, 2008). Ecologically, *C. cylindracea* is able to form dense mono-specific meadows that can grow on top of the benthic assemblages, causing strong negative impacts on the native communities (Piazzi et al., 2001; Klein and Verlaque, 2008; Bulleri et al., 2017). For all these reasons, *C. cylindracea* is currently considered the most successful and harmful invasive species within the Mediterranean Sea (Klein and Verlaque, 2008; Katsanevakis et al., 2016) and has been recently ranked as one of the marine invaders with the highest negative ecological impacts worldwide (Anton et al., 2019), which definitively allow us to consider *C. cylindracea* as a hyper-successful invader. The reasons explaining its invasive success can be several such as the production of herbivore deterrent metabolites, its high growing rates or the presence of vegetative propagation mechanisms. However, over the last years, several meadows have suffered steep declines after a period of successful dominance (Klein and Verlaque, 2008; García et al., 2016; Cefalì et al., 2020; Santamaría et al., 2021), which suggests that natural communities might be developing effective resistance mechanisms against *C. cylindracea* invasion over time. In this sense, due to the accurate data available on the *C. cylindracea* invasion and to the fact that several native organisms have been observed feeding on the invasive alga (Ruitton et al., 2006; Cebrian et al., 2011; Terlizzi et al., 2011; Tomas et al., 2011b; Santamaría et al., *submitted*), *C. cylindracea* is an ideal species to study how the novel predator-prey interactions might develop and to assess whether the strength of those interactions shifts throughout the invasion process and/or in relation to the abundance of the invader at the study site.

Regarding the studied fish species, *Sarpa salpa* is the only true herbivorous fish in the study area (Verlaque, 1990; Gianni et al., 2017) and it plays an important role in structuring seagrass and macroalgae communities (Tomas et al., 2005; Vergés et al., 2009). This species is quite abundant in the shallow communities along the NW Mediterranean Sea (Bell, 1983; Dufour et al., 1995; Reñones et al., 1997; Tomas et al., 2005) and spends most of its time above 20 m (Bell, 1983; Tomas et al., 2011b), where it feeds on a wide variety of species (Verlaque, 1990; Havelange et al., 1997). *S salpa* regularly consumes *C. cylindracea* (Ruitton et al., 2006; Tomas et al., 2011b) and a previous study has reported that it even prefers the invasive alga over many native species (Tomas et al., 2011b).

3.3.2 Study sites

This study was conducted in 3 regions in the NW Mediterranean Sea: the Cabrera Archipelago, Menorca Island and the Catalan Coast (**Figure 3.1**), based on the previous long-term knowledge on the *Caulerpa cylindracea* invasion in these regions.

In the Cabrera Archipelago, *C. cylindracea* was detected in 2003 at 30 m deep (Cebrian and Ballesteros, 2009) and quickly expanded through the entire Archipelago. Nowadays it can be found in most of the benthic habitats at depths from 0 to 65 m, where it can be the dominant species (Cebrian et al., 2011). However, in the past few years, the abundance of the invasive alga has experienced a decline in the Archipelago, mainly at shallow depths (Santamaría et al., 2021).

In Menorca, *C. cylindracea* was first detected in 2006, in photophilic assemblages (≈20 m deep) from the south of the island (Illa de l´Aire) (Pons-Fàbregas et al., 2007), and then it started to move northwards and to shallower depths. From 2010 onwards, *C. cylindracea* can be found in many locations around the entire island and in some places it dominates benthic assemblages at depths from 5 to 45 m (Massutí et al., 2015), although recent reports show that its abundance is decreasing (Cefalì et al., 2020)

In the Catalan Coast, *C. cylindracea* was first detected in 2008 in the southern part, at depths from 20 to 50 m (Ballesteros et al., 2008). The invasive alga maintained this restricted distribution until 2013, when it was detected towards the north, in Blanes (García et al., 2016). Since then, it has followed with its expansion northwards and nowadays it can be found in several locations in the northern part (María García, *personal communication*).

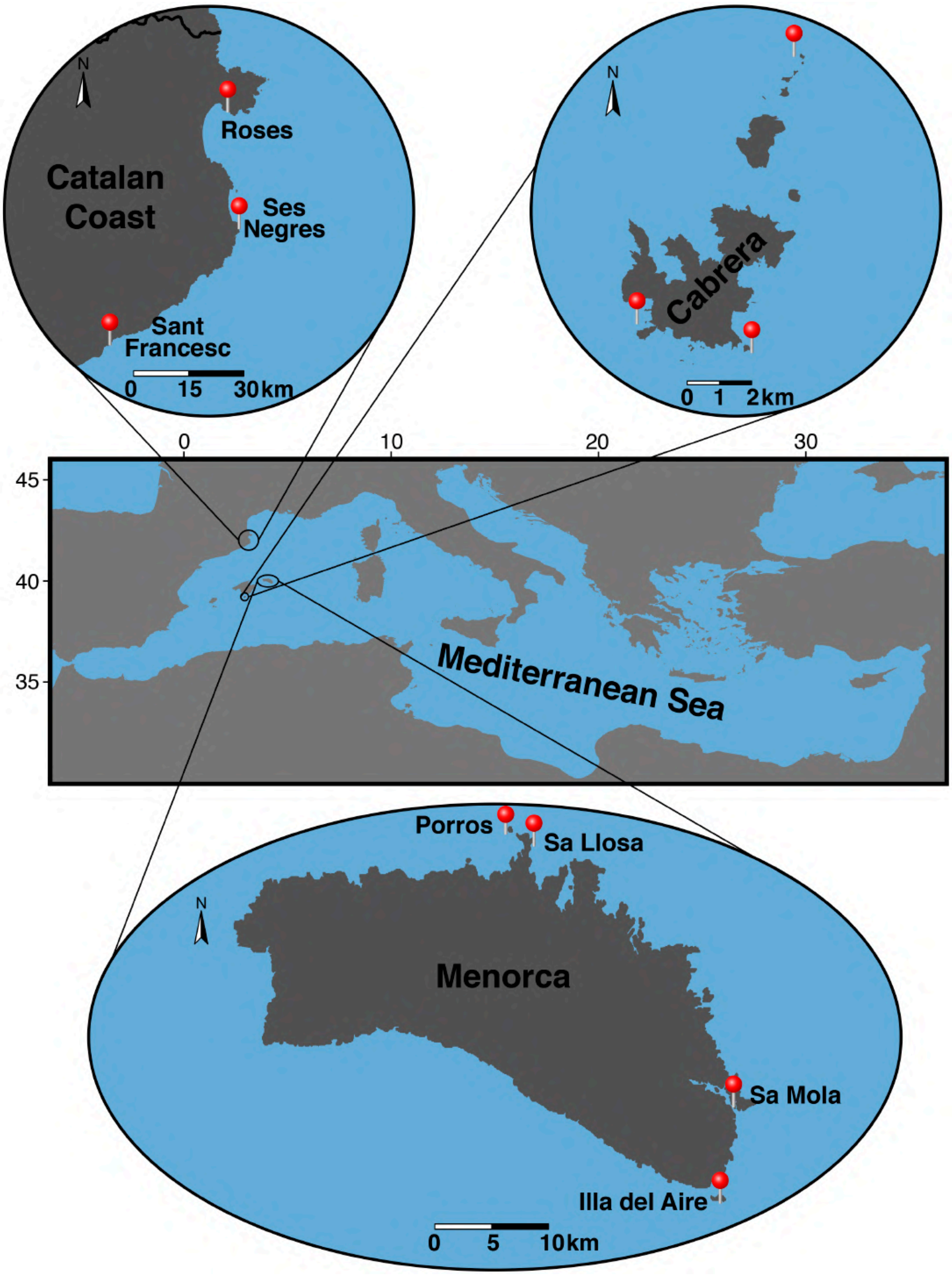

Figure 3.1. Location of the 3 regions where the study was carried (the Catalan Coast, the Cabrera Archipelago and Menorca). The points show the specific locations where different assessments were done. Shapefile for the Mediterranean Sea downloaded from www.naturalearthdata.com, for the Catalan Coast and Menorca downloaded from the Global Self-consistent, Hierarchical, High-resolution Geography Database (GSHHG) and for the Cabrera Archipelago downloaded from www.miteco.gob.es.

3.3.3 Preference assessment

To assess whether *Sarpa salpa*´s preference for *Caulerpa cylindracea* changes in relation to time since invasion and/or in relation to the abundance of the invader in the assemblage, paired-choice feeding experiments were performed in different locations in Menorca and the Catalan Coast. These regions were chosen because, due to the particularities of *C. cylindracea* invasion in both regions, it is possible to find locations with contrasting abundances of the invader and with different times since the invasion.

To determine time since invasion in a certain location, long-term records of *C. cylindracea* presence, available from the research team, were consulted. Based on year since first detection, locations were classified as "Old" when *C. cylindracea* was first detected more than 5 years ago, "or "Recent" when *C. cylindracea* was detected less than 5 years ago. Then, the abundance of *C. cylindracea* at depths from 10 to 15 m was assessed by means of scuba diving. At each location, thirty 25 x 25 cm^2 quadrats, divided into 25 subquadrats of 5 x 5 cm^2 (Sala and Ballesteros, 1997) were randomly positioned and the number of subquadrats where *Caulerpa* was present was used as the unit of abundance. Then the mean *C. cylindracea* abundance was calculated for each location. In this sense, if the abundance of *C. cylindracea* was higher than 30%, locations were classified as "High", whereas if the abundance was lower than 30%, locations were classified as "Low".

A total of 8 locations were chosen for the preference assessment and were classified following the previous criteria as follows: 4 locations from Menorca – Illa del Aire (Old-High), Sa Mola (Old-Low), Porros (Old-High) and Sa Llosa (Recent-Low); and 4 locations from the Catalan Coast – Sant Francesc (Old-Low), Roses 2019 (Recent-Low), Roses 2018 (Recent-High) and Ses Negres (Recent-Low) (**Appendix B: Table B.1**).

In each location, paired-choice feeding experiments were performed to compare the relative palatability of *C. cylindracea* vs. two native macroalgae species:

Cystoseira compressa, an important habitat-forming alga that is highly palatable (Vergés et al., 2009) and that is commonly found in *S. salpa*´s diet (Verlaque, 1990); and *Padina pavonica*, a photophilic alga that is common from the sublittoral zone in warm-temperate coasts (Sala, 1997; Bürger et al., 2017) and that is also a usual food source for *S. salpa* (Verlaque, 1990) (**Figure 3.2**). In this sense, there were 2 treatments: i) *Caulerpa* – *Cystoseira* and ii) *Caulerpa* – *Padina* and 3 controls, one per alga species. Each replicate in each treatment was formed by a couple of clothespins attached to each other with zip-tie and a piece of weight attached to the clothespins to keep them in place underwater. Controls were formed by one clothespin protected from herbivores with a plastic mesh cage (0.5 cm mesh size) and they were also attached to a piece of weight. The clothespins were used to hold the algae fragments and avoid losing them due to currents.

A total of 7 replicates for each treatment and 5 replicates for each control were deployed at each of the eight locations, always at the same depth (≈10 m) to avoid potential confounding effects, in areas where there were no sea urchins, and in patches with similar macroalgae assemblages to guarantee that the fishes were choosing between the offered food choices always under the same conditions. The replicates were carefully placed on the sea floor to guarantee that clothespins were in an upright position and that all algae were easily accessible to fishes. Replicate pairs were 1 m apart from each other, whereas treatments were less than 20 m apart to maintain constant environmental conditions between them. All the experiments were performed at the end of the summer (in 2018 and in 2019), which corresponds to the period of the year when *S. salpa* feeding activity is more intense to accumulate reserves for the winter and to be prepared for reproduction (Peirano et al., 2001). Samples were deployed in the morning and collected after 24 h; and before and after deployment, every algae fragment was pad-dried of excess water and wet weighted to the nearest 0.01g. In all pairs, similar initial weights for each alga were offered to herbivores.

Biomass consumption was estimated with the formula:

$$\left(H_i\ x\ \frac{C_f}{C_i}\right) - H_f$$

where H_i and H_f were initial and final wet weights of algae exposed to herbivory and C_i and C_f were initial and final mean wet weight in controls (Cronin and Hay, 1996; Parker and Hay, 2005; Tomas et al., 2011b). Consumption values were then transformed to percentage of consumed alga to standardize them.

3.3.4 Consumption assessment

To assess whether i) the % of fish feeding on the invader and ii) the *per capita* consumption rates on the invasive alga (total amount consumed), change in relation to time since invasion and/or in relation to the abundance of the invader in the assemblage, fish fecal pellets were collected by means of scuba diving from the same locations where the preference assessment was done (**Figure 3.2**). In each location, the day after the samples from the preference experiment were collected, schools of *S. salpa* were followed throughout their depth range and the fecal pellets were collected in the water column with individual zip bags while swimming below the fishes. Between 30 and 50 pellets were collected per location and after collection, the pellets were preserved in buffered 4% formaldehyde-seawater until the laboratory assessment. This non-invasive method was used to diminish human impact in the study areas and because it has been previously used to characterize *S. salpa*´s feeding habits (Tomas et al., 2011b).

Caulerpa cylindracea presence (% of fish feeding in the invader) and abundance (*per capita* consumption rates) in fecal pellets was determined in the laboratory by using a reticulated Petri dish under a stereomicroscope Stemi 2000-C (Carl Zeiss, Berlin, Germany). Pellet content was spread in the dish and the relative abundance of *C. cylindracea* in each pellet was estimated as the mean percentage cover that it occupied in relation to the rest of the content.

		Old-High			Old-Low		Recent-High			Recent-Low			
Variable of Interest	Type of Assessment / Locations	Cabrera (2020)	Illa del Aire	Porros	Sa Mola	Sant Francesc	Cabrera (2007)	Cabrera (2008)	Roses (2018)	Sa Llosa	Roses (2020)	Roses (2019)	Ses Negres
Preference	Pair-choice Experiment	✗	✓	✓	✓	✓	✗	✗	✓	✓	✗	✓	✓
Incidence on Herbivore *Per capita* consumption	Content in pellets	✗	✓	✓	✓	✓	✗	✗	✓	✓	✗	✓	✓
Electivity	Ivlev´s Electivity Index	✓	✗	✗	✗	✗	✓	✓	✓	✗	✓	✓	✗

Figure 3.2. Schematic representation of the different methodologies used in this study to assess the predator-prey interaction between the native herbivore *Sarpa salpa* and the invasive alga *Caulerpa cylindracea* in different locations characterized by contrasting exposures times and abundances of the invader in the community. The green tick indicates that the assessment was performed in that location, whereas the red cross indicates that the assessment was not performed in that location. Illustrations were obtained and modified from the IUCN, Henry Bradbury´s and Greville´s original illustrations (Algae Britannicae), devianart and freepik.

3.3.5 Assessment of the electivity towards *Caulerpa cylindracea* throughout the invasion

To assess whether *S. salpa*´s electivity towards *C. cylindracea* changes in relation to time since invasion, the Ivlev´s Electivity Index (E) (Ivlev, 1961) was calculated in two locations: Roses, at the 2nd, 3rd and 4th year after *C. cylindracea* invasion – first record in 2016; and the Cabrera Archipelago, at the 4th, 5th and 17th year after the arrival of *C. cylindracea* – first record in 2003 (**Figure 3.2**).

To determine E at each location in each time period, the following formula was used:

$$E = \frac{(d_i - a_1)}{(d_i + a_i)}$$

where d_i = % of *C. cylindracea* in the stomach content of *S. salpa* (see the consumption assessment section) and a_i = % of *C. cylindracea* available in the environment (see the preference assessment section). The values of the Ivlev´s Index (E) can range from -1 (complete avoidance of the food item) to +1 (exclusive selection of the item), with positive values indicating that the food item is selected and eaten more than it is encountered by chance in the environment (Ivlev, 1961).

3.3.6 Statistical analysis

To assess whether *S. salpa* preference changes with time since invasion and/or with the abundance of *C. cylindracea* in the assemblage, the data from the paired-choice assays was analyzed in the statistical environment R (R version 3.6.3) (R Core Team, 2018), with paired Student t-tests when data was normal and homoscedastic, and with Wilcoxon signed-ranks paired tests when data was not normal or was heteroscedastic. Replicates in which fish did not feed on any of the algae were discarded from the statistical analyses, because they do not provide any information on preference.

To evaluate the effect of time since invasion and abundance of the invader on the % of fishes feeding on the invader and on the *per capita* consumption rates of *C. cylindracea*, generalized linear models (GLMs) were fitted to the fecal pellet data. In this case, binomial models were used because the response variables were measured either as 0-1 data (presence/absence of *C. cylindracea* in the pellets - % of fishes feeding on the invader) or as proportions (abundance of *C. cylindracea*: % of the pellet content that corresponded to the species – *per capita* consumption rates) and could be approximated to a logistic distribution. Two models were fitted in R, one for the presence/absence data and the other for the abundance data. In both models, the factors "time since invasion" and "abundance of the invader" were included and if the interaction between them was significant, it was also included in the model. Tukey *post hoc* tests were performed using the functions "pairs" and "emmeans" from the *emmeans* package (Lenth, 2018) to compare effects in the time since invasion factor ("recent" and "old") at each level of abundance ("high" and "low") when the interaction between the factors was significant.

To assess whether there were differences in the Ivlev´s Electivity Index values between years at each of the studied locations (Roses and the Cabrera Archipelago), Kruskal-Wallis tests were performed due to the lack of normality in the data (Kruskal and Wallis, 1952). Then, to compare effects between years, Dunn´s *post hoc* tests (Dunn, 1964) were performed using the *FSA* package in R (Ogle et al., 2020), correcting the p-values with the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

3.4 Results

3.4.1 Preference assessment

The preference of *Sarpa salpa* towards *Caulerpa cylindracea* is influenced by time since invasion but not by the abundance of the invader in the community. In this sense, when the invasive alga has been in the community for more than 5 years, *S. salpa* has a total preference for *C. cylindracea*, no matter whether the abundance of the invader in the community is high or low (**Figure 3.3, A and B**). In fact, the native fish showed at least a 2.5-fold preference for *C. cylindracea* over any of the native species (**Figure 3.3, A and B**), both in places with high and with low abundance of the invader.

However, in populations where the invasion of *C. cylindracea* is recent, *S. salpa* does not exhibit a preference for the invader, neither when *C. cylindracea* abundance in the community is high, nor when it is low (**Figure 3.3, C and D**). In this sense, although *S. salpa* does not show a preference for either of the offered algae, it generally consumed more from the native species than it did from the invasive one (**Figure 3.3, C and D**).

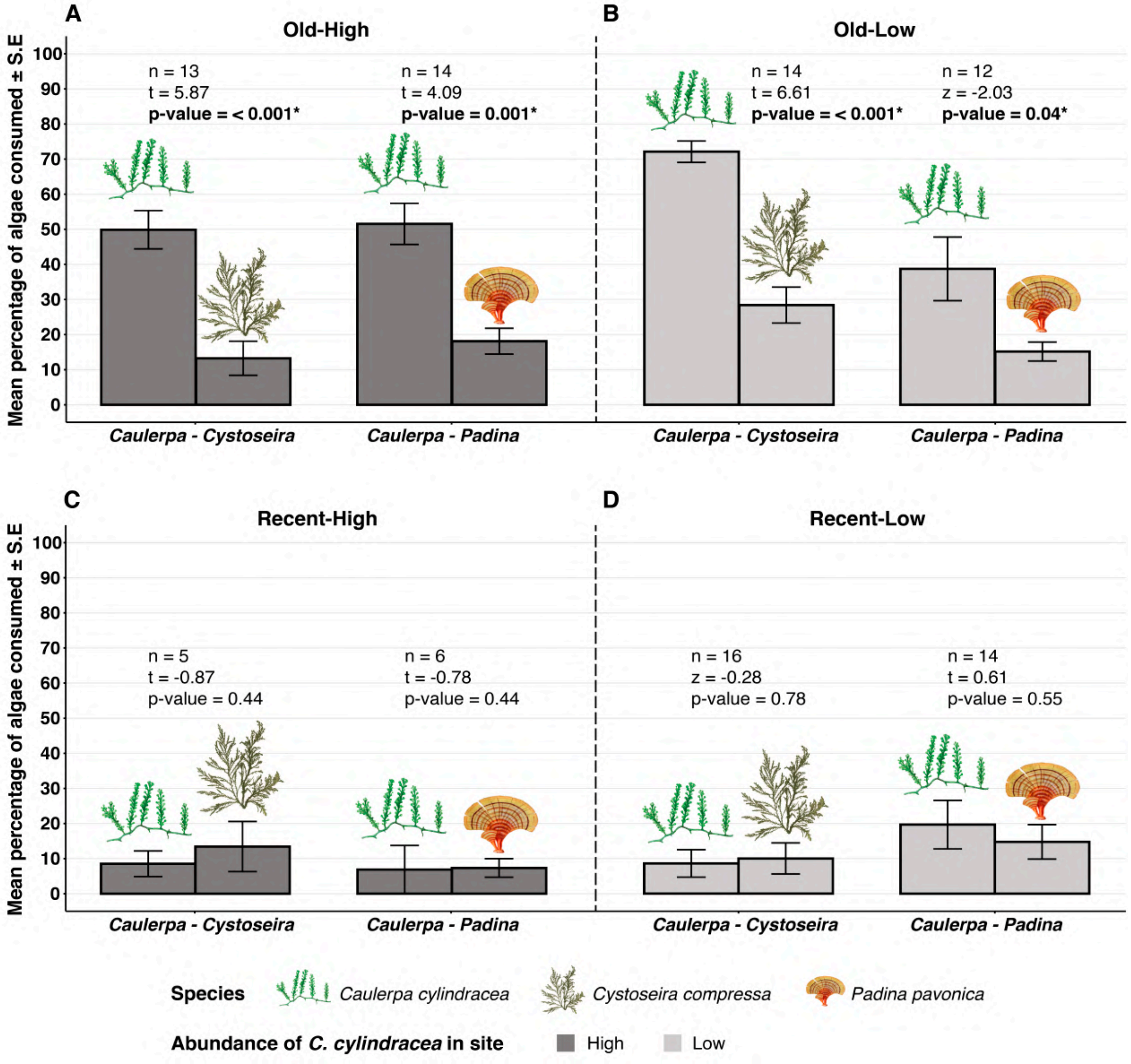

Figure 3.3. Results from the paired-choice feeding experiments performed at: **A, B)** locations that have been invaded by *Caulerpa cylindracea* for more than 5 years (Old locations) and where the abundance of the invader in the community was either **A)** high (dark gray bars) or **B)** low (light grey bars); and **C, D)** locations that have been invaded by *Caulerpa cylindracea* for less than 5 years (Recent locations) and where the abundance of the invader in the community was either **C)** high (dark gray bars) or **D)** low (light grey bars). Bars represent the mean percentage of algae consumed ± S.E.. The illustrations represent the algae species used in the experiments: invasive species (*Caulerpa cylindracea*), native species (*Cystoseira compressa* and *Padina pavonica*). Text on top of the bars refers to the number of replicates (n), statistics from either paired Student T-tests (t) or Wilcoxon signed-ranks paired tests (z) and the probability values for those statistics (p-value). P-values in bold highlight the pairs where significant differences were detected. (Algae illustrations were obtained and modified from the IUCN, Henry Bradbury´s and Greville´s original illustrations (Algae Britannicae)).

3.4.2 Consumption assessment

The % of fishes feeding on *C. cylindracea* varied significantly by the interaction term (p-value < 0.01; **Appendix B: Table B.2**), indicating that the number of *S. salpa* feeding on *C. cylindracea* is dependent both on the time since the invasion and on the abundance of the invader in the community. In this sense, at each level of exposure time to the invader, the number of fish eating *C. cylindracea* was significantly higher when the abundance of the invader was high (p-value < 0.05; **Appendix B: Table B.3, A**), with at least 2 times more fishes targeting the invader than in populations where *C. cylindracea* abundance was low (**Figure 3.4, A**). Additionally, time since invasion significantly influenced the number of fishes targeting the invader in places with high abundances (p-value < 0.001; **Appendix B: Table B.3, B**), with 2 times more fishes consuming *C. cylindracea* in the Old-High populations than in the Recent-High populations (**Figure 3.4, A**). In summary, more than 90% of the *S. salpa* individuals consumed the invasive alga in the populations that had a high abundance of *C. cylindracea* and that had been invaded for a long time; whereas only 21% of the *S. salpa* individuals consumed *C. cylindracea* in populations that were invaded recently and where the abundance of the invader was low (**Figure 3.4, A**).

Similarly, the *per capita* consumption rates of *C. cylindracea* by *S. salpa* are also dependent on both, the time since the invasion and the abundance of the invader in the community (significant interaction term (p-value < 0.001); **Appendix B: Table B.4**). In this sense, *S. salpa* significantly consumed more *C. cylindracea* in communities with a high abundance of the invader, regardless on time since invasion (p-value < 0.001; **Appendix B: Table B.5, A**)(**Figure 3.4, B**). However, a significant difference in the *per capita* consumption rates of *C. cylindracea* between old and recent populations was only detected under high abundances of the invader (p-value < 0.001; **Appendix B: Table B.5, B**). In particular, in communities with a high abundance of *C. cylindracea*, consumption of the invader was almost 7 times higher in the old populations than in the recent populations, whereas in communities with a low abundance of *C. cylindracea*, consumption in old and in recent populations was similar (**Figure 3.4, B**).

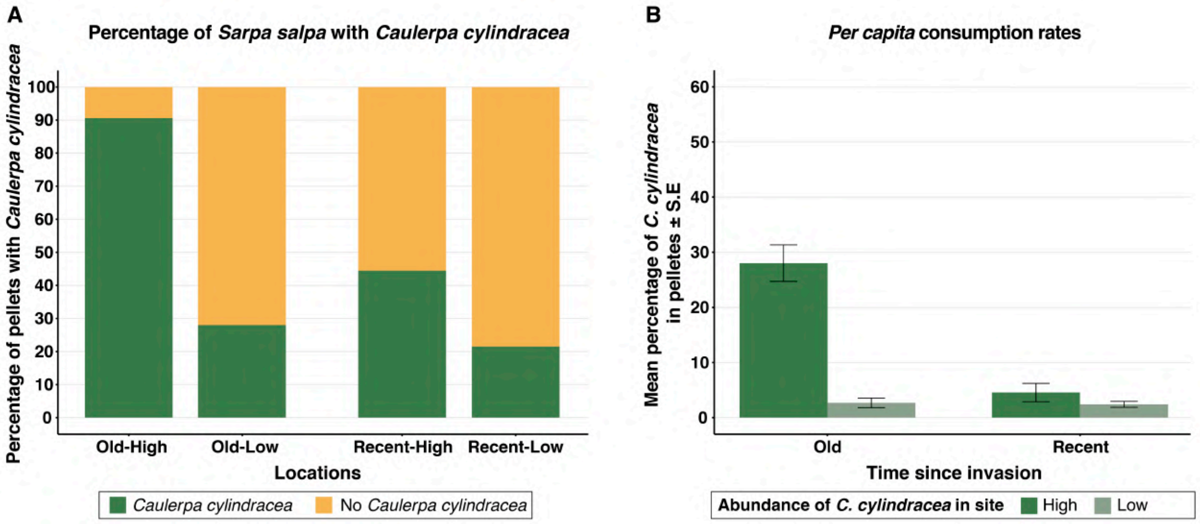

Figure 3.4. A) Percentage of *Sarpa salpa* invidual feeding on *Caulerpa cylindracea* (percentage of pellets with the invader) in old and in recent locations, with high and with low abundances of the invader in the community. **B)** *Per capita* consumption rates of *Sarpa salpa* on *Caulerpa cylindracea* (mean percentage of *C. cylindracea* in pellets ± S.E.), in old and in recent locations, with high and with low abundances of the invader in the community.

3.4.3 Electivity assessment

In Roses, where the invasion of *C. cylindracea* just started a few years ago, *S. salpa* showed a negative electivity for *C. cylindracea* (**Appendix B: Table B.6**), but the electivity towards the invader has increased throughout the invasion (**Figure 3.5-Roses; Appendix B: Table B.6**). Despite this, no significant differences were detected in the Ivlev´s Index values between years (p-value > 0.05; **Appendix B: Table B.7**).

In the Cabrera Archipelago, where *C. cylindracea* has been well established for a long time (~17 years), the electivity of *S. salpa* towards *C. cylindracea* has increased throughout the invasion and nowadays the native fish has a positive electivity for the invader (**Figure 3.5-Cabrera; Appendix B: Table B.6**). Actually, the Kruskal-

Wallis test detected significant differences in the Ivlev Index between years (p-value < 0.001; **Appendix B: Table B.7**), being the Electivity Index significantly higher in 2020 than in 2007 and 2008 (p-value < 0.01; **Appendix B: Table B.8**).

Overall, we observe that independently on the invader abundance, the number of specimens showing a total avoidance of *C. cylindracea* (-1 values; **Figure 3.5** – first years of the invasion) decreases through the invasion and a higher proportion of specimens moves towards a positive electivity for the invader (positive values; **Figure 3.5** – year 17 after the invasion).

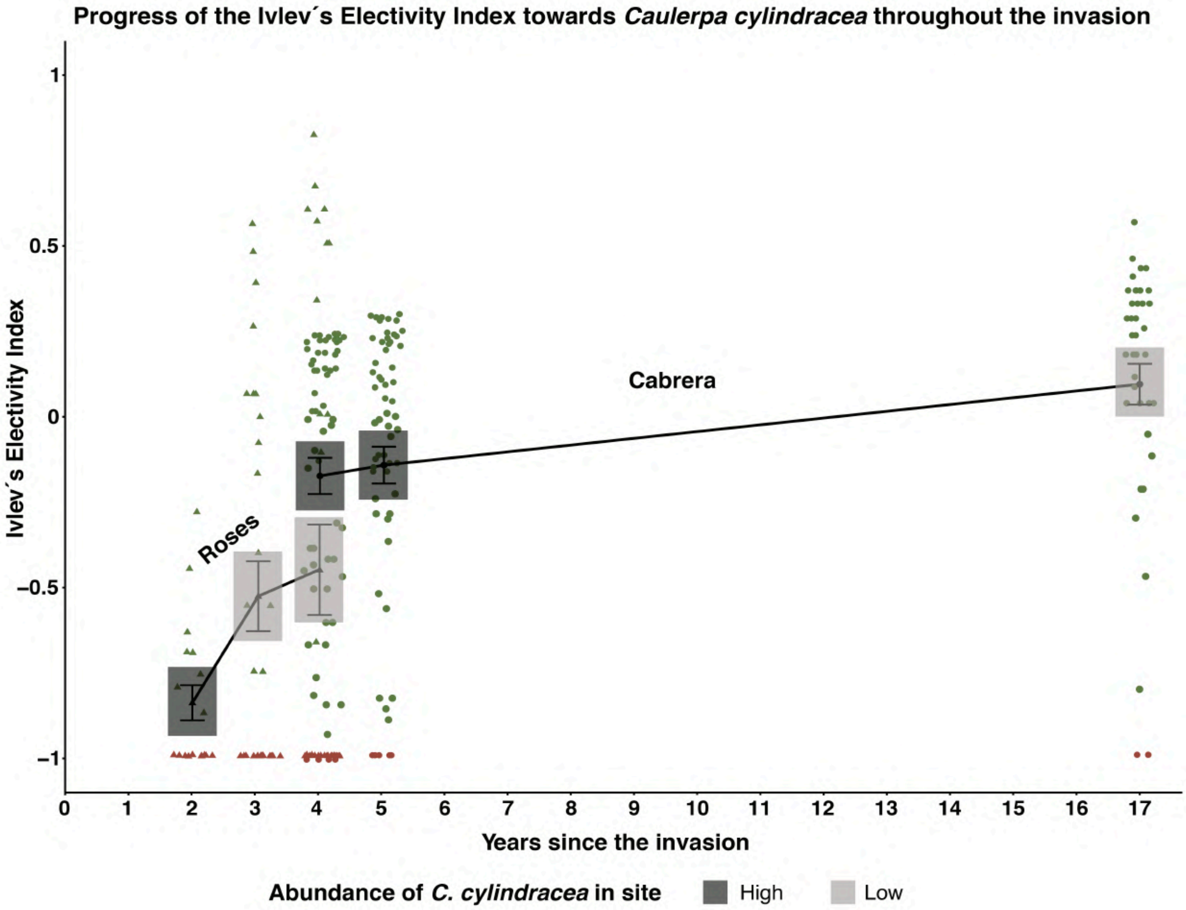

Figure 3.5. Progress of the Ivlev´s Electivity Index towards *Caulerpa cylindracea* through time in two locations that differ in their exposure time to the invader: Cabrera, where *C. cylindracea* was detected 17 years ago and Roses, where *C. cylindracea* was detected 4 years ago. The line connecting the points does not represent any relationship between them and was only added to help interpretation of the figure. Dark grey rectangles highlight the sampling times when the abundance of *C. cylindracea* in the community was high; and light grey rectangles highlight the sampling times when the abundance of *C. cylindracea* in the community was low.

3.5 Discussion

Invasive species, particularly invasive producers (plants and algae), are commonly reported to be released from biotic control due to their ability to scape native enemies (Maron and Vilà, 2001; Keane and Crawley, 2002; Colautti et al., 2004; Liu and Stiling, 2006). However, considering the dynamic nature of ecological interactions, can we expect an invader to maintain its novelty and to scape biotic control forever? Our results definitively suggest that novelty is lost during the invasion process and that the strength of predator-prey interactions increases as the invasion progresses, which in turn might intensify the biotic resistance of native communities through time.

In agreement with Tomas et al. (2011b), we show that *S. salpa* prefers to feed on *C. cylindracea* rather than on native algae, however, this preference for the invasive alga does not develop from the very beginning of the invasion, but it needs some exposure time to emerge. In this sense, a total preference for the invader was only evident in locations where *S. salpa* has had a long exposure – at least 6 years – to the invader, whereas in populations with a recent exposure to *C. cylindracea*, no preference for the invader was detected. Remarkably, preference for the invader in the old populations was maintained even when the abundance of *C. cylindracea* in the study area was low, which seems noteworthy considering that both *P. pavonica* and *C. compressa* are highly palatable and are commonly found in *S. salpa*´s diet (Verlaque, 1990; Vergés et al., 2009), and definitively suggests that *C. cylindracea* has some characteristics that make it a preferred food source. So far, the reasons behind *S. salpa*´s feeding preference for *C. cylindracea* are still unknown, but they might be based on several traits such as the nutritive value or the morphology of the invader, similarly to what has been described from other generalist herbivores (Littler and Littler, 1980; Mattson, 1980; Lodge David M., 1991; Cronin, 1998; Schädler et al., 2003).

In contrast, the % of herbivorous fishes feeding on the invader and the *per capita* consumption rates were not only influenced by the exposure time but also by the availability of the invader in the community. In this sense, only when the invader reached certain abundance, the number of herbivores and the *per capita* consumption rates increased significantly, especially in sites historically invaded compared with recent invaded areas. This clearly suggests that despite the importance that exposure time might have for the development of novel predator-prey interactions (Carlsson and Strayer, 2009; Carlsson et al., 2011; Schultheis et al., 2015; Kurr and Davies, 2017), other characteristics of the invasion (e.g. availability of the invader) and the synergy between them, might have a critical influence in the development of such novel interactions. However, we cannot disregard that low invader abundances (<30%) could partially mask the effect of time for the development of the novel predator-prey interaction, since low availabilities of the invasive alga could inherently prevent high consumptions even when the fish population is highly experienced with the invader. Actually, the electivity of *S. salpa* towards *C. cylindracea* increased through time, even when the abundance of the invader had suffered important declines (**Figure 3.5**). So it seems that once the herbivore has identified the invasive alga as a suitable food item and has incorporated it to its diet, *S. salpa* will target *C. cylindracea* even under low invader abundances due to the high preference that the herbivore has towards the invader.

Consumption of *C. cylindracea* by native herbivores might be unexpected, considering that the invasive alga produces caulerpenyne, a secondary metabolite with herbivore deterrent properties (Paul et al., 2007). However, the low metabolite concentrations in *C. cylindracea* (Box et al., 2010) and previous exposition to caulerpenyne through the consumption of native *Caulerpa* species (Marco-Méndez et al., 2017), might have allowed the fish to generate tolerance to it. Actually, several studies confirm that the phylogenetic similarity between native and invasive species can explain the failure of invasive plants to scape naïve herbivores (Hill and Kotanen, 2009; Pearse et al., 2013).

In nature, mechanisms such as learning and social transmission can influence the adaptive processes of predators to novel prey at different time scales, from days to years and even generations since first exposition (Cox, 2004; Strauss et al., 2006; Carlsson et al., 2009). In this particular case, the mechanisms by which *S. salpa* starts to consume *C. cylindracea* remain speculative, but they potentially operate at two different time scales, a longer one involving many generations that allows *S. salpa* to generate tolerance to *C. cylindracea* defenses through the subsequent exposition to *C. prolifera* (phylogenetic similarity with the invader), and a shorter one that allows *S. salpa* to identify *C. cylindracea* as a suitable prey and to develop a taste for the newcomer, probably following steps similar to the predation cycle (Jeschke et al., 2008; Saul and Jeschke, 2015) (**Figure 3.6**). Thus, although the first interaction with the invader might be accidental and might only be done by few individuals (low % of individuals consuming the invader in recent populations); they will then target the invader due to its high nutritional value, while the rest of the population will learn to target the invader by mimicking their conspecifics. Actually, social learning by which some individuals learn behaviors and acquire information such as what to eat and what to avoid through observation, is important in fish (Brown and Laland, 2003; Reader et al., 2003; Warburton and Hughes, 2011) and can explain the fast transmission of search images between demonstrators, those that know how to feed on the novel prey, and bystanders, those that observe and learn to target the new prey (White and Gowan, 2014). Complementary, and in support of our results, the time needed for the development of the novel predator-prey interaction will be influenced by the abundance of the invader in the community, because the formation and transmission of search images within the predators will be faster the higher the abundance of novel prey is (Allen, 1988; Saul and Jeschke, 2015).

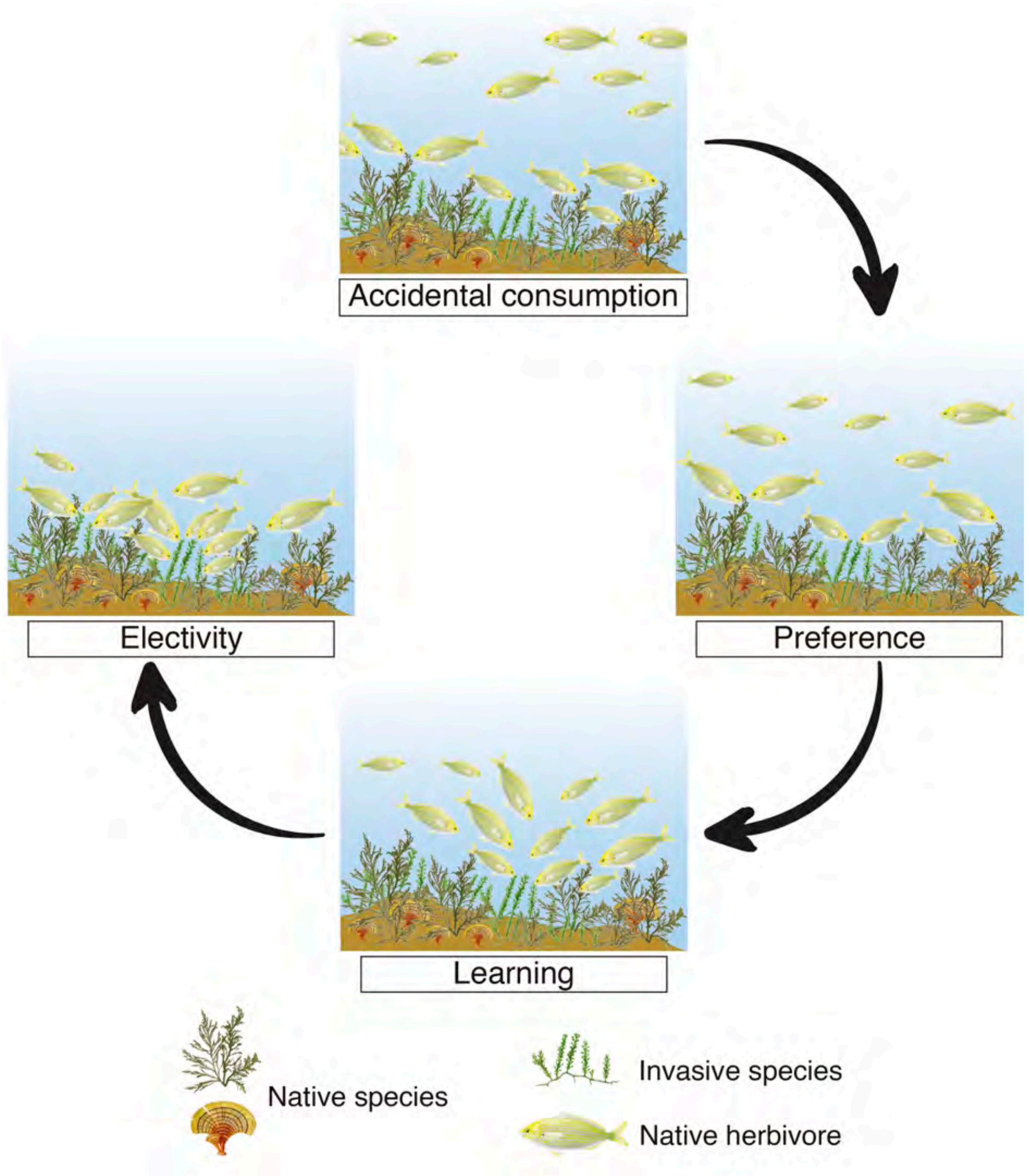

Figure 3.6. Diagram showing the phases necessary for the development of the effective predator-prey relationship between the native herbivore *Sarpa salpa* and the invasive alga *Caulerpa cylindracea*. In the first phase, consumption of the invader is accidental and will only be done by few individuals. Then, those individuals that accidentally consumed the invader will start to target it and to have a preference towards it due to its highly nutritious value. Following that, the rest of the population will learn by observing the individuals that target the invader and the search image for *C. cylindracea* will be transferred to the entire fish population. Finally, most of the fish population will target the invader, increasing the electivity towards it as the invasion progresses. Illustrations were obtained and modified from the IUCN, Henry Bradbury´s and Greville´s original illustrations (Algae Britannicae) and devianart.

Overall, our study suggests that throughout an invasion, novel predator-prey interactions can become an effective biotic resistance mechanism against an invader. However, given that some time is needed for the effective development of these interactions, invaders might scape biotic control at the beginning of the invasion and this will allow them to attain large abundances and to impact native communities, similarly to what has been observed for the invasion of *C. cylindracea* (Piazzi et al., 2001; Klein and Verlaque, 2008; Katsanevakis et al., 2016). Actually, this release from enemies might be in part responsible for the booms that many invaders suffer when they first arrive to a new habitat (Strayer et al., 2017), whereas the increase in predation after enough exposure time might cause population busts after a period of successful dominance (Carlsson et al., 2009, 2011; Strayer et al., 2017). In fact, shifts in the strength of biotic resistance through time are suspected to be the main reason behind the steep declines in abundance that several hyper-successful invaders have previously suffered (Simberloff and Gibbons, 2004; De Rivera et al., 2005; Carlsson et al., 2010, 2011; Santamaría et al., 2021). This reinforces the idea that the biotic resistance against an invader might not be a static entity and might fluctuate through the invasion, increasing its strength as the invasion progresses (Hawkes, 2007; Carlsson and Strayer, 2009; Diez et al., 2010; Mitchell et al., 2010; Carlsson et al., 2011; Dostál et al., 2013; Stricker et al., 2016). For this reason, studies that only consider a specific time-frame might provide an unreal and biased estimation of the importance of biotic resistance towards an invader as the outcomes might be greatly influenced by the time considered – recent invasion vs. old invasion, partially explaining previous contradictory patterns found in the ability of native communities to develop effective biotic resistance mechanisms towards invaders (Maron and Vilà, 2001; Colautti et al., 2004; Mitchell et al., 2006; Kimbro et al., 2013; Papacostas et al., 2017; and references therein). Therefore, long-term studies or studies assessing the resistance responses of native communities at different times since invasion – chronosequences – and at different invader intensities, should be favored to help us understand the long-term dynamics of certain invaders (Strayer, 2012; Strayer et al., 2017).

In short, this study adds evidence to the growing literature that suggests that native communities need time to develop resistance mechanisms against invasive species (Hawkes, 2007; Lankau et al., 2009; Diez et al., 2010; Carlsson et al., 2011; Dostál et al., 2013; Stricker et al., 2016). In this sense, although studies on terrestrial ecosystems refer to time exposures of several decades and even centuries for the development of effective resistance mechanisms through time (Siemann et al., 2006; Hawkes, 2007; Diez et al., 2010; Dostál et al., 2013; Stricker et al., 2016), in our case, however, an exposure time of less than a decade was enough to observe an increase in the biotic resistance of the native community. Actually, the fast behavioral change of the native herbivore that we report here, perfectly explains the recent decrease in the abundance of the invader after a period of successful dominance (Santamaría et al., 2021). Therefore, even if the time needed for the development of effective resistance mechanisms varies depending on the particularities of the studied system, previous findings stress the importance of preserving native communities in a good status to allow them to develop resistance mechanisms through time. Particularly, taking into account the role that herbivory might have in the long-term dynamics of an invader, the maintenance of healthy herbivore assemblages can be a useful strategy to naturally manage invasions in the long-term.

3.6 Acknowledgments

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Chapter 4

Herbivory on the invasive alga *Caulerpa cylindracea*: The role of omnivorous fishes.

Jorge Santamaría, Fiona Tomas, Enric Ballesteros, Emma Cebrian

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Cover image: Several *Diplodus* species thriving in a Marine Protected Area in the North-Western Mediterranean Sea.

Author: Enric Sala Gamito.

4.1 Abstract

Herbivory has long been considered an important component of biotic resistance against macroalgae invasions in marine habitats. However, most of the studies on herbivory of invasive algae refer only to consumption by strictly herbivorous organisms, whereas consumption by omnivorous species has been largely ignored and rarely quantified. In this study, we assess whether the commonest omnivorous sparid species in the Mediterranean Sea are consuming the highly invasive alga, *Caulerpa cylindracea*, and determine both, its importance in their diet and their electivity towards it as a source of food. Our results confirm that three of the four fish species studied regularly consume *C. cylindracea*, but in most cases, the importance of *C. cylindracea* in the diet is low. Indeed, the low electivity values indicate that all species avoid feeding on the invasive alga and that it is probably consumed accidentally. However, despite animals and detritus being the main food for these sparid species, several individual specimens were found to have consumed high amounts of *C. cylindracea*. This suggests a potential role that these fish species, being really abundant in shallow rocky bottoms, may play in controlling, to some extent, the abundance of the invader.

4.2 Introduction

Invasive macroalgae are one of the most successful and conspicuous groups of invaders in marine systems (Schaffelke et al., 2006; Thomsen et al., 2016), where they contribute to the homogenization of marine habitats and affect the structure of native assemblages by reducing both native species biomass and the overall assemblage diversity (Williams and Smith, 2007; Schaffelke and Hewitt, 2008; Thomsen et al., 2009, 2016). In the receiving community, the establishment and persistence of invasive algae can be reduced and affected by biotic and abiotic factors (Dunstan and Johnson, 2007; Catford et al., 2009; Thomsen et al., 2009; Kimbro et al., 2013; Papacostas et al., 2017), thus determining the invasive success of the invader. Among biotic factors, herbivory has long been considered as a potential biotic resistance mechanism and many studies have been conducted worldwide to assess the role of this mechanism on invasive macroalgae success (see references within Kimbro et al., 2013 and Papacostas et al., 2017). Until now, assessments on the role of herbivory as a limiting factor for macroalgae invasion have mainly considered strictly herbivorous species (e.g.,Ruitton et al., 2006; Wikström et al., 2006; Lyons and Scheibling, 2008; Britton-Simmons et al., 2011; Cebrian et al., 2011; Tomas et al., 2011b; Hammann et al., 2013; Enge et al., 2017; Noè et al., 2017; Caselle et al., 2018), reporting contrasting results depending on the assemblage and the invasive species considered (Boudouresque et al., 1996; Trowbridge and Todd, 1999; Scheibling and Anthony, 2001; Stimson et al., 2001; Davis et al., 2005; Wikström et al., 2006; Monteiro et al., 2009; Steinarsdóttir et al., 2009; Tomas et al., 2011b, 2011a; Nejrup et al., 2012; Caselle et al., 2018).

In the Mediterranean Sea, the most successful and widespread invasive macroalga is *Caulerpa cylindracea* (Klein and Verlaque, 2008; Katsanevakis et al., 2016), a green alga native of the Southwestern coast of Australia that was first detected in Mediterranean waters in Libya in 1990 (Nizamuddin, 1991). Since then, it has colonized marine communities throughout the entire Mediterranean basin (Piazzi et al., 2005; Klein and Verlaque, 2008), where it can exert strong detrimental effects on native communities (Piazzi et al., 2001; Klein and Verlaque, 2008; Bulleri et al., 2016, 2017). However, despite its rampant success, several *Caulerpa cylindracea* meadows have suffered sudden steep declines in abundance some years after the onset of the invasion (Klein and Verlaque, 2008; García et al., 2016; Santamaría et al., 2021), which may indicate the existence of certain resistance mechanisms against this invasive species. Among these, herbivory on *C. cylindracea* has been described and assessed mainly in relation to the strictly herbivorous species present in the Mediterranean Sea, such as the fishes *Sarpa salpa* and *Siganus luridus* (Azzurro et al., 2004; Ruitton et al., 2006; Tomas et al., 2011b; Santamaría et al., 2021), and the sea urchins *Paracentrotus lividus*, *Sphaerechinus granularis* and *Arbacia lixula* (Ruitton et al., 2006; Bulleri et al., 2009; Cebrian et al., 2011; Tomas et al., 2011a). Nevertheless, there are omnivorous fish species, such as *Diplodus sargus*, *Boops boops*, and *Spondyliosoma cantharus*, that have been occasionally observed feeding on *C. cylindracea* before (Ruitton et al., 2006; Box et al., 2009; Terlizzi et al., 2011). Unfortunately, information is scarce on whether *C. cylindracea* is a common food source for omnivorous fish species, or whether these fish actively elect to feed on it. Since some of these species are the dominant fish in the shallow, infralittoral rocky habitats of the Mediterranean Sea (García-Rubies, 1997; Sala and Ballesteros, 1997; García-Charton et al., 2004), information on their consumption of *C. cylindracea* is needed to assess whether they can potentially contribute to limit the abundance of the invader.

In this study, the diets of four of the most abundant omnivorous sea bream species (Sparidae) were examined in order to determine (i) whether they feed on the invasive alga *C. cylindracea*, (ii) whether *C. cylindracea* is important in their diet and (iii) whether they actively select or avoid *C. cylindracea* as a source of food

4.3 Materials and methods

4.3.1 Study area

The samples for this study were collected in the Cabrera Archipelago National Park (North-Western Mediterranean Sea; 39°12'21" N, 2°58'44" E) (**Figure 4.1**) in 2008. This marine-terrestrial protected area was established in 1991 and since then it has maintained an exceptional conservation status for its marine habitats (Sala et al., 2012; Coll et al., 2013; Guidetti et al., 2014). *Caulerpa cylindracea* was recorded for the first time in the National Park in 2003 at a depth of 30-35 m and since then its distribution has expanded to cover most of its benthic communities at depths of between 0 and 65 m (Cebrian and Ballesteros, 2009).

4.3.2 Analysis of *Caulerpa cylindracea* consumption

To determine whether non-strictly herbivorous fish species consume *C. cylindracea*, specimens for this study were captured by artisanal long-lines and gillnets on several occasions during June and July 2008, at different sites across the Archipelago, at Ses Rates and Foradada Islets (**Figure 4.1**). Fishing campaigns were performed in the summer because it corresponds to the period of the year when the activity of the targeted fishes is higher. The main fishes targeted belong to the family Sparidae: white sea bream (*Diplodus sargus*), annular sea bream (*Diplodus annularis*), two-banded sea bream (*Diplodus vulgaris*) and black sea bream (*Spondyliosoma cantharus*). These species were chosen because they are common representatives of the fish assemblages found in the Western Mediterranean, they are not herbivorous but can feed on macroalgae (Sala and Ballesteros, 1997) and some of them have been observed feeding on *C. cylindracea* before (Box et al., 2009; Terlizzi et al., 2011). These four species have different abundances within the National Park, the least abundant of them being *S. cantharus*, with 1.7 individuals per 250 m^2; then *D. annularis*, with 1.9 individuals per 250 m^2; then *D. sargus* with

5.3 individuals per 250 m^2; and the most abundant being *D. vulgaris*, with up to 42 individuals per 250 m^2 (Reñones et al., 1997; Coll, 2020).

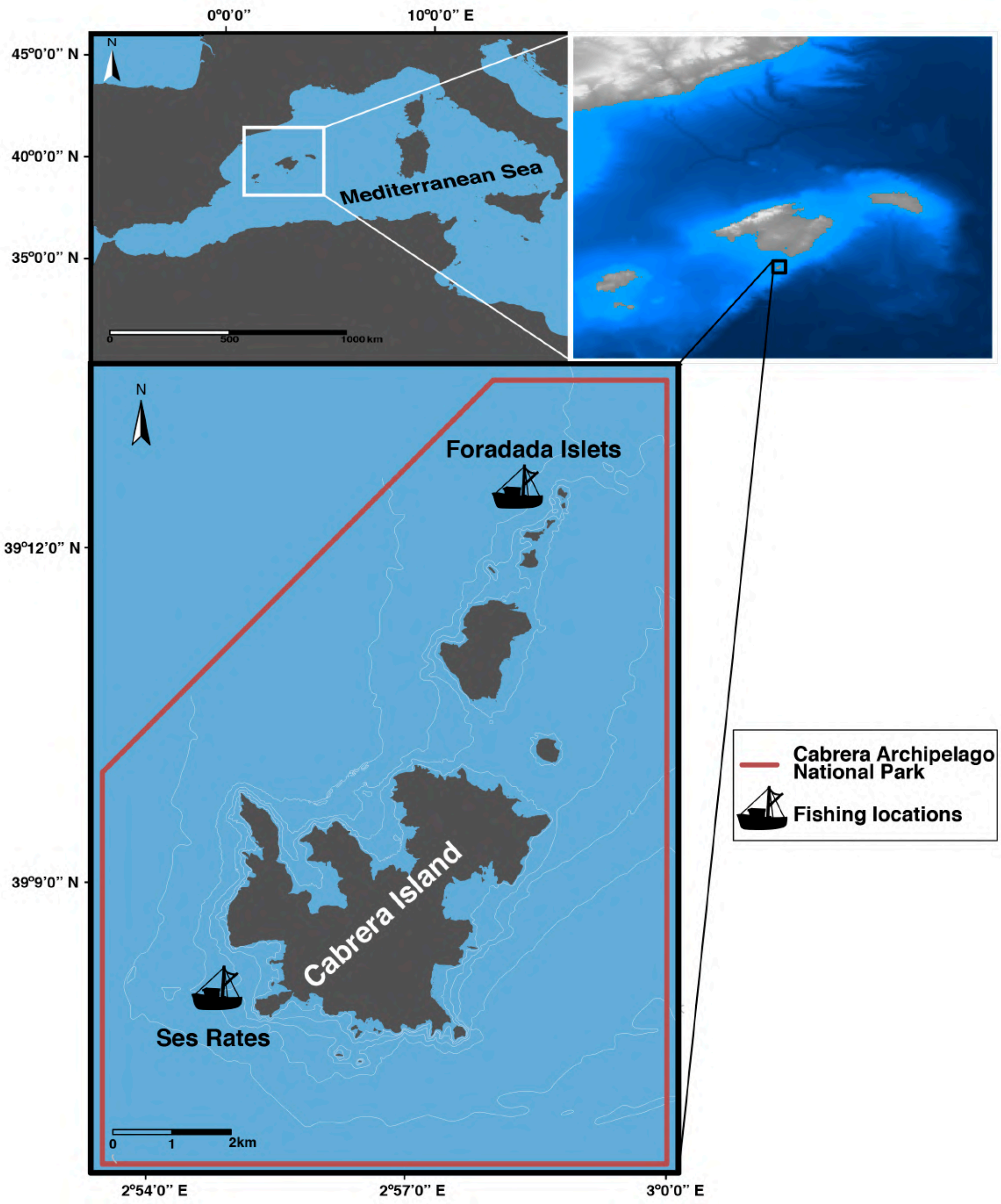

Figure 4.1. Location of the 3 regions where the study was carried (the Catalan Coast, the Cabrera Archipelago and Menorca). The points show the specific locations where different assessments were done. Shapefile for the Mediterranean Sea downloaded from www.naturalearthdata.com, for the Catalan Coast and Menorca downloaded from the Global Self-consistent, Hierarchical, High-resolution Geography Database (GSHHG) and for the Cabrera Archipelago downloaded from www.miteco.gob.es.

The long-lines and gillnets, two gears commonly used in artisanal fishing, were deployed in the morning at depths of between 10 and 30 m. Every time a targeted fish species was hauled in, it was gutted and its stomach was stored and preserved in buffered 4% formaldehyde-seawater solution for later analysis of its content. Once in the laboratory, the species composition and abundance of the food items in each fish stomach was determined under a Stemi 2000-C stereomicroscope (Carl Zeiss, Berlin, Germany). The content of each stomach was spread onto a reticulated Petri dish and the food items were classified to the lowest taxonomic level possible. Both surface area and weight measurements can reflect the dietary contribution of food items (Hyslop, 1980; Macdonald and Green, 1983), but in this case, and to avoid biases that could be derived from the small quantities present in the stomach contents, surface area measurements were preferred over weight measurements to quantify the dietary contribution of each food item. As such, the abundance of a particular food item was estimated as the percentage cover on the reticulated fields of the Petri dish in relation to the cover of the whole stomach content. When a species had a minimal presence and its cover could not be determined, a value of 0.1% of relative coverage was assigned.

When calculating the relative measures of prey quantity (RMPQ), the stomach contents were divided into the following five food categories: *Caulerpa cylindracea*, Other algal content, Seagrasses, Animal content and Detritus. Subsequently, for each fish species, the percentage frequency of occurrence of each food category (FO_i) was calculated as:

$$FO_i = \left({}^{S_i}/_{S_t}\right) \cdot 100$$

where S_i is the number of stomachs containing the food category and S_t is the total number of stomachs analyzed for that particular fish species. The FO_i value is a measure of the consistency with which a species selects a given food category and was used to calculate two dietary indices that allow to compare the diets between species: the Combined Index (Q_i) and the Geometric Index of Importance (GII).

The combined index, Q_i, was chosen to assess the relative importance of each food category for each fish species. This index standardizes the abundance of each category and increases the importance of frequent smaller items while reducing the importance of occasional larger items (Nilssen et al., 2019). It was calculated as:

$$Q_i = \frac{V_i \cdot FO_i}{\sum_{i=1}^{m}(V_i \cdot FO_i)}$$

where V_i refers to the percentage surface of a food category, FO_i refers to the frequency of occurrence of the given food category, and m is the total number of food categories.

On the other hand, the Geometric Index of Importance, GII, represents the degree of feeding specialization on a particular food type (Assis, 1996; Preti et al., 2001) and allows us to classify the food categories as: "Primary prey", "Secondary prey" and "Occasional prey" in relation to the larger discontinuities in a decreasing sequence of values (Assis, 1996; Tripp-Valdez et al., 2015). It was calculated as:

$$GII_j = \frac{\sum_{i=1}^{n}(V_i + FO_i)}{\sqrt{2}}$$

Finally, the degree to which the four fish species tend to elect to feed on *C. cylindracea*, was assessed by Ivlev's electivity Index (E) (Ivlev, 1961). This Index was determined by:

$$E = \frac{(d_i - a_1)}{(d_i + a_i)}$$

where d_i = % of *C. cylindracea* in the stomach content and a_i = % of *C. cylindracea* available in the environment (see following section). The values of the Ivlev's Index (E) can range from -1 (complete avoidance of the food item) to +1 (exclusive selection of the item), with positive values indicating that the food item is selected and eaten more than it is encountered by chance in the environment (Ivlev, 1961).

4.3.3 Assessment of the abundance of *Caulerpa cylindracea* in the community

The abundance of *C. cylindracea* at the sampling sites where fish specimens were captured was assessed by means of scuba diving, also in the summer 2008. At each site, a perpendicular transect to shore was done, at depths of 10 to 30 m, so as to cover the same bathymetric range as that of the fishing gear used to collect fish samples. To estimate *C. cylindracea* abundance, a total of thirty quadrats measuring 25 x 25 cm^2 were randomly positioned within each 10 m-depth range (total of 90 quadrats per sampling site). These quadrats were divided into 25 subquadrats of 5 x 5 cm^2 and the number of subquadrats where *C. cylindracea* appeared was used as the unit of abundance (Sala and Ballesteros, 1997; Cebrian and Ballesteros, 2004; Sant et al., 2017). Subsequently, the average *C. cylindracea* abundance for the study area was calculated and this value was used in the calculation of the Ivlev's electivity Index.

4.3.4 Statistical analyses

Differences in the specific composition of stomach contents between fish species were assessed through multivariate techniques such as non-metric multi-dimensional scaling plots (NMDS plots), analysis of similarities (ANOSIM) and similarity percentage analysis (SIMPER). All of these techniques were performed within the *vegan* package (Oksanen et al., 2018) in the R environment (R Core Team, 2018). First, in order to visualize and represent stomach content composition, a NMDS (Clarke and Warwick, 1994; Cox and Cox, 2000) based on the Bray-Curtis dissimilarity matrix of the square-root transformed data was plotted and the most important species that determine the least stressful ordination were detected using the envfit function within the *vegan* package. Then, the statistical differences in the food items consumed by the fish species were assessed using ANOSIM (Clarke, 1993), applied to the Bray-Curtis dissimilarity matrix, with fish species as a fixed factor. Additionally, a pairwise ANOSIM was performed by modifying the pairwise.adonis function (https://github.com/pmartinezarbizu/pairwiseAdonis) and the R-values obtained were used as an indication of diet similarity, with values near 1 indicating separation in diet composition and values near 0 indicating diet similarity (Rogers et al., 2012). Finally, a SIMPER analysis based on the Bray-Curtis dissimilarity index was used to assess the relative contribution of each food item to the overall differences between fish species diets.

4.4 Results

During the sampling events, a total of 93 fishes were captured, with *D. sargus* being the most abundant (n=51) followed by *S. cantharus* (n=22), *D. vulgaris* (n=13) and *D. annularis* (n=7). All the stomachs examined contained ingested material of some kind, which, as a whole, was composed of a high diversity of taxonomic groups, with 73 different prey items identified, 32 of them to the species level (**Appendix C: Table C.1**).

Differences in stomach content were observed between species in terms of the dominant prey categories, although detritus and animal content were certainly prominent in all four species (**Table 4.1**). In this sense, the Combined Index (Q_i) and the Geometric Index of Importance (GII), identified the category "Detritus" as the primary food item for *S. cantharus*, while the category "Animal content" was the primary prey for the other three fish species (**Figures 4.2 and 4.3**). Despite this predominance for "Animal" and "Detritus" items, algae and seagrasses were found in all the species, being the stomach contents of both *D. sargus* and *D. annularis* particularly rich in algae, with values of around 18% and 30% respectively (**Table 4.1**).

Table 4.1. Summary of the stomach content data for each fish species.

Diplodus annularis	Mean ± S.E. (%)
Other algal content	4.69 ± 3.10
Caulerpa cylindracea	**25.73 ± 11.80**
Seagrasses	0.26 ± 0.21
Animal content	43.28 ± 16.00
Detritus	26.05 ± 15.03

Diplodus sargus	Mean ± S.E. (%)
Other algal content	15.10 ± 3.67
Caulerpa cylindracea	**3.56 ± 0.96**
Seagrasses	9.36 ± 2.84
Animal content	48.31 ± 5.53
Detritus	23.64 ± 5.08

Diplodus vulgaris	Mean ± S.E. (%)
Other algal content	8.80 ± 6.91
Caulerpa cylindracea	**0**
Seagrasses	0.29 ± 0.25
Animal content	54.75 ± 12.30
Detritus	36.16 ± 11.14

Spondyliosoma cantharus	Mean ± S.E. (%)
Other algal content	1.88 ± 0.90
Caulerpa cylindracea	**5.15 ± 1.94**
Seagrasses	0.40 ± 0.26
Animal content	14.10 ± 4.81
Detritus	78.47 ± 5.12

The ingested food items are grouped into categories and the values given are mean percentages ± S.E. for each fish species.

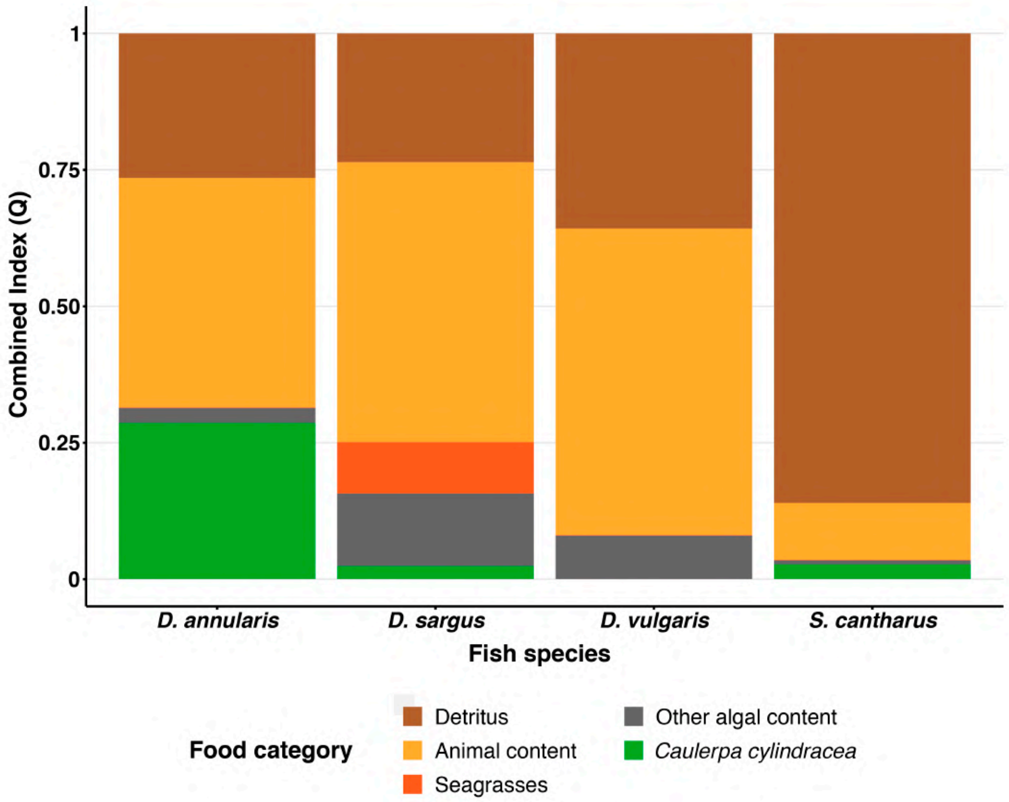

Figure 4.2. Combined Index (Q) for each fish species. Each color represents one of five food categories (Detritus, Animals, Seagrasses, Other algae and *Caulerpa*).

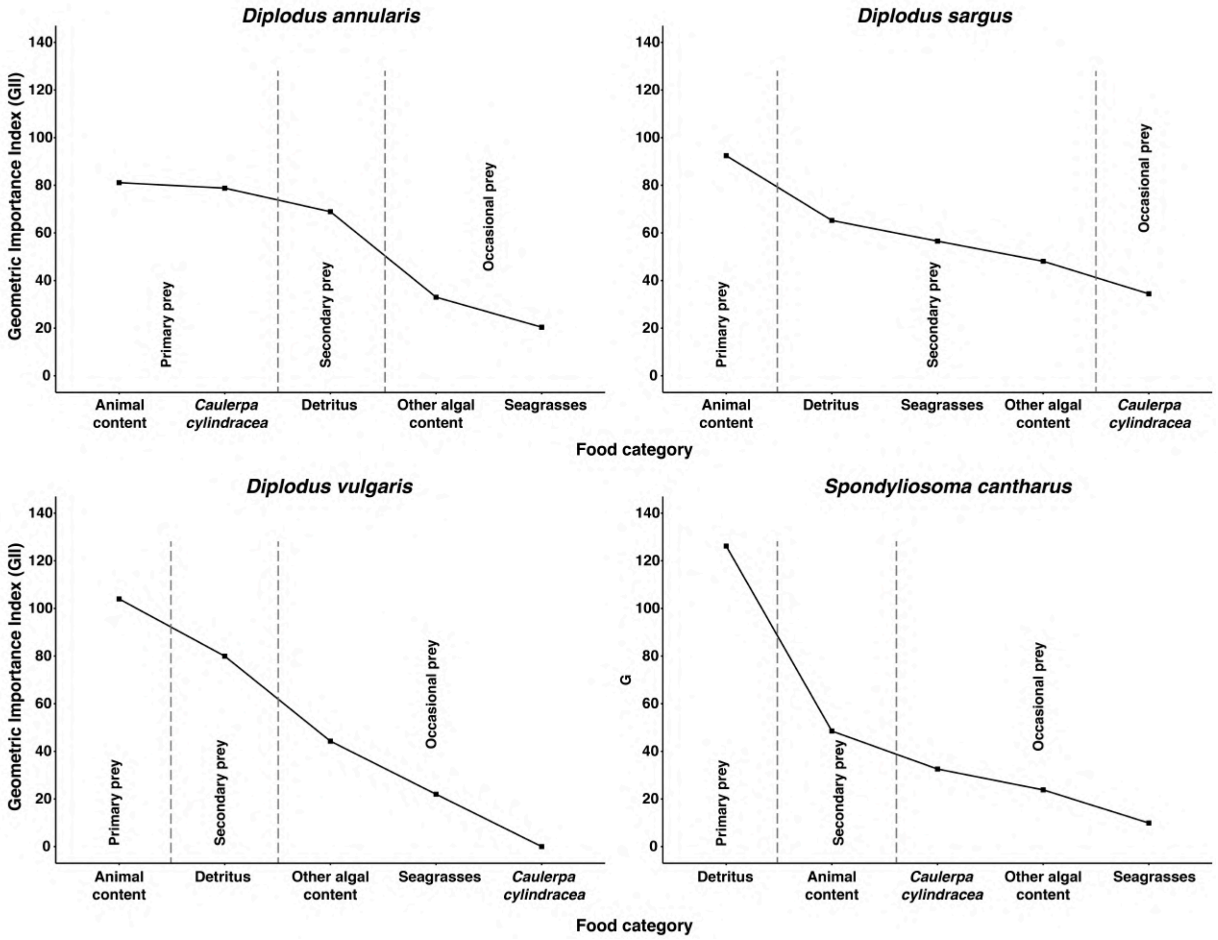

Figure 4.3. Geometric Importance Index (GII) for each fish species. Food items are classified as: "Primary prey", "Secondary prey" or "Occasional prey" according to their relative importance to the diet of each fish species. The line connecting the points was added to help interpretation of the figure.

Regarding the consumption of the invasive alga *C. cylindracea*, 81%, 45% and 41% of the *D. annularis*, *D. sargus* and *S. cantharus* specimens contained *C. cylindracea* in their stomachs respectively; whereas *D. vulgaris* was the only fish species that had not consumed the invader. However, the contribution of *C. cylindracea* to the total stomach content was generally low, except for *D. annularis*, in which 26% of the stomach content corresponded to the invasive species (**Table 4.1**). Actually, both dietary indexes, Q_i and GII, classified *C. cylindracea* as a primary food item for *D. annularis*, being the second most common food category in the stomachs and having values similar to animal content (**Figures 4.2 and 4.3**). In contrast to this, *C. cylindracea* was classified as an occasional food item for both *D. sargus* and *S. cantharus* (**Figures 4.2 and 4.3**), but most of the algae content in the stomachs of *S. cantharus* corresponded to the invasive species (≈74%; **Table 4.1**). However, even with the high proportion of *C. cylindracea* found in some of the studied species, the negative values obtained for the Ivlev´s Electivity Index suggest that *C. cylindracea* is generally avoided by all the species (**Table 4.2**).

Table 4.2. Mean ± S.E. values for Ivlev's electivity Index (E), as a measure of the electivity of the four sparid fish species studied towards the invasive alga *C. cylindracea*.

$\mathbf{E}_{\textit{Caulerpa}}$	
D. annularis	-0.52 ± 0.18
D. sargus	-0.90 ± 0.02
D. vulgaris	-1
S. cantharus	-0.86 ± 0.05

An E value approaching -1 indicates that the food item is avoided; whereas an E value approaching 1 indicates the species only feeds on that item.

Considering the whole diet of the four sampled sea bream species, the graphical ordination suggested that there might be some overlap between diets (**Figure 4.4**), yet the ANOSIM detected significant differences in the stomach content composition between all fish species (ANOSIM; p-value < 0.05; **Appendix C: Table C.2**), with two exceptions: *D. sargus* – *D. vulgaris* and *D. sargus* – *D. annularis* (p-value > 0.05; **Appendix C: Table C.2**). In this sense, the greatest dissimilarities in diet were found between *S. cantharus* and two of the *Diplodus* species, *D. annularis* and *D. vulgaris* (R=0.63 and R=0.47 respectively, **Appendix C: Table C.2**), since the diet of *S. cantharus* was more homogeneous (**Figure 4.4**) and it was dominated by detritus rather than by animal content (**Table 4.1**, **Figures 4.2, 4.3 and 4.4**). Actually, the SIMPER analysis identified "organic detritus" as the biggest contributor to the diet dissimilarities between the four fish species, with values ranging from 19% to 40% (**Appendix C: Table C.3**). Remarkably, *C. cylindracea* was one of the species most strongly influencing the graphical ordination

(**Figure 4.4**) and the SIMPER analysis identified the invasive alga as the second most important food item in terms of explaining the diet dissimilarities between *D. annularis* and the other fish species (**Appendix C: Table C.3**).

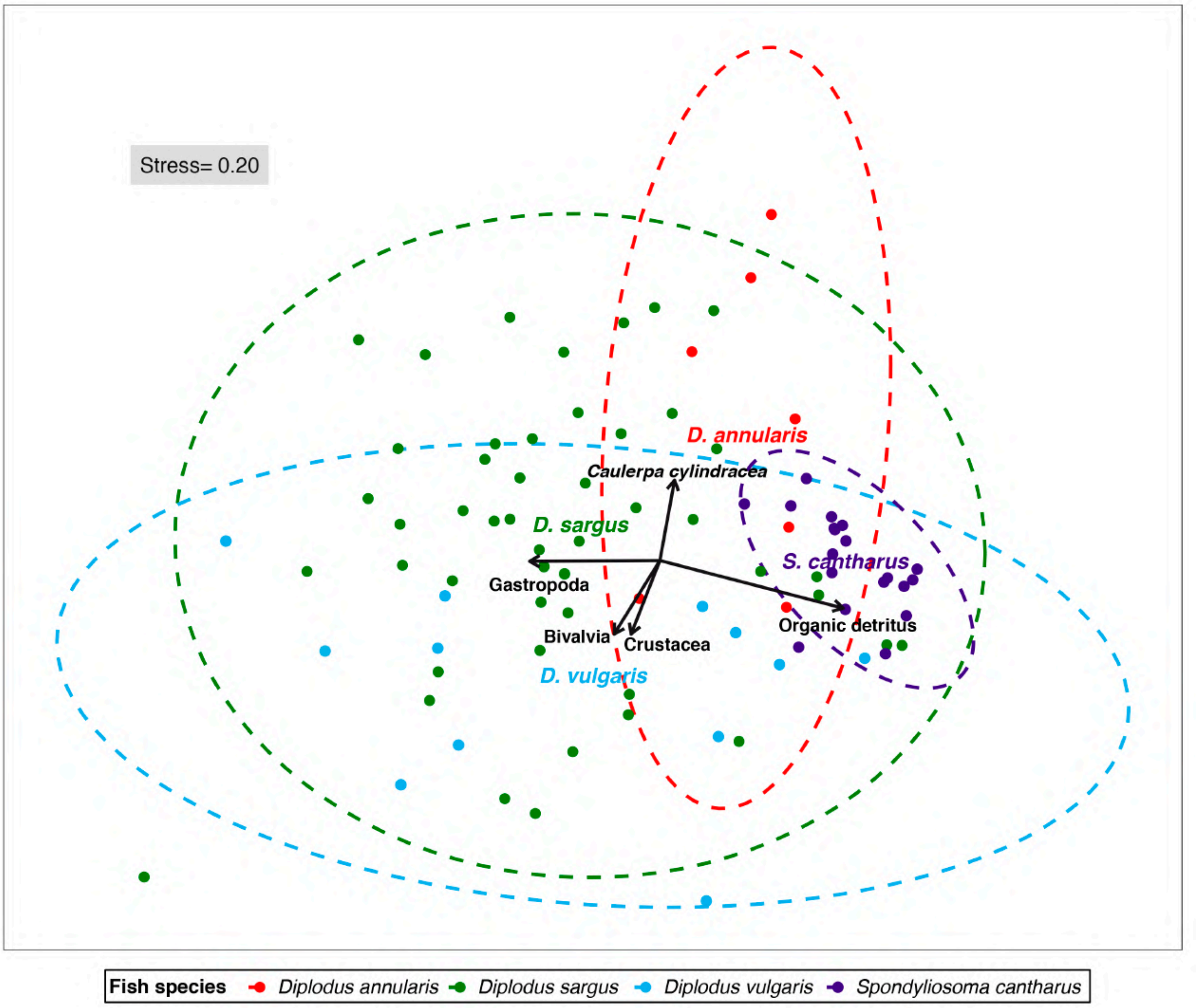

Figure 4.4. Non-metric MDS for the stomach content of the different fish species. Each ellipse surrounds the points for one fish species and the black arrows represent the most significant food items determining the ordination. For these variables, only the ones with a p-value lower or equal to 0.001 were represented.

4.5 Discussion

Stomach content analysis of four of the commonest species in the shallow rocky infralittoral communities in the western Mediterranean Sea, revealed that despite these species having a diet predominately based on animal and detritus content (Sala and Ballesteros, 1997; Gonçalves and Erzini, 1998; Pita et al., 2002; Box et al., 2009; Terlizzi et al., 2011; Felline et al., 2012, 2017), they can also feed on considerable amounts of algae, with values exceeding those previously reported for non-strictly herbivorous sea-bream species (Sala and Ballesteros, 1997; Sánchez-Jerez et al., 2002; Leitão et al., 2007; Box et al., 2009). Remarkably, the invasive alga *C. cylindracea* was consumed by 3 of the 4 studied fish species, even being the most prominent algae for 2 of the fish species, *D. annularis* and *S. cantharus*. This clearly suggests that, being *C. cylindracea* a widespread and regionally abundant alga (Klein and Verlaque, 2008; Katsanevakis et al., 2016), it can potentially become a recurrent food item for omnivorous fish species, similarly to what has happened with the herbivorous fish *S. salpa*, which has adopted the invader as a preferred food item (Tomas et al., 2011b). Actually, several studies involving lower sample sizes, had previously reported the ability of *D. sargus*, *S. cantharus* and *D. vulgaris* to occasionally feed on *C. cylindracea* on several locations in the Mediterranean Sea (Box et al., 2009; Terlizzi et al., 2011; Felline et al., 2012, 2017; Gorbi et al., 2014). In our assessment, *C. cylindracea* was a primary food item for *D. annularis*, whereas it was an occasional one for both *S. cantharus* and *D. sargus*. Interestingly, our findings are, to our knowledge, the first evidence of *D. annularis* feeding on *C. cylindracea* in the Mediterranean Sea, but given that almost all the fished individuals had consumed the invader and that they had high amounts of *C. cylindracea* in their stomachs, this fish species should be included in subsequent assessments to further elucidate whether this feeding behavior is common across the Mediterranean region.

Despite previous studies reporting the consumption of *C. cylindracea* by omnivorous fishes, none of them report the availability of *C. cylindracea* in the environment, making it impossible to assess whether the omnivorous fishes are actually targeting the invader. In this sense, in our study area, *C. cylindracea* was found to be the dominant species in the benthic communities where the fish species were fished, with mean coverage values close to 55%. However, electivity of the fish species for *C. cylindracea* suggests that all the species were avoiding feeding on the invader and so, their consumption was more occasional than that of the strictly herbivorous fishes, which show a positive electivity for the invader (Tomas et al., 2011b). Surprisingly, despite this general avoidance of *Caulerpa cylindracea*, high amounts of the invader were found in the stomach contents of some individuals, with values reaching over 35%. This suggests that the invasive alga is probably consumed accidentally when the fish are trying to feed on other prey living within the dense meadows of *C. cylindracea*. In fact, polychaetes, mollusks and decapods – which are the preferential prey for most of the sea breams studied (Bauchot and Hureau, 1986; Sala and Ballesteros, 1997; Gonçalves and Erzini, 1998; Pita et al., 2002; Leitão et al., 2007) – have been found to be very abundant under the stolons of *C. cylindracea* (Carriglio et al., 2003; Galil, 2007; Box, 2008; Klein and Verlaque, 2008). Furthermore, the suggestion that consumption of the alga is accidental is also supported by the low assimilation of *C. cylindracea* in the stomach contents, as in most cases it was found intact and undigested (**Figure 4.5**). However, taking into account that some of the sea breams considered here have small home ranges and show strong site fidelity (D'Anna et al., 2011; March et al., 2011; Alós et al., 2012; Di Lorenzo et al., 2014), it cannot be ruled out that they might also be forced to feed on *C. cylindracea* in heavily colonized areas, and this might have a negative impact on the physiology of the fish species. In fact, previous evidence relates *C. cylindracea* consumption to a decrease in certain essential fatty acids in fish tissues and liver (Felline et al., 2014), to an increase in the levels of antioxidants and in pro-oxidant effects (Box et al., 2009; Terlizzi et al., 2011; Felline et al., 2012), to a decrease in the condition factor (Terlizzi et al., 2011) and to a decrease in the gonadosomatic-index (Felline et al., 2012), all of which may negatively affect the fish fitness. It is not yet clear what causes these physiological responses, although they could be caused by the accumulation of some of the compounds produced by *C. cylindracea*, such as caulerpenyne, a toxic, secondary metabolite that has herbivore-deterrent properties (Paul et al., 2007). However, considering that *Caulerpa prolifera*, a native species in the Mediterranean Sea, has much higher caulerpenyne concentrations than *C. cylindracea* (Box et al., 2010) and that sea breams can often consume the native *Caulerpa* species (**Appendix C: Table C.1**) (Chaouch et al., 2013, 2014; Marco-Méndez et al., 2017), it is likely that the fish might have developed a certain tolerance and effective detoxification pathways for the toxic metabolites, as other herbivores do (Cornell and Hawkins, 2003; Sotka and Whalen, 2008; Sotka et al., 2018). In any case, more studies are needed to understand the possible long-term consequences of *C. cylindracea* consumption on the health of fish assemblages and whether these consequences could propagate throughout the food-web, potentially affecting the functioning of the ecosystem through cascading effects.

Figure 4.5. Macroscopic view of *Caulerpa cylindracea* fragments, as found in the stomach contents.

Overall, our findings confirm that the invasion of *Caulerpa cylindracea* in the Mediterranean Sea has the potential to influence the feeding habits of the omnivorous fish species as it has already done with some of the strictly herbivorous organisms (Azzurro et al., 2004; Ruitton et al., 2006; Cebrian et al., 2011; Tomas et al., 2011b, 2011a). In this sense, the assessment of *C. cylindracea* consumption by omnivorous (i.e., non-strict herbivores) fish presented here is noteworthy since most of the previous research into the effects of herbivory on invasive algae has focused only on the strict herbivores disregarding the effect that omnivorous organisms might have (Scheibling and Anthony, 2001; Davis et al., 2005; Wikström et al., 2006; Ruitton et al., 2006; Lyons and Scheibling, 2008; Vermeij et al., 2009; Britton-Simmons et al., 2011; Cebrian et al., 2011; Tomas et al., 2011b, 2011a; Nejrup et al., 2012; Hammann et al., 2013). Furthermore, while some of the previous studies have highlighted the contribution of some of these herbivores to limit the abundance of invasive algae, our findings suggest that non-strict herbivores could also potentially have a similar, albeit less important contribution. For instance, given the high amounts of *C. cylindracea* found in some specimens and considering that omnivorous sparid fishes dominate the shallow rocky infralittoral habitats in the Mediterranean Sea (García-Rubies, 1997; Sala and Ballesteros, 1997; García-Charton et al., 2004; Coll et al., 2013) with abundances of up to 60 individuals per 250 m^2 and biomasses of more than 40 g/m^2 (García-Rubies and Zabala, 1990; Sala and Ballesteros, 1997; Di Franco et al., 2009; Guidetti et al., 2014; Coll, 2020), it seems that they could certainly have some impact on the abundance of *C. cylindracea*. Therefore, we suggest that the lower impact exerted by omnivorous fish can complement the higher impact exerted by strictly herbivorous organisms (Santamaría et al., 2021) and that, taken together, they might significantly reduce the abundance of *C. cylindracea* in shallow habitats. So definitively the role of herbivory by omnivorous organisms should be researched more in depth to better assess and quantify the impact of these organisms on the success of *C. cylindracea*. Finally, considering that sea breams are highly targeted by fisheries and have already suffered important declines in the Mediterranean basin (Sala et al., 1998; Coll et al., 2004; Sala, 2004; Morales-Nin et al., 2005; Guidetti, 2006; Lloret et al., 2008), places that promote their recovery, such as well-enforced marine protected areas (MPAs) (Mosquera et al., 2000; Micheli et al., 2005; Claudet et al., 2006; Guidetti, 2006; Guidetti and Sala, 2007; Guidetti et al., 2008, 2014; Sala et al., 2012; Coll et al., 2013), might also foster the strength of this complementary control mechanism on the abundance of *C. cylindracea*.

4.6 Acknowledgments

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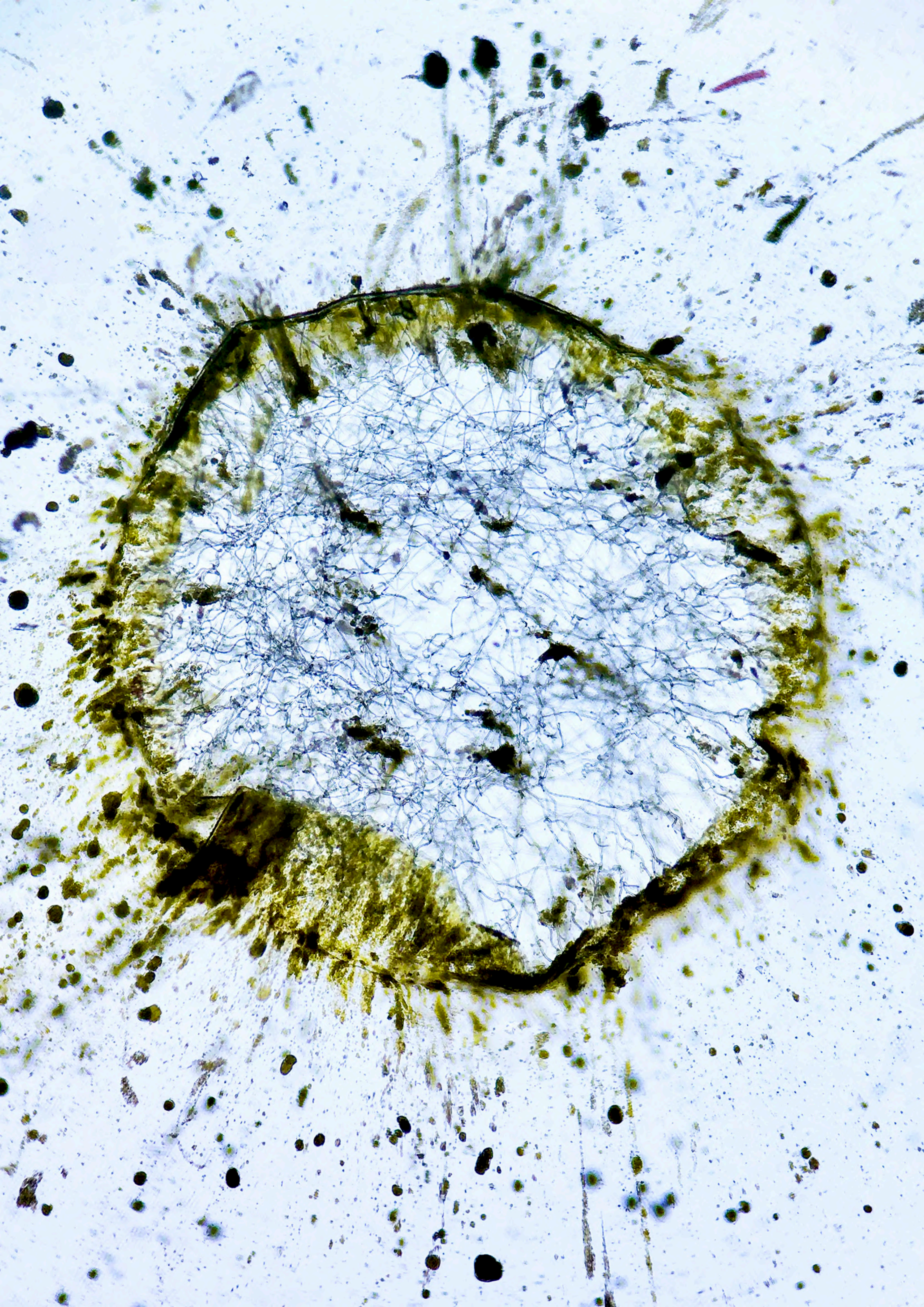

Chapter 5

Stressful Conditions Give Rise to a Novel and Cryptic Filamentous Form of *Caulerpa cylindracea*

Jorge Santamaría, Raül Golo, Emma Cebrian,
María García, Alba Vergés

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Cover image: Cross-section of a *Caulerpa cylindracea* filament as observed under the microscope. The strands traversing the lumen of the filament are trabeculae, physiological structures characteristic of the genus *Caulerpa*.
Author: Jorge Santamaría Pérez.

5.1 Abstract

Morphological plasticity can enable algae to adapt to environmental change and increase their invasibility when introduced into new habitats. Nevertheless, there is still a lack of knowledge on how such plasticity can affect the invasion process of an invasive species. In this context, the high plasticity in the genus *Caulerpa* is well documented. However, after an extremely hot summer, a previously unreported filamentous morphology of *Caulerpa cylindracea* was detected; indeed, this morphology could only be confirmed taxonomically after in-depth morphological characterization and molecular analysis with the genetic marker *tuf*A. We describe an *ex situ* culture experiment which showed that stressful conditions, such as high temperatures, can trigger this morphological change. Almost all of the thalli maintained at a constant extreme temperature of 29ºC died, but after being returned to optimum temperature conditions, the filamentous morphology began to develop from the surviving microscopic tissue. In contrast, thalli at a control temperature of 21ºC maintained the regular morphology throughout the experiment. When *C. cylindracea* develops this filamentous morphology, it may act as a cryptic invader because it is difficult to detect in the field. Furthermore, the filaments likely improve *C. cylindracea´*s invasive capabilities with regard to resistance, persistence and dispersion and may have an important role in the re-colonization process, after a population disappears following a period of stressful conditions. Possibly, *C. cylindracea´*s ability to respond plastically to stressful conditions might explain its remarkable success as an invasive species.

5.2 Introduction

Biological invasions refer to the process by which different organisms, commonly known as invasive species, can arrive and establish in a new habitat, where they disrupt the normal functioning of the system. Currently, these invasions are considered one of the main drivers of global change due to their adverse effects on biodiversity, habitat structure and native ecosystem functioning (Mack et al., 2000; Stachowicz and Byrnes, 2006; Simberloff et al., 2013; Bellard et al., 2016). Additionally, the establishment of invasive species it is often associated to great economic costs (Pimentel et al., 2001, 2005) due to their alteration of several ecosystem services (Pejchar and Mooney, 2009; Vilà et al., 2010). The impacts of invasive species are especially important and noticeable in marine ecosystems, where biological invasions are on the rise due to the increases in their main vectors of introductions, such as shipping traffic, the aquarium trade or the opening and widening of new corridors (Katsanevakis et al., 2013; Seebens et al., 2013; Galil et al., 2017); and in the future, the establishment of non-native species is expected to continue increasing due to climate change (Stachowicz et al., 2002; Lejeusne et al., 2010).

Species invasiveness depends on the features that enable a non-native organism to invade a certain habitat (Richardson et al., 2011) with the main influence being the life-history traits of the invader (Grotkopp et al., 2002; Pyšek and Richardson, 2008; Van Kleunen et al., 2010). Several studies have suggested that phenotypic plasticity is one of the most important of such features for invasive species (Richards et al., 2006; Davidson et al., 2011). Plants and algae can adapt by modifying, among other things, photosynthetic traits (Molina-Montenegro et al., 2012; Zanolla et al., 2015), leaf-area and shoot allocation (Arenas et al., 2002; Liu and Su, 2016) and growth form (Van Kleunen and Fisher, 2001; Monro and Poore, 2009) allowing them to respond to changes in light, temperature or herbivory pressure (Lewis et al., 1987; Monro and Poore, 2005; Nicotra et al., 2010). In this sense, understanding how this phenotypic plasticity affects the success of an invasive species is crucial to our understanding of its invasion process (Schaffelke et al., 2006; Theoharides and Dukes, 2007) and might have important implications for the successful management of the species (Hobbs, 2000; Simberloff et al., 2005).

Caulerpa cylindracea is a siphonaceous green macroalga, native to Western Australia, that has become one of the most widespread non-native algae in the Mediterranean Sea (Piazzi et al., 2005; Klein and Verlaque, 2008; Montefalcone et al., 2015). Indeed, *C. cylindracea* is currently considered the most invasive species within the Mediterranean basin (Katsanevakis et al., 2016) and has also invaded areas in the Atlantic Ocean (Verlaque et al., 2004) and in Southern Australia (Klein and Verlaque, 2008). Still, it is not clear which has been the source of the primary introduction in the Mediterranean Sea, although it is quite likely that it was through the aquarium trade, with shipping traffic and fishing gear being the main sources of the secondary introductions within the basin (Verlaque et al., 2003). Ecologically, *C. cylindracea* spread causes the homogenization of native communities due to the formation of dense and continuous meadows (Klein and Verlaque, 2008), which has negative implications for the native macroalgal assemblages and the diversity of the communities (Piazzi et al., 2001; Piazzi and Ceccherelli, 2006; Klein and Verlaque, 2011). Also, this species affects the sedimentation rates, the carbon turnover, the organic matter composition and the quality of the invaded sediments (Piazzi et al., 2007; Holmer et al., 2009; Pusceddu et al., 2016; Rizzo et al., 2017). Morphologically, *C. cylindracea* is characterized by a simple morphology, formed by creeping stolons and erect shoots with grape-like ramuli (also called branchlets) that can be arranged radially or distichously (Klein and Verlaque, 2008). In addition, it has been reported that species in the genus *Caulerpa* show a high degree of morphological plasticity in response to environmental conditions (Peterson, 1972; Calvert, 1976; Coppejans and Beeckman, 1989; Collado-Vides, 2002a), allowing these species to adapt to different environments and thus increasing their invasive potential (Collado-Vides, 2002a; Raniello et al., 2004; Smith, 2009). Several factors such as temperature, light or depth, can trigger subtle morphological changes in stolon and ramuli shape (Peterson, 1972; Calvert, 1976; Ohba and Enomoto, 1987; Ohba et al., 1992), photosynthetic traits (Raniello et al., 2004, 2006) and the interspace between erect axes (Collado-Vides, 2002a; De Senerpont Domis et al., 2003). However, more acute morphological changes have been detected for the first time in *C. cylindracea* during a recent field survey (**Figure 5.1**). The thalli of these specimens consisted only of thin vertical filaments, which were impossible to identify as *Caulerpa* species until morphological and molecular characterization confirmed their identity.

Figure 5.1. Macroscopic picture of a rock covered by a thick patch of *Womersleyella setacea* turf with the filamentous form of *Caulerpa cylindracea* growing from beneath it (black arrows).

At present, the exact conditions that trigger this morphological change are unknown but, considering that the filamentous form was found in Montenegro after the extremely warm summer of 2018 (**Figure 5.2**), it would appear that stressful conditions brought about by high temperatures could be involved. A better understanding of the conditions that trigger this morphological shift – which allows *C. cylindracea* to become a cryptic invader – will greatly enhance our understanding of the invasive process, the collapses and the recoveries of this species.

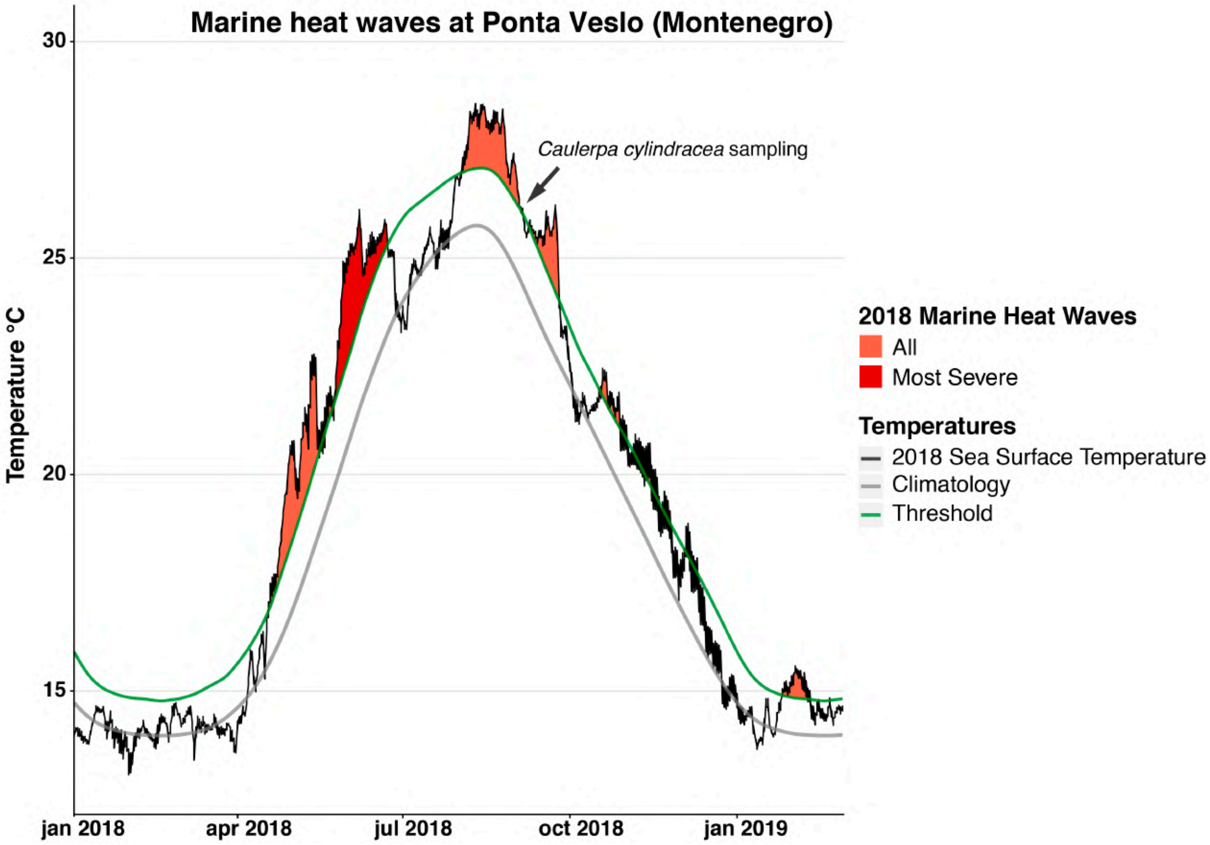


Figure 5.2. Marine heatwave (MHW) events during 2018 at Ponta Veslo, Montenegro (42º 22’ 5.15” N; 18º 36’ 22.50” E), calculated with the *heatwave*R package (Schlegel and Smit, 2018) using Reynolds Optimally Interpolated Sea Surface Temperature (OISST) data (Reynolds et al., 2007). The grey line represents the SST climatology for the last 35 years; the green line indicates the 90th percentile MHW threshold; and the black line shows the SST during 2018. The dark red filled area indicates the most severe MHW event during 2018, while the orange filled areas indicate all the other MHW events identified over the same time period. The dark grey arrow indicates the day of the year when the filamentous morphology of *Caulerpa cylindracea* was sampled from the field (5th of September).

In this study, our aim was to determine whether extreme temperature conditions can trigger the formation of a filamentous morphology in *Caulerpa cylindracea* similar to that observed in the field. To do so, the morphological plasticity of this species was studied through culture experiments at contrasting temperatures. In addition, in order to confirm the taxonomical identity of the specimens, all thalli (from both cultured and natural populations) were genetically characterized with a chloroplast molecular marker (*tuf*A), which had been used previously for the genus *Caulerpa* (Famà et al., 2002; Kazi et al., 2013; Sauvage et al., 2013).

5.3 Materials and methods

5.3.1 Study sites and culture

5.3.1.1 Extreme temperature laboratory experiment

To study the effects of extreme temperatures on *C. cylindracea*, specimens from a population in Spain (Roses: 42° 14’ 18.26” N; 3° 12’ 25.74” E) were sampled in February 2019. Once in the laboratory, samples were cleaned with sterilized seawater to remove all the epiphytes and detritus (such as dead Posidonia oceanica rhyzomes and dead shells). For acclimation, *Caulerpa* samples were placed in aquariums (12 L) with sterile seawater and in a *Radiber AGP-360* growth chamber at 12ºC and a 12:12 (L:D) cycle at 200 µmol photons m^{-2} s^{-1} to simulate natural conditions of irradiance and temperature for one week. After the acclimation period, algal cultures were prepared for a period of 170 days under either control conditions or extreme (i.e., very warm) conditions (**Figure 5.3**). Six fragments of *C. cylindracea* (≈4 cm^2 each) were randomly transferred to six plastic beakers (1 L): three control treatments and three extreme-temperature treatments, each containing 200 g of sterilized gross sand and 0.5 L of sterilized seawater to which was added 5 ml/l of K-medium (Keller et al., 1987). The temperature treatments for the experiment were as follows: "control" (21ºC) based on the average summer seawater temperature recorded in the sampled area and "extreme" (29°C) based on abnormally high summer seawater temperatures recorded in the Mediterranean Sea (www.t-mednet.org). After an adaptation period of 7 days at 12ºC in the growth chamber, the temperature was progressively increased (by 1ºC every 2 days) in all six treatment beakers for 18 days until a temperature of 21ºC was reached in the growth chamber. At this point, the beakers were split into two *Radiber AGP-360* growth chambers, one to keep the "control" beakers and the other to keep the "extreme" beakers throughout the experiment. Following this, the "control" beakers were maintained at 21ºC for the remaining 152 days of the experiment in the growth chamber; whereas, in the case of the "extreme" beakers, the temperature was raised in the other growth chamber by 1ºC every 5 days for the next 40 days until a temperature of 29ºC was reached; this temperature was then maintained for a further 14 days and then gradually lowered by 1ºC each day back to 21ºC and kept at this temperature for the remaining 90 days of the experiment (**Figure 5.3**). Throughout the experiment, the seawater and growth medium mixture was renewed once a week.

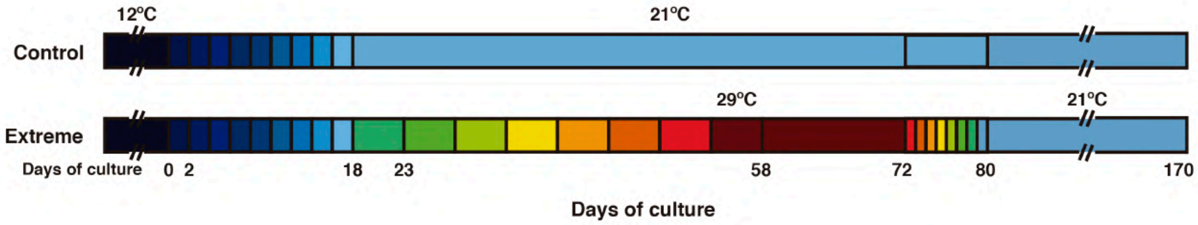


Figure 5.3. Water temperature variation over the course of the experiment in "control" conditions (above) and "extreme" conditions (below). The numbers indicate the days of culture and each change in color represents a 1ºC change in temperature.

5.3.1.2 Field sampling for morphological and taxonomical characterization

Samples of *C. cylindracea* were collected from natural populations in Spain (Roses: 42º 14’ 18.26” N; 3º 12’ 25.74” E); Croatia (Funtana: 45º 10’ 40.16” N; 13º 35’ 32.31” E and Split: 43º 30’ 28.79” N; 16º 23’ 17.56” E); Montenegro (Ponta Veslo: 42º 22’ 5.15” N; 18º 36’ 22.50” E); and Albania (Kallm: 41º 19’ 27.88” N; 19º 25’ 19.31” E) by scuba-divers at depths of between 5 and 12 m based on previous knowledge on the presence of the invasive alga in these locations. All samples were transported in zip bags within a thermal box to maintain a constant seawater temperature until the morphological characterization was performed in the laboratory. All samples showed the typical morphology of the species (**Figure 5.4, B**). These samples were also used for the taxonomic characterization of the populations (see **Molecular analysis** section).

5.3.2 Data collection and analysis

5.3.2.1 Extreme temperature laboratory experiment

The effects of the extreme temperatures we applied to *C. cylindracea* were assessed by measuring the macroscopic morphometric changes. The structure and area of *Caulerpa* were measured by means of macroscopic photographs taken with an Olympus TG-5 camera, which were then analyzed with Adobe Photoshop CC 2018. Living parts of *C. cylindracea* (green color) were manually selected and measured using the “analysis tool”. Then, to assess whether there were differences in the area of *C. cylindracea* between treatments at the end of the experiment, a linear mixed effect model (LMM) was fitted with “*C. cylindracea* area” as the response variable and “treatment” and “time” as the explanatory variables. The interaction between both explanatory variables was included in the model and a random term for “replicate” was used to take into account the repeated measures design

(Harrison et al., 2018). To fit this model, the package *lme4* (Bates et al., 2015) in the statistical environment R was used (R version 3.6.3) (R Core Team, 2018). Finally, to compare the effects between levels in the treatment factor ("control" and "extreme) at each time observation ("beginning" and "end"), Tukey post-hoc tests were performed using the functions "pairs" and "emmeans" from the package *emmeans* (Lenth, 2018).

5.3.2.2 Morphological characterization and comparison

The morphological characterization of the cultured stolons and filaments was assessed by means of microscopic photographs, taken using a Zeiss AXIO Imager A.2 (Carl Zeiss, Berlin, Germany) equipped with an AxioCam MRc5 camera and a stereomicroscope Stemi 2000-C (Carl Zeiss, Berlin, Germany) equipped with an AxioCam ERc 5s camera; and the images were analyzed with Zen2011 software (Blue Edition). Also, to account for regional morphological variability, the mean stolon thickness of 10 randomly sampled stolons from each of the natural populations (Roses, Funtana, Split, Ponta Veslo and Kallm) was compared to the thickness of filaments obtained at extreme temperatures.

To assess whether mean thickness of filaments differed from that of stolons, a linear model was fitted with "*C. cylindracea* thickness" as the response variable and "location_morphology" as the explanatory variable, in the statistical environment R. Finally, to compare between location_morphology levels ("Roses_filaments", "Roses_stolons", "Funtana_stolons", "Split_stolons", "Ponta Veslo_stolons" and "Kallm_stolons"), Tukey *post hoc* tests were performed using the functions "pairs" and "emmeans" from the package *emmeans*.

5.3.3 Molecular analysis

To determine species identity, a genetic analysis was performed on all sampled and incubated thalli, including both filamentous and typical morphology. DNA extraction was performed following the DNeasy Plant Mini Kit (QiaGen) protocol in order to obtain the best DNA quality. The primer used to amplify the genetic material was *tuf*A (elongation factor A, from the chloroplast). The reactions were performed in 25 µL volumes and the master mix contained 5 mM of $MgCl_2$, 0.3 mM of each primer, 0.2 mM of dNTPs, 0.5 units of Taq DNA polymerase and 1.0 µL of the extracted DNA. The PCR reaction consisted of 40 cycles of 94ºC for 1 min (denaturation), 52ºC for 1 min (primer annealing) and 72ºC for 2 min (extension) (Famà et al., 2002). The PCR reaction was finalized with a final 5 min step at 72ºC. The PCR products were purified and sequenced by Macrogen Spain.

Sequences analyses were performed using different R (R Core Team, 2018) packages: *MUSCLE* (Edgar, 2004) to align the sequences, and *APE* (Paradis et al., 2004) and *PHANGORN* (Schliep et al., 2017) to create phylogenetic trees based on statistical analyses (Bio neighbor-joining tree, k80 distance, with 10000 replicates).

5.4 Results

5.4.1 Extreme temperature laboratory experiment

In the model fitted to the data from the extreme temperature experiment, both "treatment" and the interaction term between "treatment" and "time" showed a significant effect on *C. cylindracea* area (p-value < 0.05; **Appendix D: Table D.1**), being the area of *C. cylindracea* equal between treatments at the beginning of the experiment but being significantly different between "control" and "extreme" conditions at the end (p-value < 0.05; **Appendix D: Table D.2**). Actually, after 72 days, the mean surface area of the *Caulerpa cylindracea* thalli under "control" conditions had increased by 44%, whereas that of the specimens exposed to the "extreme temperature" treatment (at 29ºC for final 14 days of this 72-day period) had decreased significantly by 87% (**Figure 5.4, A**). Furthermore, while the control specimens maintained the typical morphology of the species – characterized by thick rhizomes with some vertical vesicular fronds (**Figure 5.4, C**) – almost all the thick parts of the specimens in the "extreme temperature treatment" had disappeared after 14 days at 29ºC (**Figure 5.4, E**).

When the cultures were returned to optimum conditions, new living parts emerged from the sediment in all the "extreme treatment" beakers, after 20 days. However, these new parts did not resemble the original *C. cylindracea*´s morphology. Instead, they presented a new type of structure characterized by erect thin filaments (**Figure 5.5, A**), which for the next three months continued growing vertically and extending throughout the liquid in the beakers (**Figure 5.5, B**).

Macroscopically, these filaments, which grow vertically from the substrate, are long (between 5 and 10 cm) and thin, and occasionally branched. Each filament has rhizoids in its basal part (which is without plastids) that serve to attach the filament to the substrate. The upper part of the filament (with plastids) is light green and in all cases devoid of rhizoids or vesicles, which contrasts with the typical morphology of *C. cylindracea* (**Figure 5.4, B** and **D**).

These filaments contrasted with the *C. cylindracea* thalli cultured under control conditions which, throughout the experiment (**Figure 5.4, B** and **D**), maintained the typical morphology described for this species: the thalli were characterized by thick, prostrate stolons with interspaced rhizoids for anchoring, even where they grew unattached to the substrate. Vesicular fronds could also be observed, although these were smaller than in the field.

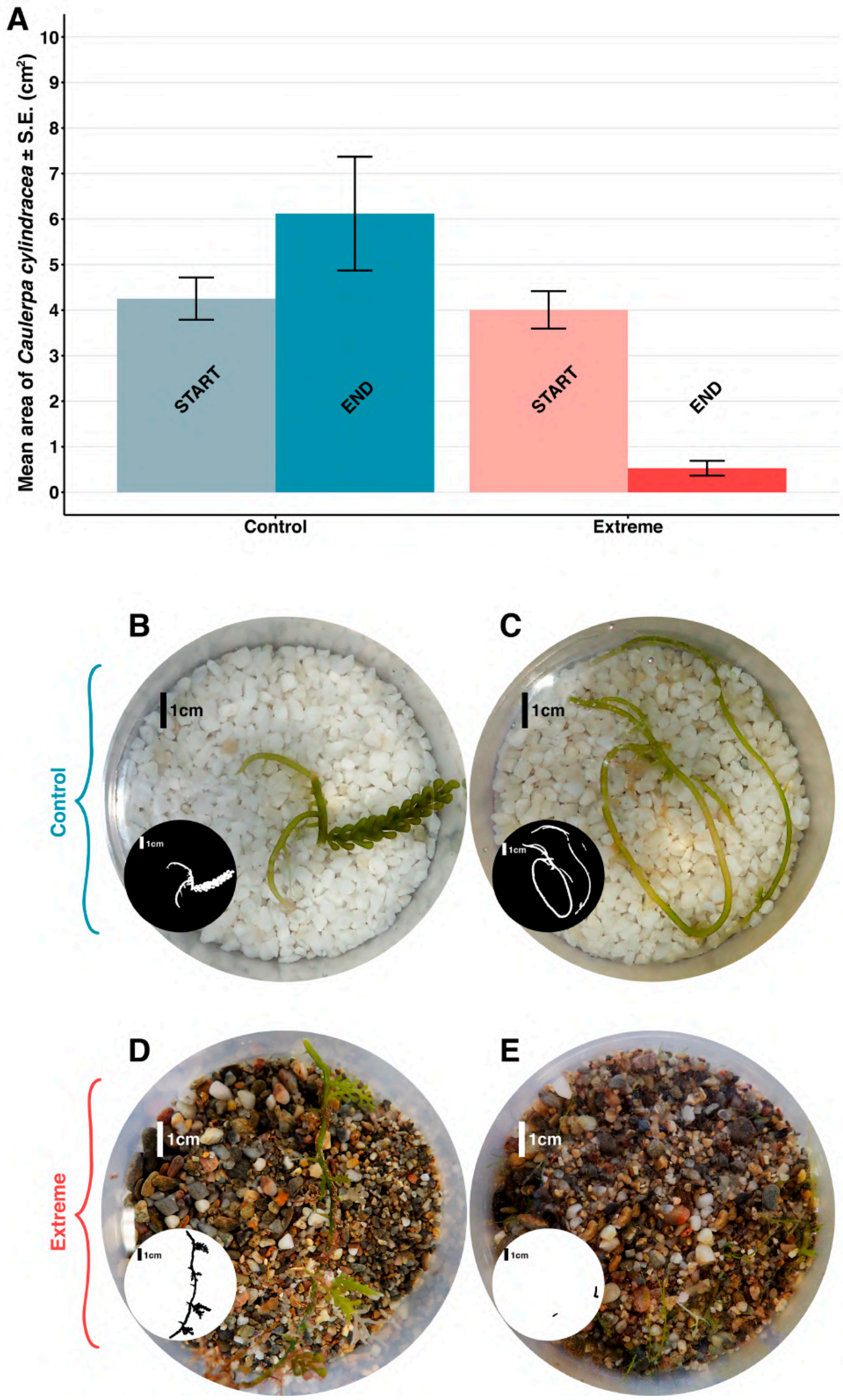


Figure 5.4. (A) Mean area ± S.E. of *Caulerpa cylindracea* in the beakers (n=6, 3 control beakers and 3 extreme temperature beakers), at the beginning of the experiment, and after 72 days. Photographs **(B)** and **(C)** show control beakers at the beginning of the treatment and after 72 days, respectively. Photographs **(D)** and **(E)** show extreme temperature beakers at the beginning of the treatment and after 72 days, respectively. The small black and white circles highlight the *C. cylindracea* present in each beaker.

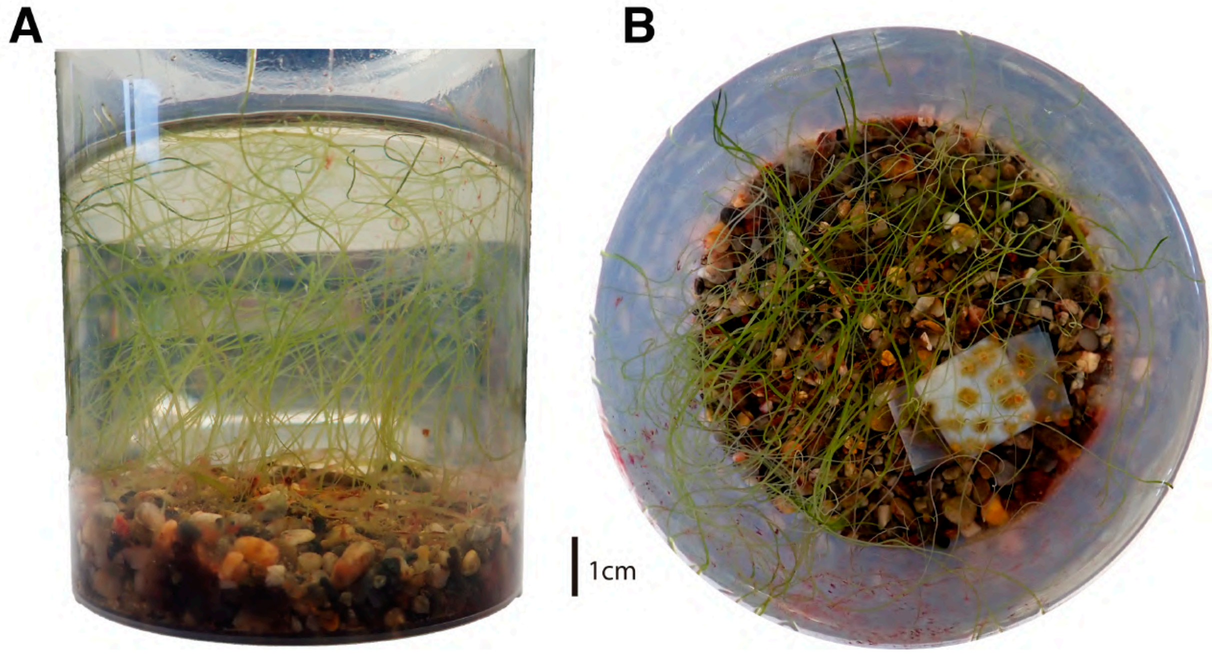


Figure 5.5. Lateral **(A)** and vertical **(B)** view of one beaker with the filamentous morphology of *Caulerpa cylindracea*. The white square on the bottom of the beaker was added at the end of the experiment, to use it as a scale.

5.4.2 Morphological characterization

In the model fitted to the morphological data, the variable "location_morphology" showed a significant effect on *C. cylindracea* thickness (p-value < 0.05; **Appendix D: Table D.3**), with filament thickness being significantly different to stolon thickness (p-value < 0.005; **Appendix D: Table D.4**) while stolon thickness was similar between populations (**Appendix D: Table D.4**). Actually, microscopically, there was an almost 10-fold difference in mean thickness between the filaments (222.42 ± 9.78 µm) in the extreme temperature beakers and the stolons (2093.38 ± 31.50 µm) sampled at different natural populations. This is amply illustrated in **Figure 5.6**.

Although erect filaments are rarely ascribed to *Caulerpa* genus, the presence of trabeculae – slender strands traversing the lumen of the thallus (Lamouroux, 1809; Womersley, 1984; Wynne and Bold, 1985) – confirms the identity of the thalli. The anatomical morphology of this structure in the filaments is identical to that of the regular stolons, as they traverse the interior of the filament going from one side to the other of a circular section and attach to the wall with a thickened structure that resembles a suction cup. However, the trabeculae in the filaments are much thinner and less numerous than in the stolons, which might explain why the filaments are so weak.

5.4.3 Taxonomical characterization

For the genetic analysis, a total of 14 high quality *tuf*A sequences of up to 820 bp. were obtained and amplified from 5 specimens with the filamentous shape, 8 specimens from the Mediterranean Sea with the common morphology of *Caulerpa cylindracea* and an additional sequence from *C. sertularioides*, which was obtained from the Caribbean Sea (**Appendix D: Table D.5**). In addition, several *C. cylindracea* sequences were obtained from GenBank, together with sequences from *C. taxifolia* and *C. prolifera*, in order to establish differences in the phylogenetic tree. In the

Neighbor Joining tree (**Figure 5.7**), all sequences from *C. cylindracea* formed a highly supported cluster, grouped close together, which included both filamentous and typical thalli, thus indicating that they are the same entity.

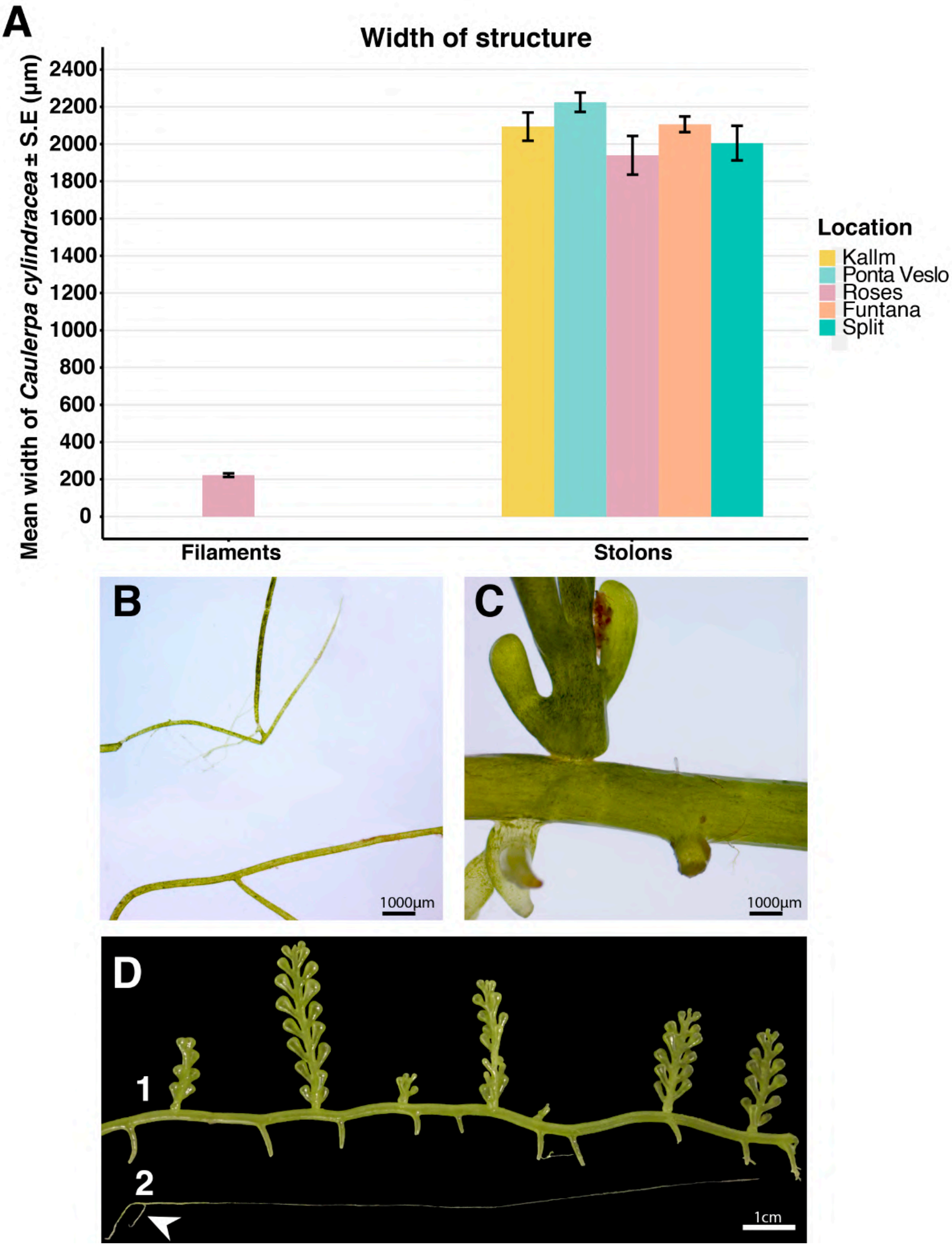


Figure 5.6. (A) Mean width ± S.E. of *Caulerpa cylindracea* filaments (left) and stolons (right) from different populations (n=10 for each population). **(B)** View of two filaments

under the stereomicroscope at 1.0x. **(C)** View of a stolon with a vertical vesicular frond under the stereomicroscope at 1.0x. **(D) 1)** Macroscopic view of *C. cylindracea* with the regular morphology and **2)** macroscopic view of the filamentous form of *C. cylindracea*. The white arrow shows the rhizoids that the filaments use for attaching themselves to the substrate.

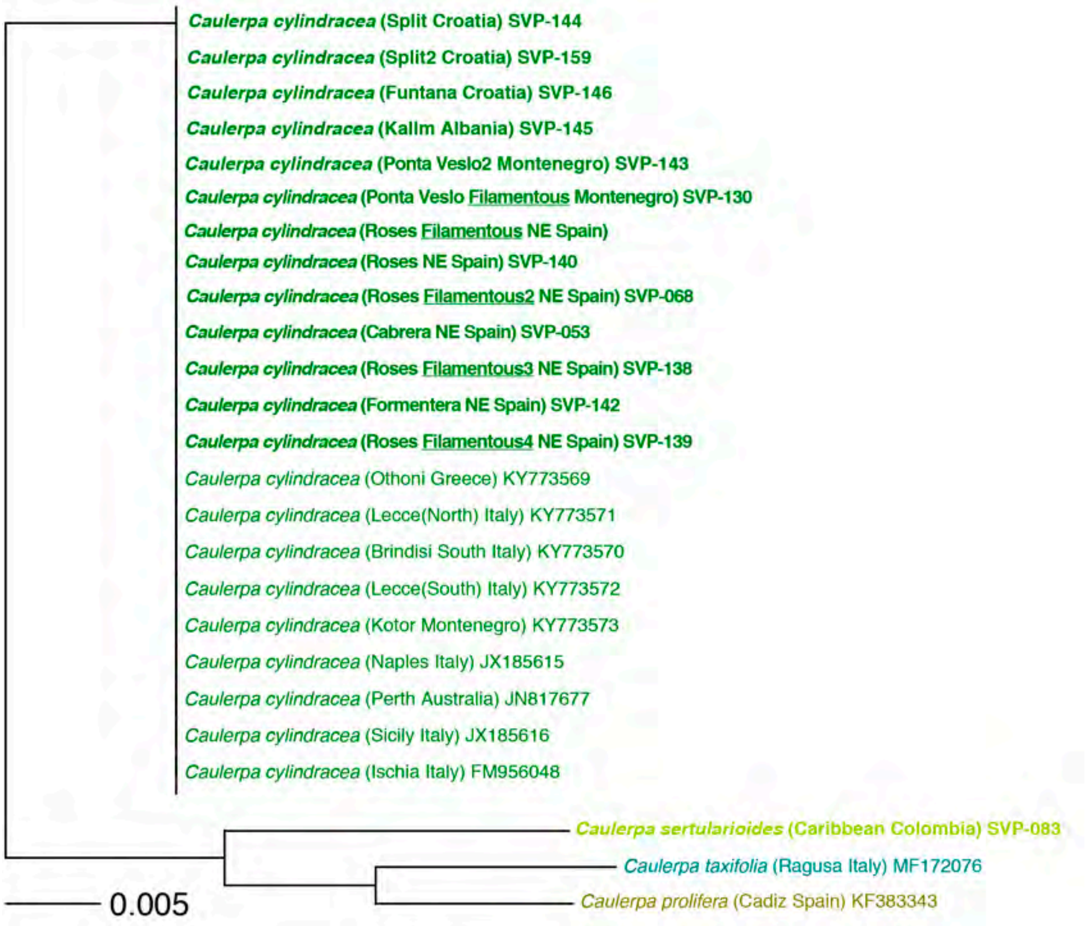


Figure 5.7. Phylogenetic Neighbor Joining (NJ) tree for *Caulerpa cylindracea*, obtained using the *tuf*A marker. Three other species of *Caulerpa* (*C. sertularioides, C. taxifolia and C. prolifera*) were used as closely related species to establish differences. GenBank accession numbers are given for each sequence. Values at nodes derived from the NJ. The sequences produced in the present study are highlighted in bold.

5.5 Discussion

Our culture experiments showed that extreme environmental conditions, in this case high temperatures maintained for long periods of time (i.e. 14 days at 29ºC), affected the survival of *Caulerpa cylindracea* and triggered the development of a new morphology characterized by long, thin vertical filaments from the surviving tissues. This new morphology of *C. cylindracea* has also been found in the field and is described here for the first time.

The laboratory experiment showed the remarkable capacity of *C. cylindracea* to survive under stressful culture conditions as the temperature treatment chosen (29ºC) is much higher than the usual temperatures found at both the native and the invaded range (Klein and Verlaque, 2008). Additionally, *C. cylindracea* showed a great ability to adapt to environmental change, because it developed a new filamentous morphology from the damaged tissue that survived to the unfavorable culture conditions, i.e., extreme temperatures. These morphological changes during vegetative development are a common strategy in sessile organisms such as plants (Dorn et al., 2000; Puijalon et al., 2008; Nicotra et al., 2010) and algae (Kübler and Dudgeon, 1996; Garbary et al., 2004; Monro and Poore, 2005; Fowler-Walker et al., 2006) to tolerate environmental change, and to improve their competitive and survival capacity (Bradshaw, 1972; Harper et al., 1986; Price and Marshall, 1999; Collado-Vides, 2002b). Actually, more or less acute morphological changes have been previously observed and described in different algae species (e.g.: some *Caulerpa* species, *Ulva prolifera*, *Chondrus crispus*, *Asparagopsis armata*, *Padina jamaicensis*, *Codium fragile* or *Ecklonia radiata* among others) under different culture conditions (Ohba and Enomoto, 1987; Ohba et al., 1992; Kübler and Dudgeon, 1996; De Senerpont Domis et al., 2003; Garbary et al., 2004; Monro and Poore, 2005; Gao et al., 2016) and in the field (Lewis et al., 1987; Meinesz et al., 1995; Collado-Vides, 2002a; Garbary et al., 2004; Fowler-Walker et al., 2006), as a response to changes in temperature, salinity, hydrodynamism, light or herbivory pressure.

Taking into account that *Caulerpa cylindracea* may be one of the most widely-studied invasive algae species around the world, the lack of a previous description of the filamentous morphology demonstrates that when *C. cylindracea* adopts this form, it clearly goes undetected. For instance, the filamentous morphology was sampled – accidentally – in the field within a macroalgae benthic community (**Figure 5.1**) and was only identified as *Caulerpa* after the samples were analyzed under the microscope. Thus it is clear that *C. cylindracea* filaments also develop under natural conditions. The variables that trigger such filaments in the field may, however, be multiple and in general, remain uncertain. Nevertheless, the culture experiments and field observations described here strongly indicate that stressful conditions may induce the formation of these structures.

The phenotypic plasticity observed in *C. cylindracea* and its ability to withstand extreme conditions (high temperatures) for a long time are characteristics that clearly influence the invasiveness of this species, and improve its persistence and resistance to stress. Actually, this resistance, together with the difficulty of observing the filaments underwater, might explain why *C. cylindracea* populations sometimes seem to disappear only to bounce back after a few months (García et al., 2016). In such situations, it may be that while most of the population dies following unfavorable conditions (**Figure 5.8, E**) (Klein and Verlaque, 2008), some small remnants survive, most probably hidden and undetectable in the sediment or sand. Then, as was the case in our culture experiment, the surviving parts may produce filaments that go unnoticed by divers and researchers (**Figure 5.1**), and, subsequently, the regular morphology of *C. cylindracea* returns when favorable conditions allow the population to recover (**Figure 5.8, G**). Typically, colonization of a new area by *C. cylindracea* can take approximately 3 years, but the presence of this cryptic and resistant form of the species would explain the marked reduction – from 3 years to less than one year – in the time it takes *C. cylindracea* to re-colonize areas in which it had disappeared (García et al., 2016), thus highlighting the importance of this morphology in the re-colonization process (**Figure 5.8**).

Furthermore, when adopting this cryptic phase, identification is extremely difficult or simply impossible, which has further implications for the management of the species, since early detection is one of the most important requirements for the successful management of invasive species (Lodge et al., 2006; Vander Zanden et al., 2010; Giakoumi et al., 2019a). This misidentification of the filaments can also affect current estimations of *C. cylindracea* in the Mediterranean Sea as this species may be present at several locations in this latent, cryptic form. Since traditional methods of direct observation may not always be reliable in detecting filaments of *C. cylindracea*, as they can be misidentified or missed entirely, methods involving environmental DNA (Taberlet et al., 2012; Kelly et al., 2014; Thomsen and Willerslev, 2015) could be useful in detecting this species, as has been the case with the detection of other invasive species (Dejean et al., 2012; Ardura et al., 2015; Simmons et al., 2015).

Dispersion is another process that might also be favored by *C. cylindracea* filaments, because these long and weak vertical structures are more easily broken upon contact or by water movement than the regular thallus. This will release living fragments that can be transported by currents or attached to drifting objects, favoring dispersal of *C. cylindracea* and the potential for secondary introductions. Actually, in *Caulerpa* species, the fragmentation process is one of its most important reproductive strategies (Fralick and Mathieson, 1972; Ceccherelli and Cinelli, 1999; Smith and Walters, 1999; Ceccherelli and Piazzi, 2001) and allows these species to spread really fast.

To conclude, this newly-identified filamentous morphology of *C. cylindracea* could act as a potential jack-of-all-trades that further improves this species' capacity as an invader. The filaments described here are involved in the persistence, resistance and dispersion of this invasive species and have characteristics that allow this species to withstand harsh abiotic conditions and which may help to explain its successful expansion in the Mediterranean Sea.

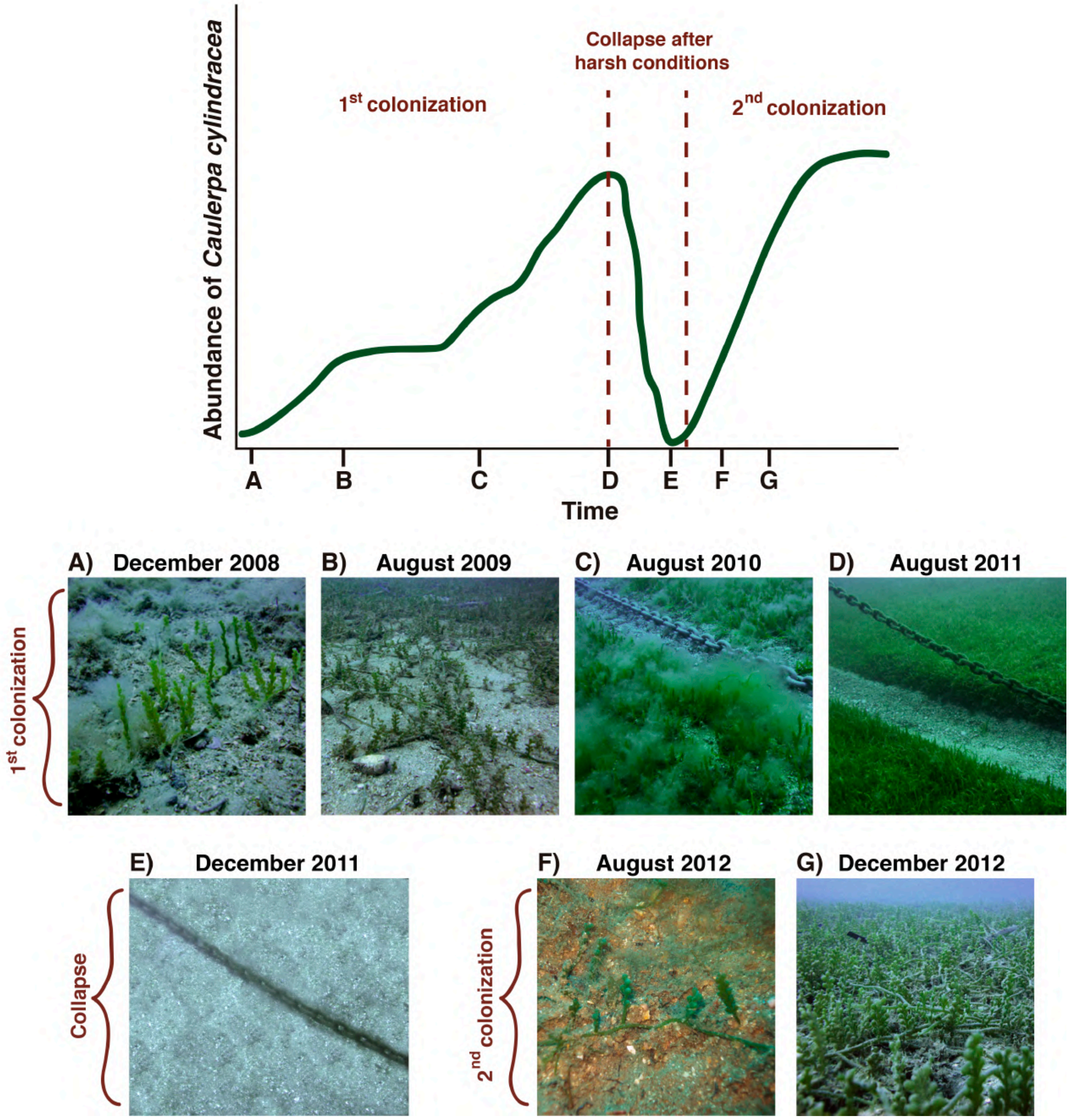


Figure 5.8. Evolution of the abundance of *Caulerpa cylindracea* over a period of 4 years in a population under study, close to Vilanova i la Geltrú (Spain). During the first colonization, *C. cylindracea* was first detected in December 2008 **(A)**; by August 2009, *C. cylindracea* had expanded and had a patchy heterogeneous distribution **(B)**; in August 2010, *C. cylindracea* covered a larger area forming a continuous meadow **(C)**; and in August 2011, the abundance of *C. cylindracea* reached a peak, forming a dense and continuous meadow over more than 1000 m^2 **(D)**. In December 2011, the population had collapsed after an exceptionally warm autumn and no sign of *C. cylindracea* could be found **(E)**. The first report of *C. cylindracea* in the second colonization was in August 2012 **(F)**; and just three months later, in December 2012, it was already forming a continuous meadow over a large area **(G)**, with greater abundance than that of August 2010 **(C)**.

5.6 Acknowledgments

The authors would like to thank Jana Verdura, Ljiljana Iveša, Petra Lučić, Vesna Mačić and Jerina Kolitari for support during field collection of the samples. We also acknowledge the role of the project AFRIMED (EASME/EMFF/2017/1.2.1.12/S4). Support for this work was provided by the Spanish Ministry of Science, Innovation and Universities (project ANIMA, ref. CGL2016-76341-R), J.S. received the support of a fellowship from "La Caixa" Foundation (ID 100010434) with code (LCF/BQ/DE17/11600001) and R.G benefitted from a pre-doctoral fellowship from the Spanish Ministry of Science (ref. BES-2017-079907). J.S., R.G., E.C. and A.V. are members of the Marine Conservation Research Group (www.medrecover.org; 2017 SGR 1521) from the Generalitat de Catalunya.

Chapter 6

General Discussion

Cover image: The nudibranch *Flabellina affinis* on top of a pair of *Caulerpa cylindracea* fronds.

Author: Enric Ballesteros Segarra.

The role that specific factors might have for the success of well-established invaders and whether native habitats can develop resistance mechanisms against invasive species still remains poorly understood, making it difficult to disentangle the long-term dynamics of most invaders and to propose effective management measures to reduce their impacts. Therefore, this dissertation is an attempt to unravel the mechanisms that influence the success and the invasion history of the invasive alga *Caulerpa cylindracea*, with the objective of improving our understanding of the invasive process and of the mechanisms influencing the dynamics of invaders once they have been successfully established. Overall, we believe that the insights reported in this thesis can be useful across systems and species and could help us find effective management measures to hinder the consequences of invasions once prevention is no longer possible.

In this general discussion, considering that the discussions of each specific chapter were addresses in a very broad and general way, we present the main findings mainly focusing on marine invasions, in order to provide a view of their application and contribution to marine invasion ecology, as well as to discuss the implications that they might have for the conservation of marine native habitats and diversity.

6.1 Biotic resistance mechanisms. Are they effective against marine invasive algae?

Previous evidence, mostly from terrestrial ecosystems, suggests that both competition and herbivory can affect invader success and reduce the fitness and the abundance of the invasive species (e.g., Levine et al., 2004; Parker and Hay, 2005; Cushman et al., 2011; Frankow-Lindberg, 2012; Pearson et al., 2012; Byun et al., 2013; te Beest et al., 2018; Zhang et al., 2018). However, studies from marine ecosystems quite often report the inability of native communities to limit the performance of invaders, particularly that of invasive algae (Kimbro et al., 2013; Enge et al., 2017; Papacostas et al., 2017 and references therein), with only 16% and 30% of the assessed studies reporting a limiting role of competition and herbivory respectively (Papacostas et al., 2017). In contrast to this, in **Chapter 2** we provide evidence that biotic mechanisms can significantly limit the abundance of *C. cylindracea*, a well-established and successful marine alga that thrives in the Mediterranean Sea.

In our particular case, **competition** mediated by the dominance of canopy-forming and erect species had a significant effect on the abundance of the invader throughout its entire bathymetric distribution, probably because the presence of certain traits among the community members (e.g., large size, perennial habits and complex morphology) reduced the availability of light and avoided the reattachment of invader fragments through the branch-sweeping of the substratum (Bulleri and Benedetti-Cecchi, 2008; Bernardeau-Esteller et al., 2015; Piazzi et al., 2016). In this sense, our findings agree with previous evidence that reports the ability of complex communities – those dominated by canopy-forming species with a large size and usually perennial habits – to strongly influence the success of invasive algae (Ceccherelli et al., 2002; Arenas et al., 2006; Britton-Simmons, 2006; Bulleri et al., 2010; Vaz-Pinto et al., 2012) and highlight the role of competition as an important biotic resistance mechanism also in marine systems. In fact, competition can prevent the invasion of the system or totally suppress the invader in well-preserved communities as has been demonstrated by the inability of *C. cylindracea* to establish in healthy *Posidonia oceanica* meadows (Katsanevakis et al., 2010; Bulleri et al., 2011; Tamburello et al., 2014; Bernardeau-esteller et al., 2020) or by the suppression of *Sargassum muticum* in mature kelp forests within an old marine protected area (MPA) (Caselle et al., 2018). So, it seems that by canopy-forming and erect species mainly modulating the availability of light and space, the most important limiting resources for algae (Sousa, 1979; Carpenter, 1990), competition can significantly influence invader success.

Surprisingly however, if the abundance of invaders can be limited by competition, how can it be possible that many of the marine studies report the inability of this mechanism to limit invasive algae success? We believe that this could be likely related to the fact that many marine habitats, due to the strong cumulative impacts they have been exposed to (e.g., extensive habitat loss, over-grazing by herbivores and climate change), have shifted from being dominated by marine foundation and canopy-forming species to habitats dominated by turf, encrusting and filamentous species (Wernberg et al., 2013; Filbee-Dexter and Scheibling, 2014; Ling et al., 2014; Vergés et al., 2014b; Filbee-Dexter et al., 2016; Wernberg et al., 2016; Filbee-Dexter and Wernberg, 2018). In turn, these oversimplified systems have little or no capacity to outcompete invasive algae and can even promote the attachment and spread of invaders (Ceccherelli et al., 2002; Arenas et al., 2006; Bulleri and Benedetti-Cecchi, 2008; Vaz-Pinto et al., 2012). Successively, due to the dominance by turf species and invaders, the native species might not be able to colonize back, allowing the invader to continue dominating the system and maintaining the degraded stable state even when the pressures that brought it to that state are no longer there (Scheibling and Gagnon, 2006). Putting all this together, it seems that the fact that many marine studies report the lack of strong competitive effects against algae invaders it is actually a symptom of the degradation, deforestation and oversimplification of macroalgal communities worldwide, definitively a more worrying scenario than that imposed by marine invasions.

Regarding the effect of **herbivory** on *C. cylindracea* success, we report the ability of herbivores, specifically that of the native fish *Sarpa salpa*, to limit *C. cylindracea* abundance. However, contrary to what we observed for competition, herbivory only influenced the abundance of the invader in shallow habitats, due to the constrained distribution and activity of the herbivore to depths shallower than 30 m (Tomas et al., 2011b). This is the first evidence of herbivory limiting the abundance of *C. cylindracea* and contrasts with many of the previous studies assessing the role of herbivory on invasive algae success, where herbivores have been reported to be ineffective for the control of invaders (e.g., Boudouresque et al., 1996; Wikström et al., 2006; Monteiro et al., 2009; Forslund et al., 2010; Tomas et al., 2011a; Cebrian et al., 2011; Nejrup et al., 2012; Enge et al., 2017; but see: Stimson et al., 2001; Lyons and Scheibling, 2008; Britton-Simmons et al., 2011; Caselle et al., 2018). However, providing that exclusion experiments have been seldom done, it is difficult to deduce the role of herbivory on the success of invasive algae from previous assessments, as they mainly rely on pair-choice experiments that exclusively assess preference. Actually, although these assessments serve as an approximation of the alga performance under herbivory pressure, they cannot be used to infer the role of herbivores on the population dynamics of invaders under natural conditions. That is so, because in natural communities, apart from the herbivory effect itself, invader success will also be influenced by the direct effect of interspecific competition and by the abiotic conditions (Mitchell et al., 2006; Catford et al., 2009; Lockwood et al., 2013). Therefore, *in situ* herbivore exclusion experiments like the one we have performed, might be the only reliable way of assessing the influence of herbivory on the population dynamics of invaders (Liu and Stiling, 2006; Pearson et al., 2012) and they are urgently needed if we are to better assess the actual role of herbivory on the success of invasive algae (Enge et al., 2017). Taking this into account, it is quite likely that the herbivory effect observed in our exclusion experiment it is also reflecting the competition by the canopy-forming and erect species occurring in the assemblage, which might hinder the ability of *C. cylindracea* to compensate herbivory damage, a mechanism that has been attributed to the invader to explain its invasive success (Ruitton et al., 2006; Bulleri and Malquori, 2015). Remarkably, this **interactive effect** between competition and herbivory was also detected when the abundance of the species was assessed through its bathymetric distribution and it definitively explains why the invader is significantly more limited in the high complexity assemblages at shallow depths, where both biotic mechanisms act at their maximum intensity. This is, to our knowledge, the first time that the interaction between biotic mechanisms has been reported for marine ecosystems and our findings agree with those reported from terrestrial ecosystems, where the interaction between competition and herbivory yielded the strongest limitation of invader success (Suwa and Louda, 2012; Li et al., 2014; Zhang et al., 2018). In fact, while single biotic mechanism assessments still predominate in invasion ecology, several authors have highlighted the urgent need for assessments that consider the interaction between biotic mechanisms in order to obtain more reliable estimations of the actual role and strength of biotic resistance against invaders (Levine et al., 2004; Mitchell et al., 2006; Alofs and Jackson, 2014; Papacostas et al., 2017; Caselle et al., 2018; Petruzzella et al., 2020),

Until now, assessments of the role of herbivory on the success of invasive algae had mainly considered and assessed invertebrate organisms (e.g., Sumi and Scheibling, 2005; Wikström et al., 2006; Lyons and Scheibling, 2008; Forslund et al., 2010; Cebrian et al., 2011; Tomas et al., 2011a; Enge et al., 2012; Hammann et al., 2013; but see: Stimson et al., 2001; Davis et al., 2005), following the general belief of sea urchins and gastropods being the keystone herbivores in temperate systems (Hawkins and Hartnoll, 1985; Ling et al., 2014). However, recent evidence on the influence of fish herbivory on the abundance and distribution of temperate algae (Verlaque, 1990; Vergés et al., 2009; Taylor and Schiel, 2010; Poore et al., 2012; Franco et al., 2015; Gianni et al., 2017), suggests that the role of herbivorous fish for the control of invaders might have been underestimated. Actually, we provide evidence of the significant role that one herbivorous fish species, *S. salpa*, has for the control of the abundance of *C. cylindracea* (**Chapters 2 and 3**), suggesting that fishes can strongly influence the success of invaders. So definitively, in the following studies assessing the importance of herbivory as a biotic resistance mechanism, fishes should also be considered to further disentangle their role for the success of other invasive algae. In our case, the consumption of *C. cylindracea* by *S. salpa* is probably mediated by two main particularities: (1) the strong preference that the fish species shows towards the invader (Tomas et al., 2011b and **Chapter 3**), and (2) the fact that *S. salpa* has probably generated tolerance to the toxic metabolites produced by *C. cylindracea* through the previous exposition to the native conspecific *Caulerpa prolifera* (Marco-Méndez et al., 2017). Thus, taking into account that many invasive algae are known to produce herbivore deterrent metabolites (e.g., Wikström et al., 2006; Forslund et al., 2010; Cebrian et al., 2011; Enge et al., 2012, 2017; Nejrup et al., 2012; Nylund et al., 2012), special emphasis should be focused towards understanding whether native herbivores can generate tolerance to those metabolites through the subsequent exposition to the invader, something that would highlight the importance of time for the development of biotic resistance mechanisms (**6.2 The importance of time in invasion ecology**).

Complementary, in **Chapter 4** we have reported the consumption of *C. cylindracea* by the omnivorous fishes *Diplodus annularis*, *Diplodus sargus* and *Spondyliosoma cantharus* and determined that the contribution of the invader to the diet of these sparid fishes was generally low and that the consumption seemed accidental. However, several individuals were found with high amounts of *C. cylindracea* in their stomachs, suggesting that the grazing activity of these non-strictly herbivorous organisms could potentially affect the abundance of *C. cylindracea*, particularly considering that this fish species dominate the shallow rocky infralittoral habitats in the Mediterranean Sea (García-Rubies, 1997; Sala and

Ballesteros, 1997). In this sense, we pose that the lower impact on *C. cylindracea* abundance exerted by the omnivorous fish can complement the higher impact exerted by the strictly herbivorous organisms, and together, they might significantly reduce the abundance of the invader. Therefore, considering that omnivory is extended and prevalent across marine food webs (Menge, 1995; Sala, 2004; Bruno and O'Connor, 2005; Thompson et al., 2007; Long et al., 2011), more studies assessing whether other non-strictly herbivorous organisms can feed on invasive algae, could increase our understanding of the contribution of different organisms to the success of invaders and help us disentangle the myriad of interactions that might develop between the invader and the invaded assemblage (**Figure 6.1**).

In general, our results provide evidence that the use of field monitoring together with *in situ* manipulative experiments might be essential if we are to better understand and quantify the strength of biotic resistance mechanisms against a particular invader, as they allow to assess the interaction between mechanisms and their effect in natural settings. Consequently, going back to the question that we posed at the heading of this section, are biotic resistance mechanisms effective against invasive algae? The answer is that it strongly depends on the environmental context of the invasion. For instance, we can confidently say that biotic mechanisms are effective at regulating the abundance of *C. cylindracea* in the Mediterranean Sea, whenever the assemblage is well preserved, it sustains a high benthic complexity and there is a healthy and balanced community of herbivores. Unfortunately, those conditions are rare on the Mediterranean Sea (Sala et al., 2012; Bevilacqua et al., 2020), so *C. cylindracea* can scape biotic control in many locations. Similarly, making generalizations for other species is also challenging due to the context-dependent nature of the interactions; although it seems that competition, through the limitation of essential resources, can potentially affect the success of most invaders. However, the fact that canopy-forming species are in regression worldwide, could prevent us from properly assessing the true role of this biotic mechanism towards invasive algae. On the other hand, herbivory seems to be more species and context dependent than competition, providing that it is highly influenced by the traits of the invader (e.g., production of deterrent metabolites, compensation growth) and by the behavior of the herbivore (e.g., tolerance to the metabolites, preference for a particular species). For example, in the Mediterranean Sea, two of the most wide-spread invasive algae: *Lophocladia lallemandii* and *Womersleyella setacea*, are released from herbivory due to their high toxicity (Cebrian et al., 2011; Tomas et al., 2011b, 2011a), which prevents us from using our findings to disentangle the mechanisms influencing the success of these species. This definitively highlights that we cannot rely on general hypothesis to explain the success of every invader and that in order to increase our understanding of the dynamics of invaders, more assessments considering different mechanisms and species need to be done, preferably by means of *in situ* manipulative experiments that also consider the temporal component.

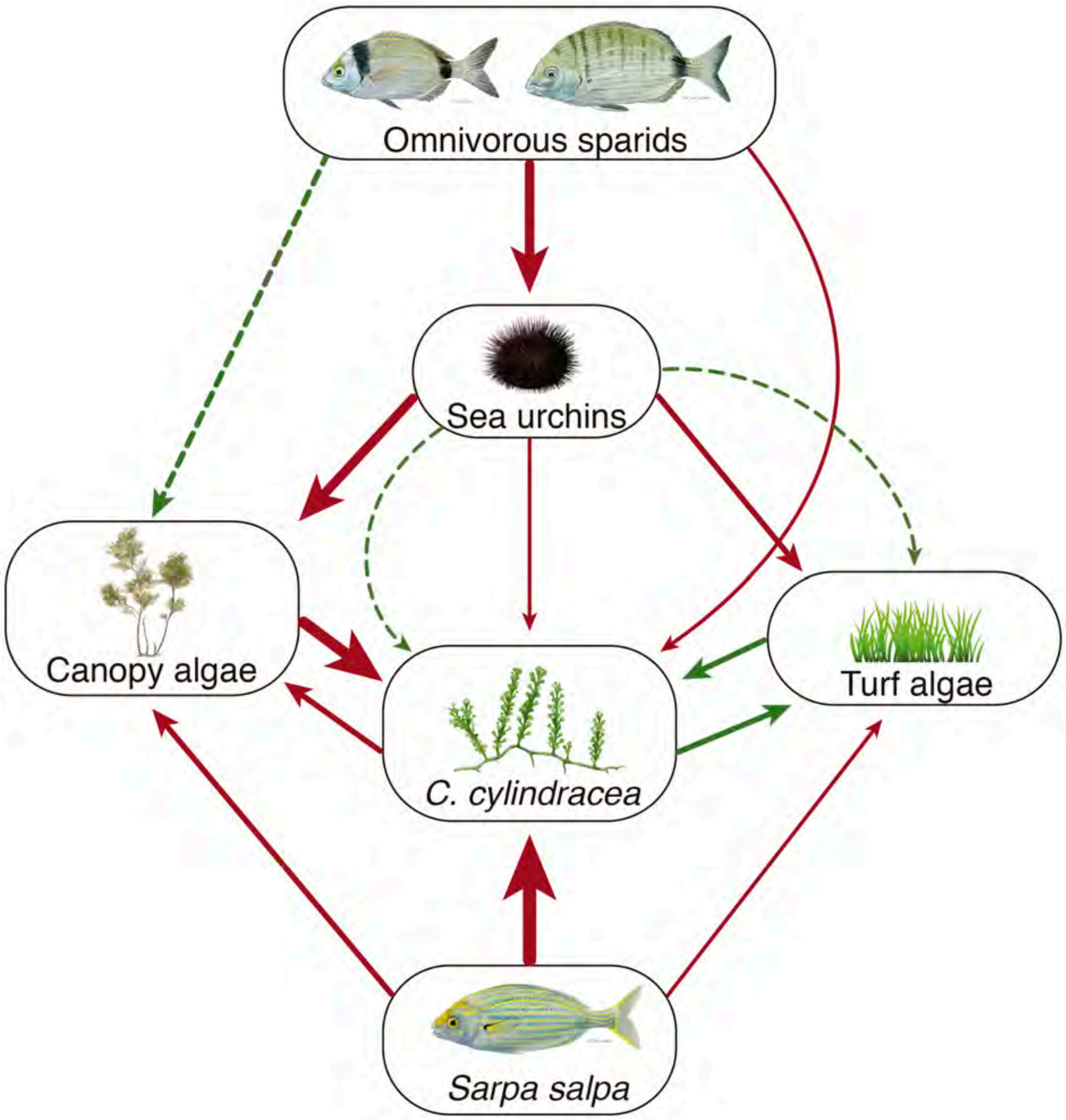


Figure 6.1. Schematic representation of some of the multiple relationships that might develop between *Caulerpa cylindracea* and different components of the invaded assemblage. There are both, direct (solid lines) and indirect (dashed lines) interactions, as well as positive (green) and negative (red) interactions. Arrow thickness represents the strength of the interaction. Scheme based on the findings reported in this thesis and on those from previous authors (Ceccherelli et al., 2002; Bulleri and Benedetti-Cecchi, 2008; Bulleri et al., 2009; Piazzi and Balata, 2009; Cebrian et al., 2011). Illustrations from Toni Llobet, the IUCN and pngaaa.

6.2 The importance of time in invasion ecology

Biological invasions are inherently dynamic ecological processes in which time plays an important role and can likely influence the strength of the interactions that develop between invaders and the invaded community (Diez et al., 2010; Carlsson et al., 2011; Lockwood et al., 2013; Strayer et al., 2017). However, probably due to temporal and economical constrains, most of the invasion ecology studies tend to focus in assessing a particular time frame in the invasion process, disregarding the importance of time and complicating the assessment of the dynamics of invaders (Strayer et al., 2006, 2017; Strayer, 2012). Consequently, in order to improve our understanding of the long-term dynamics of invaders, in **Chapters 2 and 3**, we have used two different approaches – long-term monitoring and chronosequences – that have allowed us to assess whether biotic resistance mechanisms are effective through time and to disentangle the effect of time in the development of biotic resistance mechanisms.

Specifically, the long-term monitoring of *C. cylindracea* at the Cabrera Archipelago National Park (**Chapter 2**), showed that lower abundances of the invader are always associated to the complex assemblages, and that the invader is particularly limited in the shallow complex assemblages, where herbivory and competition can complement each other. This clearly evidences that the biotic resistance mechanisms assessed in this thesis are determining the long-term dynamics of the invader at the local scale, with the pattern of *C. cylindracea* abundance and distribution reflecting the interaction outputs between the biotic mechanisms. Actually, this might be one of the first real world evidences where the effects of biotic mechanisms reported from manipulative experiments are correlated with the history of the invasion (but see: Caselle et al., 2018), allowing us to unravel the mechanisms influencing the long-term dynamics of this particular invader. Unfortunately, due to the scarcity of long-term data on invaders (Simberloff and Gibbons, 2004; Strayer et al., 2006, 2017; Strayer, 2012), particularly in marine systems, it is difficult to infer how biotic mechanisms might influence the dynamics of other invaders, which definitively must serve as a plea for more long-term monitoring programs.

Interestingly, our long-term monitoring showed that some years after the onset of the invasion, there was a decline in the abundance of the invader (**Chapter 2**), something that has also been reported for other *C. cylindracea* meadows in Menorca, the south-eastern coast of Spain and France (Klein and Verlaque, 2008; Bernardeau-esteller et al., 2020; Cefalì et al., 2020). These regressions, being marine invasions highly idiosyncratic (McQuaid and Arenas, 2009), have been usually attributed to the natural dynamics of the invader at each particular location. However, bearing in mind that biotic invasions are dynamic processes, can we disregard that the strength of biotic interactions can vary with time? Actually, several mechanisms, such as the development of allelochemical defenses by the native species, increases in the number of enemies or an increase in the feeding efficiency of predators can potentially intensify the biotic resistance of native communities through time (Siemann et al., 2006; Strayer et al., 2006; Carlsson et al., 2009; Diez et al., 2010). Specifically, in **Chapter 3**, we have demonstrated that the native herbivore *S. salpa* needs some exposure time with *C. cylindracea* before developing a preference for the invader and starting to target it, which definitively explains why the invader can attain large abundances at the beginning of the invasion, when it escapes herbivory control. But then, as the invasions progresses, herbivory by *S. salpa* becomes an effective biotic resistance mechanism and *C. cylindracea* can suffer abundance regressions such as those reported in this thesis and in previous studies. These boom and bust patterns can be common across invasions and although the reasons behind them are not fully understood yet (Lockwood et al., 2013; Aagaard and Lockwood, 2016; Strayer et al., 2017), our findings suggest that increases in biotic resistance with time can have a significant role in them. Actually, enemy accumulation and increases in the predation rates are suspected to be responsible for long-lasting busts such as the one we have observed (Strayer et al., 2017), where the abundance of *C. cylindracea* at the Cabrera Archipelago has remained below 10% for the last 3 years (2017-2020) in the habitats were herbivory pressure is high (Santamaría et al., *unpublished data*). Similarly to our case, previous declines in hyper-successful invaders have been associated to increases in biotic resistance through time (Simberloff and Gibbons, 2004; De Rivera et al., 2005; Carlsson and Strayer, 2009; Carlsson et al., 2011; Strayer et al., 2017), which highlights that our findings might not be case-specific and might be spread across other invaders and systems.

In general our findings provide strong evidence on the importance of considering the temporal component of the invasion to understand the long-term dynamics of invaders. Unfortunately however, it is difficult to predict the temporal scales that might be needed to observe the development of a novel predator-prey interaction, with time scales that can go from days to even decades since the first exposure, depending on the mechanisms involved in the adaptive process (Carroll et al., 2005; Strayer et al., 2006; Carlsson et al., 2009; Forister and Wilson, 2013; Berthon, 2015). In our case, we were "lucky" because the presence of a native conspecific alga has probably allowed *S. salpa* to already have tolerance to the toxic metabolites of *C. cylindracea*, so that a time scale of less than a decade was enough for the fish population to include the invader as a recurrent food item and to become an effective biotic resistance mechanism (**Chapters 2 and 3**). Interestingly, this fast development of the herbivore-algae interaction seems to be also mediated by *C. cylindracea* attaining large abundances from the beginning of the invasion (**Chapter 3**), which might have fostered the effective formation and transmission of search images among the *S. salpa* population. However, if the abundance of the invader remains low for a long time or if processes such as contemporary evolution, ontological changes in morphology or the development of tolerance mechanisms to overcome the prey defenses are needed (Carroll et al., 2005; Phillips and Shine, 2006; Strauss et al., 2006; Carlsson et al., 2009; Berthon, 2015), the development of the novel interactions might require definitively longer time-scales.

For instance, terrestrial studies that have assessed the development of resistance mechanisms through time refer to time exposures of several decades and even centuries to report an increase in the resistance towards the invader (Siemann et al., 2006; Diez et al., 2010; Dostál et al., 2013; Stricker et al., 2016), while Britton-Simmons et al. (2011) found that exposure times greater than 30 years were needed for a native herbivore to develop a preference for the invasive alga *Sargassum muticum*. Under such situations it might be really difficult to maintain a monitoring program for so long due to economic and temporal constrains, but chronosequences, where populations invaded at different times are assessed, can be also very useful to reconstruct the long-term dynamics of invaders and to assess the development of the novel interactions as we have demonstrated (**Chapter 3**). In particular, providing that many marine herbivores fail to control invasive algae because they produce toxic metabolites, this type of methodology might allow to assess whether the herbivores, through the subsequent exposition to the invader, are able to generate tolerance to this metabolites (Gatehouse, 2002; Cornell and Hawkins, 2003; Sotka and Whalen, 2008; Sotka et al., 2018) and can become an effective long-term biotic resistance mechanism as herbivory by *S. salpa* does.

Overall, it seems clear that disregarding the temporal component of invasions can lead to underestimations of the importance of biotic resistance mechanisms, because the biotic resistance against an invader might fluctuate through the invasion and the perceived strength of the biotic interactions can significantly change depending on the time considered (e.g. recent vs old invasions). Therefore, there is an urgent need to adopt long-term monitoring programs and chronosequences as essential tools, in order to improve our understanding of how biotic mechanisms develop and to better assess the influence of these mechanisms on the long-term dynamics of invaders.

6.3 Species traits and the role of disturbance on the success of invaders

Disturbance refers to any discrete event in time that it is able to disrupt the community structure and to change the resource and substrate availability or the physical environment (Pickett and White, 1985). These events have received a lot of interest in ecology because they play an important role in shaping the structure of natural systems (Levin and Paine, 1974), with some species taking advantage of disturbances whereas others mainly flourish after long periods free from disturbances (Meiners et al., 2015). Particularly, the study of disturbances has been quite prolific in invasion ecology, where these events have been proposed to promote invader success through the increase in the availability of limiting resources and through the reduction in the interspecific competition (Davis et al., 2000; Shea and Chesson, 2002; Scheibling and Gagnon, 2006; Lockwood et al., 2013; Jauni et al., 2015). Thus, given that global change and anthropogenic activities are altering the frequency and intensity of disturbances (Coumou and Rahmstorf, 2012; Perkins et al., 2012; Halpern et al., 2015; Trenberth et al., 2015), it has become particularly relevant to assess how communities and species might respond to such changes in disturbance regimes, in order to better predict how future climatic conditions might affect the structure of communities and whether invasive species might further dominate in a more disturbed world.

Specifically, given that the number and intensity of extreme climatic events is predicted to increase in marine systems (IPCC 2018; Oliver et al. 2018, 2019), in **Chapter 5** we simulated a marine heatwave (MHW), a discrete event characterized by anomalously warm temperatures (Hobday et al., 2016), to disentangle how *C. cylindracea* might respond to acute disturbance events. In the experiment, *C. cylindracea* showed a remarkable capacity to survive to the disturbance and to recover fast once the conditions were favorable, something that can explain its extraordinary success as an invader. Actually, MHWs, by strongly affecting native species (e.g., Garrabou et al., 2009; Wernberg et al., 2013; Rubio-Portillo et al., 2016; Smale et al., 2019; Filbee-Dexter et al., 2020; Verdura et al., 2021) and making conditions more favorable for invaders (Diez et al., 2012; Verdura et al., 2019; Atkinson et al., 2020), are likely to reduce the resilience of native assemblages through the increased mortality of foundation native species and to foster the invasibility of the system through the enhancement of the competitive performance of invaders.

In general, the ability of many invaders to tolerate disturbance has been related to the presence of certain traits such as: broad tolerance ranges, the capacity to reproduce vegetatively, the ability to disperse over long-distances, fast growth rates, rapid resource acquisition, polyploidy and high phenotypic plasticity (Pyšek and Richardson, 2008; Davidson et al., 2011; Te Beest et al., 2012; Bates et al., 2013; Higgins and Richardson, 2014; Gallagher et al., 2015; Van Kleunen et al., 2015), with *C. cylindracea* having most of them (Ceccherelli and Cinelli, 1999; Ceccherelli and Piazzi, 2001; De Senerpont Domis et al., 2003; Raniello et al., 2006; Flagella et al., 2008; Klein and Verlaque, 2008; Varela-Álvarez et al., 2012; Montefalcone et al., 2015). Interestingly, the MHW experiment in **Chapter 5** not only demonstrated the extraordinary tolerance of this species, but it also brought to light its remarkable phenotypic plasticity, as it was able to drastically change morphologically in response to the stressful conditions. In this sense, although it is the first time that this filamentous morphology has been reported for *C. cylindracea*, its capacity to respond plastically was already well known, as this species is able, for example, to change its growth form between guerrilla and phalanx typologies in response to the local environmental conditions (Bulleri et al., 2009; Uyà et al., 2017). Unfortunately, it is still not clear how the filamentous morphology might benefit the invader, although it might serve the purposes of improving the persistence, the resistance and the dispersal of the species due to the fast development, high tolerance and enhanced fragmentation of the morphology. What seems clear however is that by *C. cylindracea* adopting this morphology, it can become cryptic, which complicates the identification of the species in the field and can affect the management of the species.

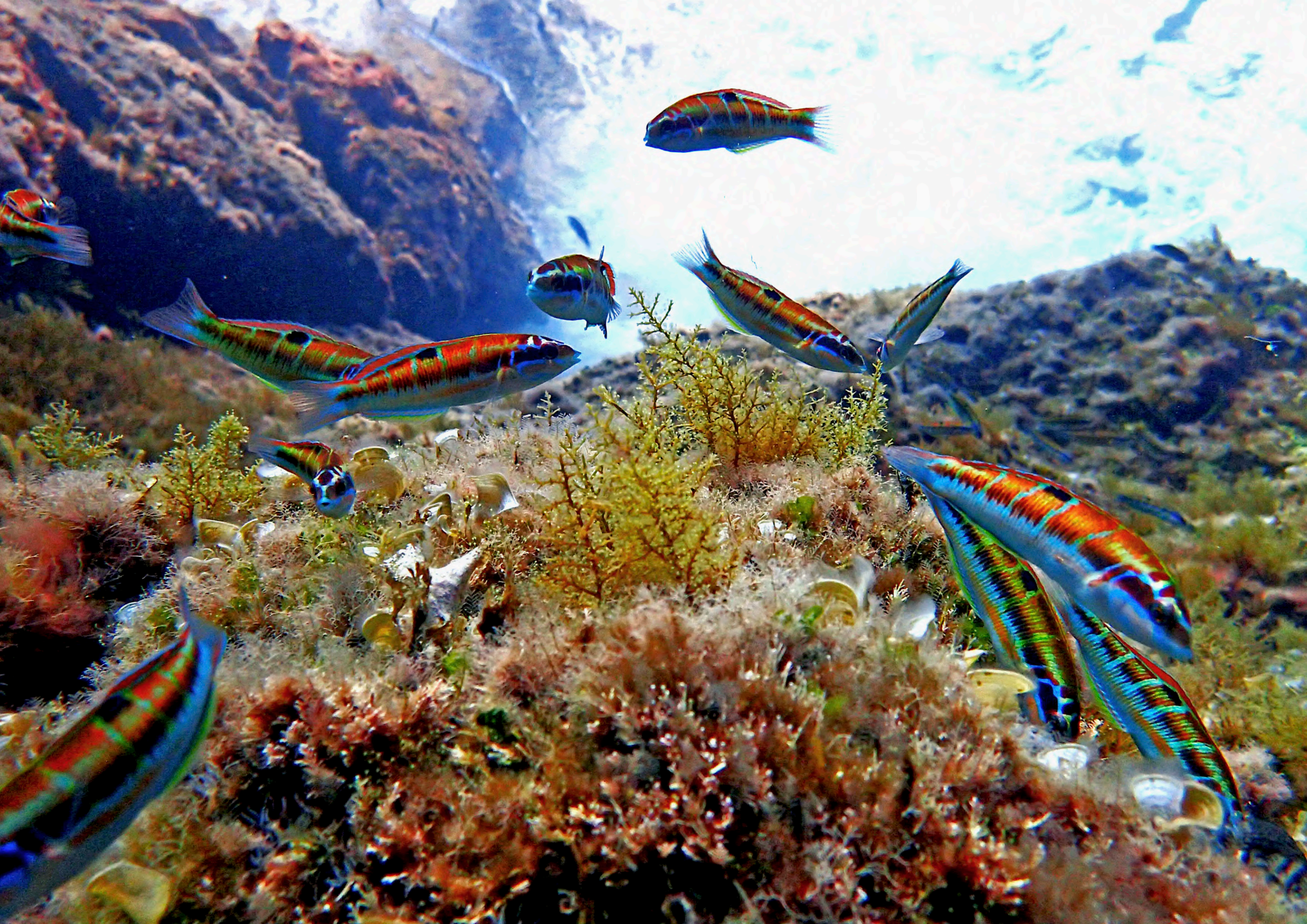
6.4 Management of invasions. Implications for marine conservation

Given the huge impact that invasive species cause in communities worldwide and the forecasted increase in invasions due to climate change and rising globalization, the management of invasive species has become one of the greatest challenges for the conservation of biodiversity (Pyšek and Richardson, 2010; Simberloff et al., 2013; Giakoumi et al., 2019a). Depending on the time since the introduction and on the distribution range of the target species, different management options are available, being those actions that are applied early in the invasive process the most successful and cost-effective (Simberloff et al., 2013). In this sense, prevention, by for example constraining pathways (e.g., treatment of ballast water or regulation of exotic organisms´ trade), is definitively the most effective option as it hinders the arrival of the species in the first place (Williams and Grosholz, 2008; Pyšek and Richardson, 2010; Simberloff et al., 2013). However, prevention is not always successful and once the species establishes in the new range, other management options such as eradication, containment or control are needed, being those interventions more successful and feasible when they are preceded by the early detection of the introduced species, before it undergoes range expansion (Pyšek and Richardson, 2010; Simberloff et al., 2013). To date, most successful eradications and control interventions have occurred on terrestrial systems (e.g., Myers et al., 2000; Simberloff, 2001, 2008; Genovesi, 2005; Robertson et al., 2017), whereas in the marine environment, most invasions are difficult to prevent, control or eradicate (Thresher and Kuris, 2004; Anderson, 2008; Williams and Grosholz, 2008; Ojaveer et al., 2015). Actually, successful eradications in marine systems have only been achieved in limited occasions, always in restricted areas, following early detection and rapid intervention (Myers et al., 2000; Willan et al., 2000; Wotton et al., 2004; Anderson, 2005). This lack of success in management efforts has been largely attributed to the open nature of the marine environment, which fosters the dispersal of species over large areas after establishment and makes successful management more challenging. Therefore, considering that the complete extirpation of marine invaders is highly unlikely, the purpose of management should be to suppress the abundance of the invader below levels that cause ecological harm, so that its impacts are lessened and the resilience of native ecosystems can be fostered (Green et al., 2014; Usseglio et al., 2017; Giakoumi et al., 2019a).

Given that the eradication of *C. cylindracea* seems unfeasible due to its high abundance, wide-distribution and high resilience, other management approaches such as the control of its abundance, might be the most effective and reliable option. Specifically, our findings show that native habitats, whenever they are well preserved and are given enough time (**Chapters 2, 3 and 4**), can develop resistance mechanisms against the invader and significantly reduce its abundance. Therefore, as MPAs are effective for the recovery of top-predators, the reestablishment of trophic cascades and the restoration of native benthic communities in decadal or multi-decadal time scales (Guidetti and Sala, 2007; Lester et al., 2009; Babcock et al., 2010; Leleu et al., 2012; García-Rubies et al., 2013), they could be used as a management tool to foster the long-term resilience and resistance of the system against *C. cylindracea*. However, it is still not clear under what situations MPAs can provide resilience and resistance to invasions or whether they can mitigate the negative impacts of invasive species (Kellner and Hastings, 2009; Burfeind et al., 2013; Giakoumi et al., 2016; Giakoumi and Pey, 2017), with some invaders being even more common inside MPAs than outside them (Byers, 2005; Klinger et al., 2006; Rilov et al., 2018; Giakoumi et al., 2019b). Therefore, there is still an urgent need to improve our knowledge on the factors that influence invader success across species and systems in order to understand under which situations natural mechanisms can hinder invasions or whether other complementary management interventions might be needed to reduce the abundance of the invader. For instance, whenever the abundance of *C. cylindracea* is really high and the native assemblage is so impacted that it cannot reduce the success of the invader, combining the targeted removal of *C. cylindracea* together with the effective protection of the assemblage can be a successful intervention to reduce the abundance of the invader and to foster the resilience of the system at the same time. Thus, based on sound knowledge on the traits of invaders, on the dynamics of invasions and on the characteristics of the invaded system, management interventions, through conservation actions, local eradications, active restorations or a combination of them, can then be prioritized to improve their effectiveness (Giakoumi et al., 2019a).

As food for thought, what should we do with non-native species in the future? To manage or not to manage that will be the question.

In the future, climate change will further reconfigure marine ecosystems worldwide (Poloczanska et al., 2013; García Molinos et al., 2016; Wernberg et al., 2016; Pecl et al., 2017), making it increasingly difficult to discern whether an species should be considered as an alien species or as an species naturally expanding its range. This situation will definitively go associated with the arduous decision of whether to focus efforts on trying to preserve the native ecosystems as we know them or whether to accept some of the arriving species as the new "normal" for the system. For instance, in some regions of the world were native assemblages have been severely impacted by anthropogenic stressors and global change, ecosystems have started to shift to a new late-successional regime (e.g. from macroalgae dominated systems to coral dominated systems) (Yamano et al., 2011; Vergés et al., 2014a; Wernberg et al., 2016; Tuckett et al., 2017; Kumagai et al., 2018), or are now dominated by alien species that could, to a certain extent, substitute the ecological function of the species they have replaced (Johnston and Lipcius, 2012; Edelist et al., 2013; Epstein and Smale, 2017; Mačic et al., 2018; Ramus et al., 2018; Rilov et al., 2018, 2019). Under such situations, when the native system has been greatly modified by climate change and human mediated disturbances, and providing that the new species could reestablish the ecosystem functions lost or support new critical functions, the wiser decision would be to tolerate the changes, to embrace the novel ecosystems and to promote the resilience of the system by safeguarding those "new" species that contribute to maintain the ecosystem functioning and services. Actually, certain alien species can have positive effects, by for example securing ecosystem functions and processes or by provisioning food and shelter (D'Antonio and Meyerson, 2002; Gozlan, 2008; Davis et al., 2011; Schlaepfer et al., 2011; Johnston and Lipcius, 2012; Bertness and Coverdale, 2013; Katsanevakis et al., 2014b; Ramus et al., 2018), so they might contribute to achieve conservation objectives in the future (Walther et al., 2009; Schlaepfer et al., 2011; Bulleri et al., 2018). However, the decision to conserve alien species should be taken carefully, and the benefits of some of the alien species should never be used as an argument to neglect the impacts of invaders, to naturalize every alien species or to promote do-nothing strategies. Instead, evidence from ecological change worldwide should be used to highlight the need for effective risk assessments and prevention measures to hinder the arrival of harmful species, to base management primarily on the ecological function and impacts of a species rather than on its origin, and to guarantee the conservation of native habitats by creating well-enforced MPAs that promote the correct functioning and the natural resilience of the system. Last but not least, providing that disturbances significantly disrupt the resilience of native ecosystems, we definitively need to focus on trying to slow down the current pace of environmental change by mitigating anthropogenic stressors and climate change, so that the successional dynamics of the systems can return to their natural rate of change. Definitively, nature seems to have the mechanisms to find the balance and tolerate environmental change whenever it has the tools and enough time to adapt. So then, the relevant question is: are we ready to change and mitigate our impacts on nature before it is too late? Or instead, are we willing to accept and to adapt to the ecological changes that will occur if we decide not the change?



Chapter 7

Conclusions

Cover image: *Thalassoma pavo* females feeding on a well-preserved assemblage dominated by the canopy-forming species *Ericaria brachycarpa*.

Author: Jorge Santamaría Pérez.

Chapter 2

- Competition by canopy-forming and erect algae species and herbivory by the fish *Sarpa salpa* significantly limit the abundance of *Caulerpa cylindracea*. Thus, the success of the invader is greatly influenced by biotic mechanisms.

- It is the synergistic effect between competition and herbivory what determines the abundance of the invader at the local scale. Therefore, assessing the interaction between biotic mechanisms might be essential to quantify the strength of biotic resistance against an invader.

- Long-term monitoring of the invader suggests that the biotic resistance of native assemblages might intensify through time as reflected by the regression in the abundance of the invader in shallow assemblages.

- The combined use of different approaches such as long-term monitoring and *in situ* manipulative experiments is needed to reliably assess the influence of biotic resistance mechanisms on the dynamics of invaders.

- The conservation of marine habitats, particularly promoting habitat complexity and balanced grazer assemblages, could be proposed as an effective management action to foster the long-term resilience of native assemblages against certain invaders.

Chapter 3

- *Sarpa salpa* preference for the invasive alga *C. cylindracea* only develops after certain exposure time to the invader. Therefore, the herbivore needs time to start targeting the invasive alga.

- Sites historically invaded support a higher proportion of herbivores feeding on *C. cylindracea* and higher *per capita* consumption rates compared to places recently invaded, particularly in sites with a high abundance of the invader, which confirms that herbivory increases its intensity through time.

- *S. salpa*´s electivity for *C. cylindracea* increases through the invasion, even when the invader suffers abundance declines, highlighting herbivory as an effective long-term biotic resistance mechanism.

- The increase in the strength of herbivory through the invasion, demonstrates that native communities need time to develop effective resistance mechanisms against invaders. Therefore, long-term studies and chronosequences are needed to unravel the mechanisms influencing the dynamics of invaders.

Chapter 4

- At least 3 of the 4 species consume *C. cylindracea*, although the contribution of the invader to the diet is generally low, except for *Diplodus annularis*.

- According to the values in the electivity index, all omnivorous sea bream species avoid to feed on the invader. Therefore, high consumptions of the invader probably happen accidentally when fish are trying to feed on other prey.

- Given the high amounts of *C. cylindracea* consumed by some species and considering that sparid fishes dominate shallow rocky habitats, these species, despite being omnivorous, could exert some control on the abundance of the invader. Thus, well-enforced MPAs, as places that support high abundances of sea breams, can foster this complementary biotic resistance mechanism.

Chapter 5

- Exposure to temperatures of 29ºC drastically reduces the survival of *C. cylindracea* thalli and triggers the development of a novel morphology when the harsh conditions subside.

- This novel morphology has been also observed in the field after acute disturbances, suggesting that stressful events trigger the morphological plasticity of the invader.

- The novel morphology is characterized by erect thin filaments that grow vertically from the substrate and that do not resemble the distinctive morphology of the invader. Thus, accurate monitoring methods are needed to reliably assess the presence of the invader.

- The great tolerance to extreme conditions and the high phenotypic plasticity of *C. cylindracea* explain its success as an invader, with the filamentous morphology likely involved in the persistence, resistance and dispersion of the invader, while also turning *C. cylindracea* into a cryptic invader.

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Cover image: Leach´s spider crab (*Inachus phalangium*) covered by the invasive alga *Caulerpa cylindracea* in an area highly colonized by the invader.

Author: Raül Golo.

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Appendices

Cover image: Detail of *Caulerpa cylindracea* vertical fronds showing the typical morphology of the species.

Author: Enric Ballesteros Segarra.

Appendix A: Chapter 2 Supporting Information

Table A.1. Algae species identified in the sampled assemblages and the trait category assigned to each of them. Epiphyte species were not considered for the complexity of the assemblage, as they generally do not compete with other algae.

Species	Trait category	Species	Trait category
Acetabularia acetabulum	Erect	*Acetabularia parvula*	Erect
Acrodiscus vidovichii	Erect	*Acrosorium venulosum*	Erect
Acrosymphyton purpuriferum	Erect	*Acrothamnion preisii*	Turf
Aglaozonia chilosa	Turf	*Aglaozonia parvula*	Turf
Alsidium corallinum	Erect	*Amphiroa beauvoisii*	Erect
Amphiroa cryptarthrodia	Erect	*Amphiroa rigida*	Erect
Anadyomene stellata	Erect	*Apoglossum ruscifolium*	Erect
Asparagopsis taxiformis	Erect	*Asperococcus bullosus*	Erect
Boergeseniella fruticulosa	Turf	*Botryocladia borgesenii*	Turf
Botryocladia botryoides	Turf	*Botryocladia chiajeana*	Turf
Bryopsis sp.	Turf	*Castagnea* sp.	Turf
Caulerpa cylindracea	Erect	*Ceramium* sp.	Turf
Ceramium tenerrimum	Turf	*Chondria* sp.	Erect
Chondria capillaris	Erect	*Chondrophycus* sp.	Erect
Chylocladia verticillata	Erect	*Cladophora coelothrix*	Turf
Cladophora hutchinsiae	Turf	*Cladophora liebetruthii*	Turf
Cladophora pellucida	Turf	*Cladophora prolifera*	Turf
Cladophora sp.	Turf	*Cladostephus hirsitus*	Erect
Codium bursa	Erect	*Colpomenia sinuosa*	Erect
Corallina elongata	Turf	*Cordylecladia erecta*	Erect
Crouania attenuata	Turf	*Cryptonemia lomation*	Erect
Cryptonemia tunaeformis	Erect	*Cystoseira balearica*	Canopy
Cystoseira compressa	Canopy	*Cystoseira foeniculacea*	Canopy
Cystoseira funkii	Canopy	*Cystoseira postulata*	Canopy
Cystoseira spinosa	Canopy	*Dasya baillouviana*	Epiphyte
Dasya sp.	Epiphyte	*Derbesia* sp.	Turf
Dictyopteris lucida	Erect	*Dictyopteris membranacea*	Erect
Dictyopteris polypodioides	Erect	*Dipterosiphonia rigens*	Epiphyte
Discosporangium mesarthrocarpum	Turf	*Dudresnaya verticillata*	Turf
Dyctiota dichotoma	Erect	*Dyctiota dichotoma* var. *intrincata*	Erect
Dyctiota fasciola	Erect	*Dyctiota linearis*	Erect
Ectocarpus sp.	Turf	*Eledista neglecta*	Epiphyte
Enteromorpha sp.	Turf	*Erythroglosum balearicum*	Erect

Table A.1. Continued.

Species	Trait category	Species	Trait category
Eupogodon planus	Erect	*Falkenbergia hildebrandi*	Epiphyte
Feldmannophycus rayssiae	Erect	*Flabellia petiolata*	Erect
Gelidiella sp.	Turf	*Gelidium pectinatum*	Erect
Gloiocladia furcata	Erect	*Gracilaria* sp.	Erect
Griffithsia sp.	Turf	*Gulsonia nodulosa*	Epiphyte
Halicystis parvula	Turf	*Halimeda tuna*	Erect
Haliptilon virgatum	Epiphyte	*Halopithys incurva*	Erect
Halopteris filicina	Erect	*Halopteris scoparia*	Erect
Halydiction mirabile	Epiphyte	*Heterosiphonia crispella*	Epiphyte
Heterosiphonia wurdemannii	Epiphyte	*Hildenbrandia crouaniorum*	Encrusting
Hypnea cervicornis	Turf	*Hypoglossum hypoglossoides*	Erect
Jania adherens	Epiphyte	*Jania corniculata*	Epiphyte
Kallymenia sp.	Erect	*Laurencia* sp.	Erect
Leathesia mucosa	Erect	*Lithophyllum duckeri*	Encrusting
Lithophyllum expansum	Encrusting	*Lithophyllum incrustans*	Encrusting
Lithothamnion coralloides	Encrusting	*Lithothamnion fruticulosum*	Encrusting
Lithothamnion valens	Encrusting	*Lobophora variegata*	Erect
Lomentaria chylocladiella	Erect	*Lomentaria* sp.	Erect
Lophocladia lallemandii	Epiphyte	*Lyngbya* sp.	Epiphyte
Melobesia sp.	Encrusting	*Mesophyllum alternans*	Encrusting
Microdyction tenuius	Turf	*Monosporus pedicellatus*	Epiphyte
Neogoniolithon brassica-florida	Encrusting	*Neogoniolithon mamillosum*	Encrusting
Nereia filiformis	Epiphyte	*Osmundaria volubilis*	Erect
Osmundea pelagosae	Erect	*Padina pavonica*	Erect
Palmophyllum crassum	Encrusting	*Peyssonelia coriacea*	Encrusting
Peyssonelia harveyana	Encrusting	*Peyssonelia rubra*	Encrusting
Peyssonnelia bornetti	Encrusting	*Peyssonnelia dubyi*	Encrusting
Peyssonnelia rosa-marina	Encrusting	*Peyssonnelia* sp.	Encrusting
Peyssonnelia squamarina	Encrusting	*Phyllophora crispa*	Erect
Phymatolithon calcareum	Encrusting	*Plocamium cartilagineum*	Erect
Poliphysa parvula	Erect	*Polysiphonia furcellata*	Turf
Polysiphonia ornata	Turf	*Polysiphonia sebulifera*	Turf
Predaea sp.	Erect	*Pseudochlorodesmis furcellata*	Erect
Pseudolithoderma adriaticum	Encrusting	*Pterosiphonia* sp.	Erect
Pterothamnion crispum	Epiphyte	*Rhodophyllis divaricata*	Erect
Rhodymenia ardissonei	Erect	*Rhodymenia delicatula*	Erect

Table A.1. Continued.

Species	**Trait category**		**Species**	**Trait category**
Rodriguezella ligulata	Erect		*Rodriguezella pinnata*	Erect
Rodriguezella strafforelloi	Erect		*Rytiphlaea tinctoria*	Erect
Sargassum vulgare	Canopy		*Sebdenia dichotoma*	Erect
Simploca hypnoides	Turf		*Spatoglossum solieri*	Erect
Spermatochnus sp.	Epiphyte		*Sphacellaria cirrosa*	Turf
Sphacellaria plumula	Turf		*Sphaerococcus coronopifolius*	Erect
Sphondylothamnion multifidum	Turf		*Spongites brassica-florida*	Encrusting
Spongites notarisii	Encrusting		*Sporochnus pedunculatus*	Erect
Spyridia filamentosa	Turf		*Stilophora rizoides*	Erect
Taonia atomaria	Erect		*Tricleocarpa cylindrica*	Erect
Valonia macrophysa	Turf		*Valonia utricularis*	Turf
Womersleyella setacea	Turf		*Wrangelia penicillata*	Turf
Wundermania miniata	Turf		*Zonardinia prototypus*	Encrusting
Zonaria tournefortii	Erect			

Table A.2. ANOVA summary for the GLM fitted to the habitat complexity data. *Caulerpa cylindracea* coverage was modeled as a function of habitat complexity. The asterisk (*) denotes significant p-values.

Dependent variable	**Predictor**	χ^2	**Df**	**p-value**
Caulerpa cylindracea coverage	Habitat complexity	670.06	2	**<0.001***

Table A.3. Effect of habitat complexity on *Caulerpa cylindracea* coverage. Tukey tests were applied to the fitted GLM to compare the coverage of *Caulerpa cylindracea* between different habitat complexities. The asterisk (*) indicates that the p-value is significant.

Dependent variable	**Comparison**	**z ratio**	**p-value**
Caulerpa cylindracea coverage	High - Medium	-10.330	**<0.001***
	High - Low	-16.404	**<0.001***
	Medium - Low	17.530	**<0.001***

Table A.4. ANOVA summary for the GLM fitted to the habitat complexity data in relation to depth. *Caulerpa cylindracea* coverage was modeled as a function of depth, habitat complexity and the interaction between both terms. The asterisk (*) denotes the significant p-values.

Dependent variable	Predictor	χ^2	Df	p-value
Caulerpa cylindracea coverage	Depth	916.10	1	**<0.001***
	Habitat complexity	802.61	2	**<0.001***
	Depth x Habitat complexity	70.62	2	**<0.001***

Table A.5. Effect of habitat complexity and depth on *Caulerpa cylindracea* coverage. Tukey tests were applied to the fitted GLM to compare the coverage of *Caulerpa cylindracea* between different habitat complexities at each depth (**A**) and between different depths at each habitat complexity (**B**). The asterisk (*) indicates that the *p*-value is significant.

A)

Dependent variable	Complexity	Comparison	z ratio	p-value
Caulerpa cylindracea coverage	High	Shallow - Deep	2.950	**0.003***
	Medium	Shallow - Deep	18.854	**<0.001***
	Low	Shallow - Deep	17.649	**<0.001***

B)

Dependent variable	Depth	Comparison	z ratio	p-value
Caulerpa cylindracea coverage	Shallow	High - Medium	-2.218	0.068
		High - Low	-4.933	**<0.001***
		Medium - Low	13.421	**<0.001***
	Deep	High - Medium	-12.642	**<0.001***
		High - Low	-17.185	**<0.001***
		Medium - Low	11.692	**<0.001***

Table A.6. ANOVA summary for the mixed effects models fitted to the exclusion experiment data. The abundances of *Caulerpa cylindracea* per depth were modeled as a function of Treatment (cage type) and Time (beginning and end), with Plot as a random effect to account for the repeated measures over time. The table shows the summaries of the ANOVAs for the two models and the asterisk (*) denotes significant p-values.

Dependent variable	Depth	Predictor	χ^2	Df	p-value
Caulerpa cylindracea abundance	Shallow	Treatment	16.662	2	**0.002***
		Time	166.795	1	**<0.001***
		Treatment x Time	113.269	2	**<0.001***
	Deep	Treatment	0.389	2	0.949
		Time	186.588	1	**<0.001***

Table A.7. Effect of the exclusion treatment on *Caulerpa cylindracea* abundance at 10 m, at the beginning (July) and end (August) of the experiment. Tukey tests were applied to the fitted mixed models to compare the abundance of *Caulerpa cylindracea* between exclusion treatments at each time observation (**A**) and between times at each exclusion treatment (**B**). The exclusion treatments are: Control (n=5), Control Exclusion (n=5) and Exclusion (n=5). The asterisk (*) indicates that the *p*-value is significant.

A)

Dependent variable	Time	Comparison	z ratio	p-value
Caulerpa cylindracea abundance	Beginning	Control – Control Exclusion	0.435	0.930
		Control – Exclusion	-1.274	0.792
		Control Exclusion – Exclusion	-0.768	0.963
	End	Control – Control Exclusion	-3.101	**0.024***
		Control – Exclusion	9.755	**<0.001***
		Control Exclusion – Exclusion	6.265	**<0.001***

B)

Dependent variable	Treatment	Comparison	z ratio	p-value
Caulerpa cylindracea abundance	Control	Beginning - End	-14.049	**<0.001***
	Control Exclusion	Beginning - End	-9.069	**<0.001***
	Exclusion	Beginning - End	-0.683	0.523

Appendix B: Chapter 3 Supporting Information

Table B.1. Temporal and numerical characteristics of the locations studied and sampled for this study in relation to the invasion of *Caulerpa cylindracea*.

Region	Location	Year 1st detection	Year sampled	Years invaded	Time since introduction	Abundance	Abund. in site
Menorca	Illa del Aire	2006	2018	12	Old	58	High
	Porros	2010	2018	8	Old	54	High
	Sa Mola	2012	2018	6	Old	18	Low
	Sa Llosa	2014	2018	4	Recent	1	Low
Catalonia	Sant Francesc	2013	2019	6	Old	1	Low
	Roses 2020	2016	2020	4	Recent	5	Low
	Roses 2019	2016	2019	3	Recent	7	Low
	Roses 2018	2016	2018	2	Recent	45	High
	Ses Negres	2017	2019	2	Recent	9	Low
Cabrera Archipelago	Cabrera	2003	2020	17	Old	28	Low
	Cabrera	2003	2008	5	Recent	55	High
	Cabrera	2003	2007	4	Recent	62	High

Table B.2. ANOVA summary for the GLM fitted to the habitat complexity data. *Caulerpa cylindracea* coverage was modeled as a function of habitat complexity. The asterisk (*) denotes significant p-values.

Dependent variable	Predictor	χ^2	Df	p-value
Caulerpa cylindracea presence in pellets	Time since Invasion	7.84	1	**<0.01***
	Caulerpa abundance	41.77	1	**<0.001***
	Time since Invasion x *Caulerpa* abundance	8.37	1	**<0.01***

Table B.3. Effect of **A)** time since invasion and **B)** *Caulerpa cylindracea* abundance on the presence of *Caulerpa cylindracea* in the fish pellets. Tukey tests were applied to the fitted GLM to compare the presence of *Caulerpa cylindracea* in the fish pellets between different exposure times at each level of invader abundance (**A**) and between different abundances at each level of exposure time (**B**). The asterisk (*) and the text in bold indicate that the p-value is significant.

A)

Dependent variable	Time since Invasion	Comparison	z ratio	p-value
Caulerpa cylindracea presence in pellets	Old	High - Low	5.67	**<0.001***
	Recent	High - Low	2.08	**0.04***

B)

Dependent variable	*Caulerpa* abundance	Comparison	z ratio	p-value
Caulerpa cylindracea presence in pellets	High	Old - Recent	3.72	**<0.001***
	Low	Old - Recent	0.93	0.35

Table B.4. ANOVA summary for the Generalized Linear Model fitted to the data on the proportion of *Caulerpa cylindracea* found in *Sarpa salpa* pellets. The proportion of *Caulerpa cylindracea* in the fish pellets was modeled as a function of the time since the invasion, the abundance of the invader in the community and the interaction between both terms. The asterisk (*) and the text in bold denote the significant p-values.

Dependent variable	Predictor	χ^2	Df	p-value
Caulerpa cylindracea proportion in pellets	Time since Invasion	343.71	1	**<0.001***
	Caulerpa abundance	1264.82	1	**<0.001***
	Time since Invasion x *Caulerpa* abundance	198.09	1	**<0.001***

Table B.5. Effect of A) time since invasion and B) *Caulerpa cylindracea* abundance on the proportion of *Caulerpa cylindracea* found in the fish pellets. Tukey tests were applied to the fitted GLM to compare the proportion of *Caulerpa cylindracea* in the fish pellets between different exposure times at each level of invader abundance (upper table) and between different abundances at each level of exposure time (lower table). The asterisk (*) and the text in bold indicate that the p-value is significant.

A)

Dependent variable	Time since Invasion	Comparison	z ratio	p-value
Caulerpa cylindracea proportion in pellets	Old	High - Low	28.56	**<0.001***
	Recent	High - Low	5.23	**<0.001***

B)

Dependent variable	*Caulerpa* abundance	Comparison	z ratio	p-value
Caulerpa cylindracea proportion in pellets	High	Old - Recent	17.92	**<0.001***
	Low	Old - Recent	1.03	0.30

Table B.6. Mean Ivlev´s Electivity Index values ± S.E., as a measure of the electivity of the herbivorous fish *Sarpa salpa* towards the invasive alga *Caulerpa cylindracea*. Ivlev Indexes were calculated for three different years at two locations with contrasting exposure times to the invader: Cabrera (first detection in 2003) and Roses (first detection in 2016). Index values approaching -1 indicate that the food item is avoided, whereas values approaching 1 indicate that the species exclusively targets that item.

Location	Year	Ivlev Index
Cabrera	2007	-0.181 ± 0.05
	2008	-0.151 ± 0.05
	2020	0.085 ± 0.06
Roses	2018	-0.877 ± 0.04
	2019	-0.533 ± 0.10
	2020	-0.455 ± 0.13

Table B.7. Kruskall-Wallis test applied to the Ivlev´s Electivity data for different sampling years and at each location. The Ivlev´s Electivity Indexes of *Sarpa salpa* towards *Caulerpa cylindracea* were analyzed as a function of sampling year. The asterisk (*) and the text in bold denote the significant p-values.

Location	**Dependent variable**	**Predictor**	**K-W χ^2**	**Df**	**p-value**
Cabrera	Ivlev Index	Year	15.94	2	**<0.001***
Roses	Ivlev Index	Year	2.04	2	0.36

Table B.8. Effect of sampling year on the Ivlev´s Electivity Index of *Sarpa salpa* towards *Caulerpa cylindracea*. Dunn tests were applied to the Kruskall-Wallis test to compare the Ivlev´s Electivity Index towards *Caulerpa cylindracea*, between sampling years at Cabrera. P-values were adjusted with the Benjamini-Hochberg method. The asterisk (*) and the text in bold indicate that the *p*-value is significant.

Location	**Comparison**	**Z**	**p-value adjusted**
Cabrera	2007-2008	-0.44	0.66
	2007-2020	-3.75	**<0.001***
	2008-2020	-3.38	**0.001***

Appendix C: Chapter 4 Supporting Information

Table C.1. Summary of the species found in the fish stomach contents. The **+** symbol indicates that the prey item was found in one or more of the stomachs from that fish species and the **–** symbol indicates that the prey item was not found in any stomach.

Group	**Species**	***Diplodus sargus***	***Diplodus vulgaris***	***Diplodus annularis***	***Spondyliosoma cantharus***
Algae	*Acetabularia* sp.	+	-	-	-
	Acrodiscus vidovichii	+	-	-	-
	Alsidium corallinum	+	-	-	-
	Amphiroa sp.	+	+	-	-
	Arthocladia sp.	-	-	-	+
	Asperococcus bullosus	+	-	-	-
	Brown algae non-identified	+	-	-	-
	Calothrix sp.	+	-	-	-
	Caulerpa cylindracea	+	-	+	+
	Caulerpa prolifera	+	-	+	+
	Champia parvula	-	-	+	-
	Chondria sp.	+	-	-	-
	Cladophora pellucida	+	-	-	-
	Cladophora prolifera	+	-	-	-
	Cladophora sp.	+	+	-	+
	Cryptonemia lomation	+	-	-	-
	Cystoseira sp.	+	+	-	-
	Derbesia tenuissima	-	+	-	-
	Dictyopteris polypodioides	+	+	-	-
	Dictyota linearis	+	+	-	-
	Dictyota sp.	+	+	-	-
	Eupogodon planus	+	-	-	-

Table C.1. Continued.

Group	Species	*Diplodus sargus*	*Diplodus vulgaris*	*Diplodus annularis*	*Spondyliosoma cantharus*
Algae	*Feldmannophycus rayssiae*	+	-	-	-
	Flabellia petiolata	+	-	-	-
	Gracilaria sp.	+	-	+	-
	Haliptilon virgatum	+	-	-	-
	Halopteris filicina	+	+	-	+
	Halopteris scoparia	+	+	-	-
	Laurencia sp.	+	-	-	-
	Leptofauchea coralligena	+	-	-	-
	Lobophora variegata	+	-	-	-
	Microtictyon tenuius	-	+	-	-
	Padina sp.	+	-	-	-
	Peyssonnelia rubra	+	-	-	-
	Peyssonnelia sp.	+	+	-	-
	Plocamium sp.	+	-	-	-
	Polysiphonia sp.	+	-	-	-
	Polysiphonia subulifera	-	-	+	+
	Rhodymenia sp.	+	-	-	-
	Rytiphlaea tinctoria	+	-	-	-
	Sargassum sp.	+	-	-	-
	Sphacelaria cirrosa	+	+	-	+
	Sphacelaria plumula	+	+	-	-
	Valonia utricularis	+	-	-	-
Plantae	*Cymodocea nodosa*	+	+	-	+
	Posidonia oceanica	+	+	+	+

Table C.1. Continued.

Group	**Species**	***Diplodus sargus***	***Diplodus vulgaris***	***Diplodus annularis***	***Spondyliosoma cantharus***
Animalia	*Actinia* sp.	+	-	-	-
	Aphrodita aculeata	+	-	-	+
	Asteroidea	+	-	-	-
	Bivalvia	+	+	+	-
	Briozoa	+	+	+	-
	Chitonidae	+	+	-	-
	Chlamis sp.	+	+	-	-
	Clavelina sp.	+	-	-	-
	Cnidaria	+	-	-	-
	Crustacea	+	+	-	-
	Fish bones	-	+	-	+
	Fish scales	+	+	-	-
	Foraminifera	+	-	-	-
	Gasteropoda	+	+	+	-
	Holothuria sp.	+	-	-	-
	Hydraria	+	+	-	-
	Invertebrate	+	-	-	+
	Mollusc eggs	-	-	-	+
	Ophiuroidea	+	+	-	-
	Paracentrotus lividus	+	+	-	-
	Pherusella tubulosa	+	-	+	-
	Polychaeta	+	-	-	-
	Sea snail eggs	+	-	-	-
	Sipunculida	-	+	-	-
	Tunicate	+	-	-	-
Other	Organic detritus	+	+	+	+
	Inorganic detritus	+	+	-	-

Table C.2. Results of the ANOSIM and the pairwise comparisons between fish species to detect diet similarities between species under 999 permutations. The asterisk (*) indicates statistical significance (p-value < 0.05).

Test	Fish species	R Statistic	Significance
Global test	All species	0.201	**0.001***
Pairwise test	*D. annularis* vs *D. sargus*	0.184	0.306
	D. annularis vs *D. vulgaris*	0.398	**0.006***
	D. annularis vs *S. cantharus*	0.625	**0.006***
	D. sargus vs *D. vulgaris*	0.156	0.102
	D. sargus vs *S. cantharus*	0.171	**0.012***
	D. vulgaris vs *S. cantharus*	0.473	**0.006***

Table C.3. Results of the SIMPER analysis to detect the most important prey items contributing to the diet dissimilarities between fish species. Only the 3 most important food items are shown.

Fish species	Item	Cumulative contribution to dissimilarities
D. annularis vs. *D. sargus*	Organic detritus	0.187
	Caulerpa cylindracea	0.327
	Eggs	0.466
D. annularis vs. *D. vulgaris*	Organic detritus	0.215
	Caulerpa cylindracea	0.359
	Eggs	0.495
D. annularis vs. *S. cantharus*	Organic detritus	0.399
	Caulerpa cylindracea	0.568
	Eggs	0.732
D. sargus vs. *D. vulgaris*	Organic detritus	0.214
	Gasteropoda	0.394
	Crustacea	0.518

Table C.3. Continued.

Fish species	Item	Cumulative contribution to dissimilarities
D. sargus vs. *S. cantharus*	Organic detritus	0.394
	Gasteropoda	0.503
	Non-identified invertebrate	0.554
D. vulgaris vs. *S. cantharus*	Organic detritus	0.382
	Gasteropoda	0.548
	Crustacea	0.695

Appendix D: Chapter 5 Supporting Information

Table D.1. ANOVA summary for the LMM fitted to the extreme temperature culture data. *Caulerpa cylindracea* area was modeled as a function of Treatment ("control" and "extreme"), Time (start and end) and their interaction, with Replicate as a random effect to account for the repeated measures over time. The asterisk (*) denotes significant p-values.

Dependent variable	Predictor	F value	Df	p-value
Caulerpa cylindracea area	Treatment	18.965	1	**0.005***
	Time	1.446	1	0.275
	Treatment x Time	15.896	1	**<0.007***

Table D.2. Effect of temperature treatment on the area of *Caulerpa cylindracea*, at the beginning and end of the culture experiment. Tukey tests were applied to the fitted mixed model to compare the area of *Caulerpa cylindracea* between temperature treatments at each time observation. The temperature treatments are: Control (n=3) and Extreme (n=3). The asterisk (*) indicates that the p-value is significant.

Dependent variable	Time	Comparison	Df	t ratio	p-value
Caulerpa cylindracea area	Start	Control - Extreme	6	0.260	0.803
	End	Control - Extreme	6	5.899	**0.001***

Table D.3. ANOVA summary for the LM fitted to the *Caulerpa cylindracea* thickness data. *Caulerpa cylindracea* thickness was modeled as a function of Location_Morphology ("Roses_filaments", "Roses_stolons", "Funtana_stolons", "Split_stolons", "Ponta Veslo_stolons" and "Kallm_stolons"). The asterisk (*) denotes significant p-value.

Dependent variable	Predictor	F value	Df	p-value
Caulerpa cylindracea thickness	Location_Morphology	116.84	5	**<0.001***

Table D.4. Effect of Location and Morphology on the thickness of *Caulerpa cylindracea*. Tukey tests were applied to the fitted mixed model to compare the thickness of *Caulerpa cylindracea* between locations and morphologies. The asterisk (*) indicates that the p-value is significant.

Dependent variable	Morphological Comparison	Population comparison	t ratio	p-value
Caulerpa cylindracea thickness	Filament vs. Stolon	Filament vs. Funtana	-18.893	**<0.001***
		Filament vs. Kallm	-18.770	**<0.001***
		Filament vs. Ponta Veslo	-20.082	**<0.001***
		Filament vs. Roses	-17.226	**<0.001***
		Filament vs. Split	-17.881	**<0.001***
	Stolon vs. Stolon	Funtana vs. Kallm	-0.123	1
		Funtana vs. Ponta Veslo	1.189	0.840
		Funtana vs. Roses	-1.667	0.559
		Funtana vs. Split	1.012	0.912
		Kallm vs. Ponta Veslo	-1.312	0.777
		Kallm vs. Roses	1.544	0.638
		Kallm vs. Split	0.889	0.948
		Ponta Veslo vs. Roses	2.856	0.064
		Ponta Veslo vs. Split	2.201	0.254
		Roses vs. Split	-0.654	0.986

Table D.5. *Caulerpa* sequences used to build the phylogenetic tree. The underlined names show the sequences that were amplified in this study. The rest of the sequences were obtained from GenBank.

Species	Accession Number	Herbarium Code	Site
Caulerpa cylindracea	MT274435	Svp-144	Split, Croatia
Caulerpa cylindracea	MT274436	Svp-159	Split, Croatia
Caulerpa cylindracea	MT274442	Svp-146	Funtana, Croatia
Caulerpa cylindracea	MT274446	Svp-145	Kallm, Albania
Caulerpa cylindracea	MT274444	Svp-143	Ponta Veslo, Montenegro
Caulerpa cylindracea	MT274443	Svp-130	Ponta Veslo, Montenegro
Caulerpa cylindracea	MT274447	Svp-053	Cabrera, Spain
Caulerpa cylindracea	MT274445	Svp-142	Formentera, Spain
Caulerpa cylindracea	MT274440	Svp-068	Roses, Spain
Caulerpa cylindracea	MT274441	Svp-140	Roses, Spain
Caulerpa cylindracea	MT274439	Svp-138	Roses, Spain
Caulerpa cylindracea	MT274438	Svp-139	Roses, Spain
Caulerpa cylindracea	KY773569		Othoni, Greece
Caulerpa cylindracea	KY773571		Lecce, Italy
Caulerpa cylindracea	KY773570		Brindisi, Italy
Caulerpa cylindracea	KY773572		Lecce, Italy
Caulerpa cylindracea	KY773573		Kotor, Montenegro
Caulerpa cylindracea	JX185615		Naples, Italy
Caulerpa cylindracea	JX185616		Sicily, Italy
Caulerpa cylindracea	FM956048		Ischia, Italy
Caulerpa cylindracea	JN817677		Perth, Australia
Caulerpa sertularioides	MT274434	Svp-083	Colombia
Caulerpa taxifolia	MF172076		Ragusa, Italy
Caulerpa prolifera	KF383343		Cádiz, Spain

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Cover image: Aerial view of our study site in Cala Sant Francesc, Blanes.

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MR. JORGE SANTAMARÍA (Orcid ID : 0000-0003-4425-6297)

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The role of competition and herbivory in biotic resistance against invaders: a synergistic effect

Jorge Santamaría[1], Fiona Tomas[2,3], Enric Ballesteros[4], Juan M. Ruiz[5], Jaime Bernardeau-Esteller[5], Jorge Terrados[2], Emma Cebrian[4,6]

[1] Marine Resources and Biodiversity Research Group (GRMAR), Institute of Aquatic Ecology, University of Girona, Girona, Catalonia, Spain

[2] Marine Ecosystems Dynamics Group – Instituto Mediterráneo de Estudios Avanzados (IMEDEA), Universitat de les Illes Balears (UIB) – Consejo Superior de Investigaciones Científicas (CSIC), Esporles, Balearic Islands, Spain

[3] Department of Fisheries and Wildlife, Oregon State University, Corvallis, Oregon, United States of America

[4] Centre d´Estudis Avançats de Blanes, Consejo Superior de Investigaciones Científicas (CEAB-CSIC), Blanes, Catalonia, Spain

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[5] Seagrass Ecology Group – Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), San Pedro del Pinatar, Murcia, Spain

[6] Corresponding Author. E-mail: emma@ceab.csic.es

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Abstract

Invasive species pose a major threat to global diversity and once they are well established their eradication typically becomes unfeasible. However, certain natural mechanisms can increase the resistance of native communities to invaders and can be used to guide effective management policies. Both competition and herbivory have been identified as potential biotic resistance mechanisms that can limit plant invasiveness but it is still under debate to what extent they might be effective against well-established invaders. Surprisingly, whereas biotic mechanisms are known to strongly interact, most studies up to date have examined single biotic mechanisms separately, which likely influences our understanding of the strength and effectiveness of biotic resistance against invaders. Here we use long-term field data, benthic assemblage sampling and exclusion experiments to assess the effect of native assemblage complexity and herbivory on the invasion dynamics of a successful invasive species, the alga *Caulerpa cylindracea*. A higher complexity of the native algal assemblage limited *C. cylindracea* invasion, probably through competition by canopy-forming and erect algae. Additionally, high herbivory pressure by the fish *Sarpa salpa* reduced *C. cylindracea* abundance by more than 4 times. However, long-term data of the invasion reflects that biotic resistance strength can vary across the invasion process and it is only where high assemblage complexity is concomitant with high herbivory pressure, that the most significant limitation is observed (synergistic effect). Overall, the findings reported in this study highlight that neglecting the interactions between biotic mechanisms during invasive processes and restricting the studied time scales may lead to underestimations of the true capacity of native assemblages to develop resistance to invaders.

Keywords: invasion ecology, biological invasions, biotic resistance, interspecific competition, herbivory, alga-herbivore interactions, *Caulerpa cylindracea*, *Sarpa salpa*

Introduction

Biological invasions are one of the main threats to biodiversity and ecosystem function worldwide, being the second most prominent cause of species extinctions and playing an important role in diversity reduction (Vilà et al. 2011, Bellard et al. 2016). Furthermore, bioinvasions can produce alterations in a number of ecosystem services and basic ecosystems processes (Pejchar and Mooney 2009, Vilà et al. 2010, Simberloff et al. 2013), often at great economic cost (Pimentel et al.

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2005). Still, our understanding of the factors that influence invasion success remains limited (Simberloff et al. 2013), complicating the development of effective management strategies to prevent and mitigate the negative effects of invasive species.

The success of an invasion is dependent on multiple processes across a wide range of temporal and spatial scales (Perelman et al. 2007, Theoharides and Dukes 2007, Eschtruth and Battles 2009a, Byun et al. 2015). Among these processes, most of the attention has fallen on biological processes, in the context of the Biotic Resistance Hypothesis (Elton 1958, Keane and Crawley 2002, Levine et al. 2004). The strength of biotic resistance against an invader is strongly influenced by the native assemblage and by the functional traits of the native species (Pokorny et al. 2005, Perelman et al. 2007, Byun et al. 2013), which modulate the interspecific competition; but also by the consumer pressure on both, the invasive and the native species (Levine et al. 2004, Parker and Hay 2005, Mitchell et al. 2006). In this sense, negative effects of competition on several invasive plants, mainly caused by the limitation of essential resources such as water, nutrients or light, have been previously reported in tropical forests (Fine 2002), saltmarshes (Amsberry et al. 2000), grasslands (Corbin and D’Antonio 2004, te Beest et al. 2018), mangroves (Li et al. 2014, Zhang et al. 2018), shrublands (Morris et al. 2015) and freshwater ecosystems (Petruzzella et al. 2018). However, competition alone might not be enough to exert a strong biotic control against a well-established invader (Levine et al. 2004, Vilà and Weiner 2004). On the other hand, herbivory has been also acknowledged as an important biotic resistance mechanism for native ecosystems (Levine et al. 2004, Parker and Hay 2005, Parker et al. 2006), although its effectiveness is controversial (Maron and Vilà 2001, Keane and Crawley 2002, Liu and Stiling 2006). In fact, herbivores can promote (Eschtruth and Battles 2009b, Relva et al. 2010, Kalisz et al. 2014), deter (Cushman et al. 2011, Pearson et al. 2012, Zhang et al. 2018), or have no effect on the dominance of invasive plant species (Stohlgren et al. 1999), which makes it difficult to understand the conditions under which herbivory can be an effective biotic resistance mechanism against an invasion. Taking into account that herbivory can be highly influenced by other factors such as native plant traits (Grutters et al. 2017) or habitat features (Alofs and Jackson 2014, Li et al. 2014, Ender et al. 2017, Zhang et al. 2018), contrasting observations on the role of biotic mechanisms in controlling invasive species may be partially explained by the fact that they are often assessed neglecting the relative importance of the interactions between

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mechanisms (Levine et al. 2004, Mitchell et al. 2006, Alofs and Jackson 2014, Zhang et al. 2018, Petruzzella et al. 2020). Indeed, the interaction between biotic mechanisms has been suggested to be responsible for an enhancement in the biotic resistance capacity of the invaded community (Suwa and Louda 2012, Li et al. 2014, Zhang et al. 2018).

In marine ecosystems, macroalgae are one of the most conspicuous and successful invaders, as well as one of the most harmful, comprising 20% of marine invasive species worldwide (Schaffelke et al. 2006) and causing potentially important ecological and economic damage (Williams and Smith 2007). Despite this, the factors that drive their invasive success remain largely unknown (Inderjit et al. 2006), although similarly to what has been observed for plant invasions in terrestrial ecosystems, both competition and herbivory are suspected to determine their invasion success (Kimbro et al. 2013, Papacostas et al. 2017). In this regard, functional traits of the native species can influence habitat resistance to algae invasion through their contribution to interspecific competition (Arenas et al. 2006, Britton-Simmons 2006, Vaz-Pinto et al. 2012). Surprisingly, however, most studies seem to confirm the inability of herbivores to limit the spread of well-established invasive macroalgae (e.g. Wikström et al. 2006, Forslund et al. 2010, Cebrian et al. 2011, Tomas et al. 2011b, Nejrup et al. 2012), despite the important role of herbivory regulating algal abundance and distribution (Vergés et al. 2009, Poore et al. 2012). Overall, previous research on biotic resistance against invasive macroalgae seems to suggest that, in most cases, the effect of single biotic mechanisms might not be enough to significantly affect invader performance (Kimbro et al. 2013, Papacostas et al. 2017). Probably, as has been suggested above, only by considering the interaction between biotic mechanisms (both competition and herbivory), more robust conclusions on the true resistance of an assemblage towards a particular invader can be obtained.

In this study, we aim to test whether herbivory interacts with competition to modulate the resistance of a marine habitat towards a particular invader. We use *Caulerpa cylindracea*, one of the most invasive macroalgae worldwide, to assess the simultaneous role that both assemblage structure and herbivory pressure have on *C. cylindracea* invasion by using *in situ* field experiments that assess herbivory and assemblage characteristics. Additionally, we monitor the abundance of the invader over time to further elucidate whether the studied biotic mechanisms and their interaction influence the long-term dynamics of the invader.

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Materials and methods

Study species

Caulerpa cylindracea is a green alga, native to the Southwestern coast of Australia (Verlaque et al. 2003), which is currently considered one of the most invasive species within the Mediterranean Sea (Klein and Verlaque 2008, Katsanevakis et al. 2016), having also invaded areas in the North Atlantic (Verlaque et al. 2004). Actually, it has recently been ranked as one of the marine invaders with the highest negative ecological impacts worldwide (Anton et al. 2019). However, despite its formidable ability to spread and grow, the abundance of *C. cylindracea* appears to differ markedly among invaded assemblages (Klein and Verlaque 2008, Cebrian and Ballesteros 2009), suggesting that there might be, in some cases, some natural mechanisms controlling *C. cylindracea* abundance. Among such mechanisms, competition may play an important role, since canopy-forming and erect algae (typical of high-complexity assemblages) can outcompete *C. cylindracea* (Ceccherelli et al. 2002, Bulleri and Benedetti-Cecchi 2008, Piazzi and Balata 2009, Bulleri et al. 2010), whereas herbivory seems to fail as a control mechanism for *C. cylindracea* when it is well established (Bulleri et al. 2009, Cebrian et al. 2011), even though several species are known to commonly feed on it (Ruitton et al. 2006, Box et al. 2009, Cebrian et al. 2011, Tomas et al. 2011b). However, it is important to consider that previous studies have focused on only one of these mechanisms (either competition or herbivory) while, in nature, both mechanisms might act together to influence the abundance of the invasive species.

Study system

The Mediterranean Sea is the largest and deepest semi-enclosed sea on Earth and it is considered a hotspot for marine biodiversity as it harbors around 17,000 marine species, 20% of them being endemic to the region (Coll et al. 2010). Due to its temperate climatic conditions, Mediterranean benthic shallow habitats are dominated by macroalgae. Unfortunately, the Mediterranean Sea is one of the areas most susceptible to the introduction of non-native species worldwide (Galil 2007) and it is considered to be a hotspot for invasive algae (Williams and Smith 2007, Thomsen et al. 2016).

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The study was conducted in the Cabrera Archipelago National Park, in the Balearic Islands (western Mediterranean; 39° 12’ 21” N, 2° 58´44” E) (Fig. 1). This maritime-terrestrial national park was established in 1991 and currently harbors some of the best-preserved benthic and fish assemblages in the Mediterranean Sea (Sala et al. 2012, Guidetti et al. 2014).

The invasive alga, *Caulerpa cylindracea*, was first detected in the area in 2003 at a depth of 30 m (Cebrian and Ballesteros 2009) and has, since then, colonized most of the park´s benthic habitats at depths from 0 to 65 m. Indeed, in some of these habitats, it has become the dominant species (Cebrian et al. 2011).

Benthic habitat sampling and assemblage complexity

To assess the role that benthic assemblage complexity might have on *C. cylindracea* coverage, different assemblages were surveyed in three sites around the Cabrera Archipelago: Ses Rates, Na Foradada and Freu de la Imperial (Fig. 1). A shallow assemblage (10 m) and a deep assemblage (30 m) were surveyed at each site to take into account the wide range of benthic assemblage complexities (Ballesteros et al. 1993) and contrasting herbivory pressures (Vergés et al. 2009, Tomas et al. 2011b) in relation to depth. Assemblages were sampled in 2005, 2006 and 2007. At each site and depth, three random samples measuring 20 x 20 cm^2 were collected, with the whole benthic cover removed using a hammer and a chisel (Boudouresque 1971, Sant et al. 2017). After removing the erect algae, and before scraping each quadrat, the cover of each encrusting species was visually estimated to obtain a more reliable value of their abundance in the encrusting layer. After collection, samples were preserved in 4% formalin in seawater, and once in the laboratory, they were sorted and all algae were identified to species level. Species coverage was calculated by placing the species specimens horizontally over a laboratory tray and measuring the area they covered (Ballesteros 1986). Then, each algal species was assigned to a different category (“Canopy-forming”, “Erect”, “Turf” and “Encrusting”) based on their morphological traits (size and morphology) (Appendix S1: Table S1). In order to avoid the effect of assemblage complexity being subject to a confounding effect of the presence of *Caulerpa cylindracea*, this species was not included in any of the previous categories. Finally, the percent cover of each category in the sample was calculated.

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Capacity of canopy-forming and erect algae to outcompete *C. cylindracea* in contrast to turf and encrusting species has been experimentally proven for coastal shallow rocky bottoms of the Mediterranean Sea (Ceccherelli et al. 2002, Bulleri and Benedetti-Cecchi 2008, Piazzi and Balata 2009, Bulleri et al. 2010). Consequently, complexity of each sample was defined based on the percentage abundance of the "Canopy-forming" and "Erect" categories. Three levels of complexity were defined for the samples based on the percentage of coverage that comprised canopy-forming and/or erect species: "high complexity" (more than 50%); "medium complexity" (between 15% and 50%) and "low complexity" (lower than 15%).

Exclusion experiment

An exclusion experiment was performed to assess whether fish herbivory could act as a biotic resistance mechanism against *Caulerpa cylindracea* invasion by reducing the abundance of the invasive alga. To this end, in order to obtain a proxy of contrasting herbivory intensities, and bearing in mind that herbivory pressure decreases strongly through the water column (Vergés et al. 2009, 2012, Steneck et al. 2017), the exclusion experiment was performed at two different depths: 10 m, where herbivory pressure is high, and 30 m, where it is low (Reñones et al. 1997, Tomas et al. 2011b, Vergés et al. 2012).

This experiment mainly targeted *Sarpa salpa*, because it is the only truly herbivorous fish in the western Mediterranean Sea (Verlaque 1990, Gianni et al. 2017), it plays an important role structuring algal communities (Vergés et al. 2009) and it regularly consumes *C. cylindracea* (Ruitton et al. 2006, Tomas et al. 2011b). This fish species is quite abundant throughout the Cabrera Archipelago, where it can reach densities up to 14 individuals per 250 m^2 above depths of 20 m (Reñones et al. 1997), spending around 90% of the time above 20 m (Tomas et al. 2011b). S*arpa salpa* is known to have a quite heterogeneous diet (Havelange et al. 1997), but it can also be very selective (Verlaque 1990, Marco-Méndez et al. 2017) and even shows a preference for *C. cylindracea* over many native species (Tomas et al. 2011b), which makes it a potential candidate for the control of *C. cylindracea*.

The exclusion experiment was set up at the end of June 2011 in Na Foradada (Fig. 1), an area where fish communities are well established, sea urchin (*Paracentrotus lividus* and *Arbacia lixula*)

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densities are very low (<0.1 per m^2) (Cebrian et al. 2011) and the highest densities of the fish *S. salpa* are found within the National Park, with more than 20 individuals per 250 m^2 (Reñones et al. 1997). Furthermore, we chose this area because both the shallow and the deep benthic habitats displayed a similar medium complexity (with coverage of erect and canopy-forming species at between 25-35%) and similar abundances of native species. This meant there was sufficient abundance of *C. cylindracea* to experimentally assess, in the field, the effect of herbivory pressure on it.

At each depth, 3 treatments were used: “Exclusion”, which consisted of cages of 50 x 50 x 50 cm^3 made of plastic netting with a mesh size of 2.5 cm; “Control-Exclusion”, consisting of cages with open sides; and “Control”, consisting of 50 x 50 cm^2 quadrats marked permanently on the corners and without a cage. A total of 5 interspersed replicates per treatment were set (15 plots per depth) within an area of <100 m^2 to avoid different abiotic conditions between plots. At the beginning (July) and at the end (August) of the experiment, pictures were taken at each plot to subsequently assess *C. cylindracea* abundance, which was calculated with the computer program *photoQuad* version 1.4 (Trygonis and Sini 2012). In each photograph, 50 random points were placed and then, each of these points was assigned to the category of either “*Caulerpa cylindracea*” or “other algae”. The proportion of points in each category was then used as a proxy of the percentage abundance for each of those two categories.

Abundance of *Caulerpa cylindracea*

The abundance of *C. cylindracea* at the Cabrera Archipelago was assessed in 2008 and then again in 2017. To do so, 16 representative sites around the archipelago were chosen (Fig. 1) and a perpendicular transect to shore was performed at each site by means of scuba diving. The depth of the transects ranged from 5 to 45 m to cover the main bathymetric range at which *C. cylindracea* can be found (Cebrian et al. 2011). The abundance of *C. cylindracea* was estimated by means of 25 x 25 cm^2 quadrats, divided into 25 subquadrats of 5 x 5 cm^2 (Sala and Ballesteros 1997, Sant et al. 2017) and the number of subquadrats where *Caulerpa* was detected, was used as a unit of abundance. A total of thirty quadrats were randomly positioned within each 5 m-depth range and then the mean *C. cylindracea* abundance per each 5 m-depth stratum was calculated. Also, to take into account the effect that assemblage complexity might have on the bathymetric abundance of *C. cylindracea*, each 5

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m-depth range at each site was classified as having either high or low complexity based on the dominant morphological categories in the assemblage (“canopy-forming”, “erect”, “turf” and “encrusting”), while ignoring the presence of *C. cylindracea* to avoid confounding effects.

Statistical analysis

The effect of benthic assemblage complexity on the cover of *Caulerpa cylindracea* was assessed with binomial generalized linear models (GLM), because the response variable was measured as a percentage (% of *C. cylindracea* coverage in each sample) and it could be approximated to a logistic distribution (e.g.: success = % coverage of *C. cylindracea*, failure = % coverage of species other than *C. cylindracea*). Two models were fitted, one to assess the role of “assemblage complexity” and another to assess the role of both “depth” and “assemblage complexity” on *C. cylindracea* coverage. In the latter, both factors were included as fixed effects and if the interaction between them was significant, it was also included in the model. To fit both models, the statistical environment R was used (R version 3.6.3) (R Core Team 2018); and to compare the effects between levels in the assemblage complexity factor itself (“high complexity”, “medium complexity” and “low complexity”), and in the assemblage complexity factor at each depth (“shallow” and “deep”), Tukey post-hoc tests were performed using the functions “pairs” and “emmeans” from the package *emmeans* (Lenth 2018).

To evaluate the effect of the exclusion treatment on the abundance of *C. cylindracea* at the end of the experiment, binomial mixed effects GLMs were used because the response variables were measured as proportions and could be approximated to a logistic distribution (e.g.: success = points that corresponded to *C. cylindracea*; failure = points that did not correspond to *C. cylindracea*), while the random terms were used to take into account the repeated measures. In the models, the factors “treatment” and “time” were included as fixed effects, whereas “plot” was included as a random effect. If the interaction between “treatment” and “time” was significant, it was also included in the fixed part of the model. Two models were fitted, one per depth (10 m or 30 m) by means of the package *lme4* (Bates et al. 2015) for R. Tukey post hoc tests, which were performed using the functions “pairs” and “emmeans” in the package *emmeans*, were used to compare effects between

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levels in the treatment factor (“Exclusion”, “Control-Exclusion”, “Control”) at each time observation (“beginning” and “end”).

The R code used to perform all the statistical analyses can be found on Zenodo: https://doi.org/10.5281/zenodo.4664432 (Santamaría 2021).

Results

Effect of benthic assemblage complexity on *Caulerpa cylindracea* coverage

Benthic assemblage complexity had a significant effect on *Caulerpa cylindracea* coverage (p<0.001, Appendix S2: Table S1), with high complexity assemblages supporting low *C. cylindracea* covers (Fig. 2, A). In fact, there were significant differences in *C. cylindracea* cover among the different levels of assemblage complexity (p<0.001, Appendix S2: Table S2), with *C. cylindracea* coverage lower than 5% in very complex assemblages, whereas in low complexity assemblages, coverage of the invasive species reached values of almost 30% (Fig. 2, A).

When the depth of the sampled assemblages was included in the model, *C. cylindracea* coverage varied significantly by depth, by assemblage complexity and by the interaction term (Appendix S2: Table S3). In fact, although the previous pattern of lower *C. cylindracea* cover in high complexity assemblages is maintained, the coverage of the invasive alga is significantly higher at deeper habitats, independently of assemblage complexity (Appendix S2: Table S4, upper table; Fig. 2, B). In particular, in shallow habitats, high complexity assemblages had 20 times less *C. cylindracea* coverage than low complexity assemblages, but only 10 times less coverage at deeper habitats (Fig. 2, B). At each depth, there were significant differences between all levels of assemblage complexity, except between high complexity and medium complexity assemblages in shallow areas (Appendix S2: Table S4, lower table).

Exclusion experiment

Significant effects of herbivory on *Caulerpa cylindracea* abundance were only detected at shallow depths (10 m), where *C. cylindracea* abundance varied significantly by treatment, by time and by the interaction between the two (Appendix S3: Table S1). While at the beginning of the experiment, all treatment areas displayed similar *C. cylindracea* abundance, both the “Control-

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Exclusion” and the “Control” treatments exhibited lower *C. cylindracea* abundances at the end of the experiment ($p<0.0001$; Appendix S3: Table S2, upper table), whereas *C. cylindracea* abundance in the “Exclusion” treatment remained constant (Appendix S3: Table S2, lower table). Indeed, at the end of the experiment, *C. cylindracea* abundance was 4.33 and 2.36 times higher in the full exclusion cages compared with the uncaged control plots and the side-open cages, respectively (Fig. 3, A).

On the other hand, at 30 m, the abundance of *C. cylindracea* varied significantly across time but not between treatments (Appendix S3: Table S1). In fact, for all three treatment levels, abundance was significantly higher (1.88 times on average) at the end of the experiment, than at the beginning (Fig. 3, B).

Abundance of *Caulerpa cylindracea*

The abundance of *Caulerpa cylindracea* in the Cabrera Archipelago decreased between 2008 and 2017 at depths of between 5 and 35 m, but remained more or less constant below 40 m (Fig. 4). In the entire bathymetric distribution, assemblage complexity showed a considerable effect on *C. cylindracea* abundance, with high complexity assemblages exhibiting lower abundances of the invasive alga than low complexity assemblages. Furthermore, it was in shallow and highly complex assemblages, from 5 to 25 m deep, where *C. cylindracea* reached its lowest abundance, with values ranging between 5 and 10% (Fig. 4, B).

Discussion

Our results indicate that while both, competition and herbivory, can provide biotic resistance to a certain extent, strong synergistic effects are observed when the two mechanisms act together. Indeed, competition (by canopy-forming and erect algae) and herbivory (by *Sarpa salpa*), significantly affect the invasion outcomes of *C. cylindracea*, particularly limiting its abundance in complex algal assemblages in which herbivory pressure is high.

Benthic assemblage complexity had a strong influence on preventing *C. cylindracea* invasion. The dominance of canopy-forming and erect species resulted in reduced cover of *C. cylindracea*, probably determining resistance to *C. cylindracea* invasion through competition mechanisms (Ceccherelli et al. 2002, Piazzi and Balata 2009), such as the reduction in light availability, which can

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limit the photosynthetic performance of *C. cylindracea* (Bernardeau-Esteller et al. 2015, Marín-Guirao et al. 2015, Bernardeau-esteller et al. 2020) and the prevention of reattachment of fragments through the branch-sweeping of the substratum (Bulleri and Benedetti-Cecchi 2008, Piazzi et al. 2016). In contrast, when the abundance of canopy-forming algae was low and the communities were dominated by turf and encrusting species, *C. cylindracea* cover was much higher. In such habitats, the colonization and spread of *C. cylindracea* seems to be facilitated because turf assemblages provide an optimal 3-D matrix which favors the anchoring of the stolons and trapping of fragments (Ceccherelli et al. 2002, Bulleri and Benedetti-Cecchi 2008, Bulleri et al. 2009). In this sense, these findings support previous evidence from marine and terrestrial ecosystems, where the presence and dominance of species with certain functional traits (e.g. growth form, size and height) among the assemblage, strongly influences the strength of the biotic resistance against invasive primary producers (Lindig-Cisneros and Zedler 2002, Arenas et al. 2006, Britton-Simmons 2006, Byun et al. 2013, Bernardeau-esteller et al. 2020) by limiting one or several essential resources for the invader.

However, the significant differences we observed in the abundance of *C. cylindracea* between shallow and deep communities, regardless of assemblage complexity, suggest that mechanisms other than assemblage complexity are playing an important role on the invasion of *C. cylindracea*, particularly given that *C. cylindracea* can readily colonize habitats from 0 to 50 m depth (Klein and Verlaque 2008, Cebrian and Ballesteros 2009). In this sense, although several factors (e.g. propagule pressure, disturbance or abiotic conditions) cannot be disregarded, we suggest that the observed differences in invader abundance mainly reflect the contrasting consumer pressures found between deep and shallow communities. Concretely, given similar assemblage complexity, when herbivory pressure was high (here shallow habitats) (Reñones et al. 1997, Vergés et al. 2009, 2012, Tomas et al. 2011b), the abundance of *C. cylindracea* was significantly lower compared to areas where herbivory pressure was low or non-existent (deeper habitats). Actually both, the exclusion experiment and the pattern of *C. cylindracea* abundance and distribution across the Cabrera Archipelago, further support that herbivory is also contributing to the biotic resistance of native assemblages to the *C. cylindracea* invasion. In this sense, where herbivory is weak, such as in deep habitats or if herbivores have been depleted, *C. cylindracea* is subject to limited control and its abundance depends largely on assemblage complexity, which results in higher abundances of the invader among the native

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assemblage (Fig. 5, A and B). Similarly, when herbivory pressure is high but assemblage complexity is low, the invader will also suffer limited control (Fig.5, C). However, when high herbivory pressure is concomitant with high assemblage complexity, both mechanisms (competition and herbivory) strongly limit the abundance of *C. cylindracea* (Fig. 5, D). Taking into account that sea urchin densities were very low in the study area (<0.1 per m^2), the herbivory pressure observed can be mainly attributed to the effect of *Sarpa salpa*, a fish which is mostly distributed at shallow depths and which has a certain preference for *C. cylindracea* (Tomas et al. 2011b). Nevertheless, considering that sea urchins have been previously reported feeding on *C. cylindracea* (Ruitton et al. 2006, Bulleri et al. 2009, Cebrian et al. 2011, Tomas et al. 2011a), additional negative effects on *C. cylindracea* abundance may occur in areas with high sea urchin abundances. These results where the interaction between biotic mechanisms yielded a stronger biotic resistance against the invader than the single mechanisms alone, agree with previous studies where habitat characteristics interacted with herbivory pressure to influence the overall biotic resistance of certain terrestrial ecosystems (Suwa and Louda 2012, Li et al. 2014, Zhang et al. 2018).

Also, and importantly, by following the long-term dynamics of *C. cylindracea* at the study area, we observe that the overall strength of the assemblage´s biotic resistance has increased over time as the abundance of the invasive alga in assemblages subjected to higher biotic resistance (i.e., communities at depths of between 0 to 25 m), has decreased over a 10-year period (Fig. 4). This regression, restricted to areas with high herbivore pressure, may be the result of either an increase in the abundance of the herbivores or to some herbivores becoming more efficient in consuming the invasive species as the invasion progresses (Strayer et al. 2006, Carlsson et al. 2009). However, given that the abundance of *S. salpa* has remained more or less stable during the assessed period (Coll 2020), we suspect that this herbivore has become more efficient at targeting the invader and has increased its *per capita* consumption rates over time (Santamaría J., unpublished manuscript). Nevertheless, other mechanisms that can increase biotic resistance, such as, for instance, allelochemical defenses deployed by native species and assemblages against the invader (Strayer et al. 2006), cannot be ruled out.

Unfortunately, in general, knowledge on whether the effects of biotic mechanisms are maintained throughout an invasion process or whether they change in relation to time since invasion

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is still quite limited, because most studies just assess a particular time frame in the invasion (Maron and Vilà 2001, Levine et al. 2004, Mitchell et al. 2006, Alofs and Jackson 2014, Papacostas et al. 2017). To our knowledge, our study is the first that reports the importance of exposure time for the development of resistance against invaders in natural ecosystems (but see Diez et al. 2010, Dostál et al. 2013 for assessments in common garden experiments) and highlights that increases in biotic resistance observed over time (due to increased consumer pressure, competitive impacts or a combination of the two), could explain why invaders that have previously been considered as hyper-successful (e.g. *Myriophyllum spicatum*, *Elodea canadiensis*, *Dreissena polymorpha*, *Carcinus maenas*, *Caulerpa* species) can suffer marked reductions in population size some years after the onset of the invasion (Simberloff and Gibbons 2004, De Rivera et al. 2005, Iveša et al. 2006, Carlsson and Strayer 2009, Bernardeau-esteller et al. 2020). Therefore, by focusing on only a small time frame or just the beginning of an invasion, we may be underestimating the true capacity of native assemblages to develop resistance to invaders (Strayer et al. 2006, 2017, Rius et al. 2014, Papacostas et al. 2017). For this reason, the use of long-term data, despite being scarce, should be prioritized whenever possible, to assess the true effect that biotic resistance mechanisms might have on the overall invasion process.

Generally, our findings highlight the importance of considering several factors and their interaction when assessing the strength of biotic resistance mechanisms against a particular invader, especially considering that herbivory and competition are universal processes that operate across ecosystems and that naturally interact with each other (Gurevitch et al. 2000, Meiners and Handel 2000, Hambäck and Beckerman 2003). In fact, it has been proposed that herbivory reinforces competition and in turn releases the chance for coexistence, favoring those species that are better competitors (Gurevitch et al. 2000). However, despite that, in invasion ecology, the interaction between these mechanisms has been rarely assessed (but see: Suwa and Louda 2012, Li et al. 2014, Zhang et al. 2018) and most studies rely on the assessment of single biotic mechanisms (Maron and Vilà 2001, Levine et al. 2004, Vilà and Weiner 2004, Kimbro et al. 2013, Papacostas et al. 2017 and references therein). This can definitively underestimate the true role of biotic processes (e.g. competition and herbivory) against invasive species and may explain why our findings, reporting a strong effect of biotic mechanisms against a successful invader, contrast with many previous studies.

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Actually, our results, together with those recently reported for mangrove ecosystems (Li et al. 2014, Zhang et al. 2018), where a successful invader (*Spartina alterniflora*) was limited and excluded due to the interaction between competition and herbivory, suggest that synergisms between biotic resistance mechanisms may be an important but overlooked process driving the invasion success of plant invaders (Fig. 6). In this sense, *in situ* field experiments, in which competition (e.g. assemblage complexity), herbivory, and the interaction between the two can be simultaneously assessed, can provide a better understanding of the true extent of biotic resistance against an invader (Levine et al. 2004, Mitchell et al. 2006, Kimbro et al. 2013, Li et al. 2014, Enge et al. 2017, Zhang et al. 2018, Petruzzella et al. 2020) and will definitively help in the understanding of the invasion success and the dynamics of different invaders.

Finally, the findings reported in this study highlight the importance of improving our knowledge regarding the factors that influence invasive species success in order to fully understand the invasion process of different species and adopt successful mitigation and management measures. As a practical example, while the removal of invasive algae has proven to be, in most cases, ineffective or infeasible (Epstein and Smale 2017, Giakoumi et al. 2019), results obtained in this and other studies (Bernardeau-esteller et al. 2020) suggest that by promoting the conservation of marine habitats and herbivorous populations, we can foster biotic resistance within an ecosystem-based approach to marine environment management and contribute to the long-term control of marine invasions.

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Supporting Information

Additional supporting information may be found online at: [link to be added in production]

Open Research

R code and data (Santamaría 2021) for this publication are available on Zenodo: https://doi.org/10.5281/zenodo.4664432

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FIGURE CAPTIONS

Figure 1. Location of the Cabrera Archipelago National Park. The points show the locations of the sampling sites where assemblage complexity was assessed (3 sites), the exclusion experiment was performed (1 site) and the scuba diving transects were done (16 sites). Shapefile for the Mediterranean Sea downloaded from www.naturalearthdata.com and for the Cabrera Archipelago National Park from www.miteco.gob.es.

Figure 2. Mean *Caulerpa cylindracea* coverage ± S.E for **A)** each level of assemblage complexity, and for **B)** each level of assemblage complexity at the two different depths. Significant differences between assemblage complexity levels (p-values from Tukey´s test with 95% confidence intervals) are indicated with letters.

Figure 3. Mean *Caulerpa cylindracea* abundance ± S.E., at each time observation and for each treatment in the exclusion experiment. **A)** Exclusion experiment at 10 m depth and **B)** exclusion experiment at 30 m depth. Significant differences between exclusion treatments (p-values from Tukey´s test with 95% confidence intervals) are indicated with letters in each graph.

Figure 4. Bathymetric abundance of *Caulerpa cylindracea* (mean ± S.E.) at the Cabrera Archipelago National Park: **(A)** in 2008 and **(B)** in 2017, on assemblages with different complexities.

Figure 5. Depiction of how different combinations of assemblage complexity and herbivory pressure determine biotic resistance against a marine invasive alga (e.g. *Caulerpa cylindracea*), representing four scenarios: **A)** low assemblage complexity and low herbivory pressure; **B)** high assemblage complexity and low herbivory pressure; **C)** low assemblage complexity and high herbivory pressure and **D)** high assemblage complexity and high herbivory pressure. (Algae illustrations obtained and modified from the Integration & Application Network (IAN) Image Library (Tracey Saxby and Joanna Woerner), the IUCN and freepik (https://www.freepik.com/macrovector); the fish illustration, by João T. Tavares, was obtained from www.deviantart.com).

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Figure 6. Schematic representation of how the interaction between competition and herbivory might determine the overall biotic resistance against an invader. Depicted illustrations correspond to *Ratus losea*, *Cervus elaphus* and *Sarpa salpa* as native herbivores; a mangrove forest, a temperate forest and a marine algal forest as native assemblages; and two grasses and an alga species (here *Caulerpa cylindracea*) as invasive species. (Illustrations obtained from the Integration & Application Network (IAN) Image Library (Tracey Saxby, Kim Kraeer and Lucy Van Essen-Fishman), the IUCN and the fish illustration, by João T. Tavares, was obtained from www.deviantart.com).

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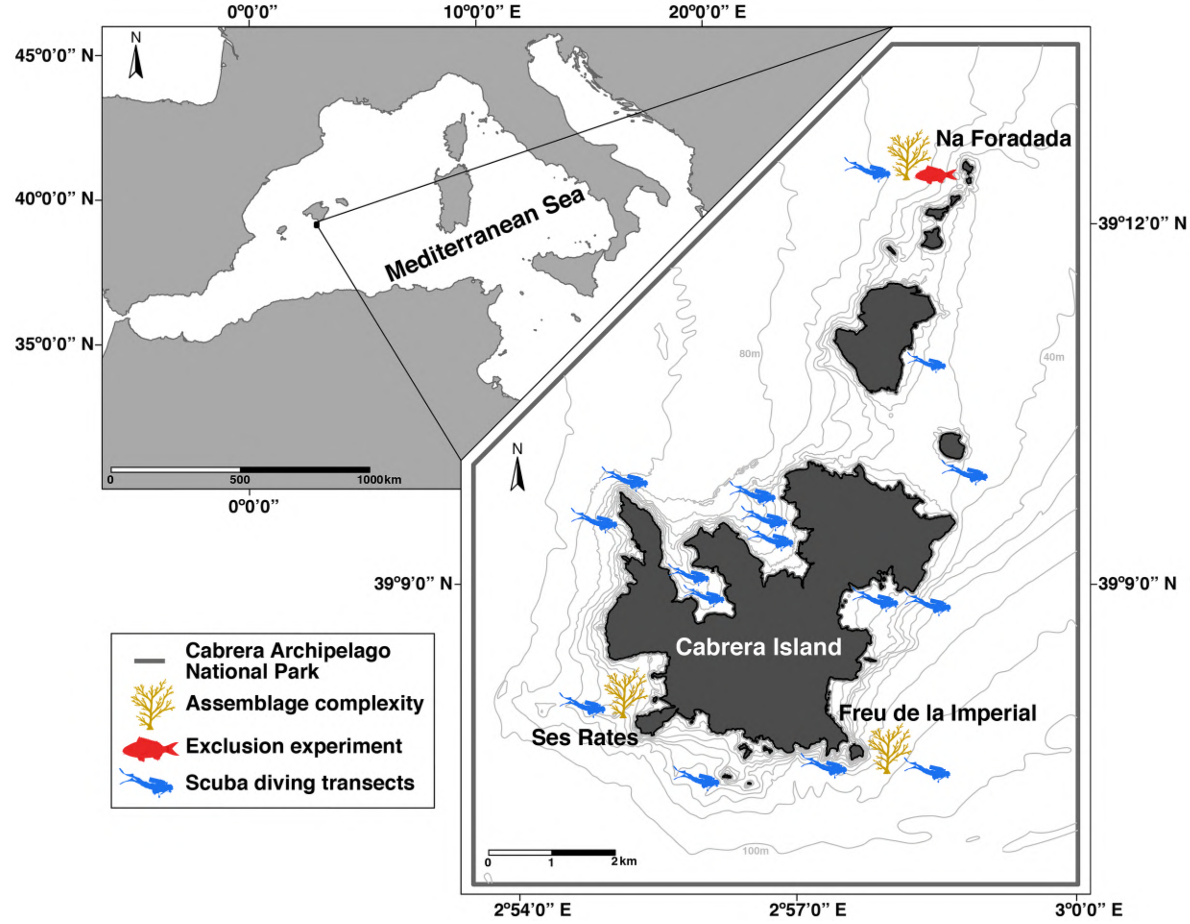

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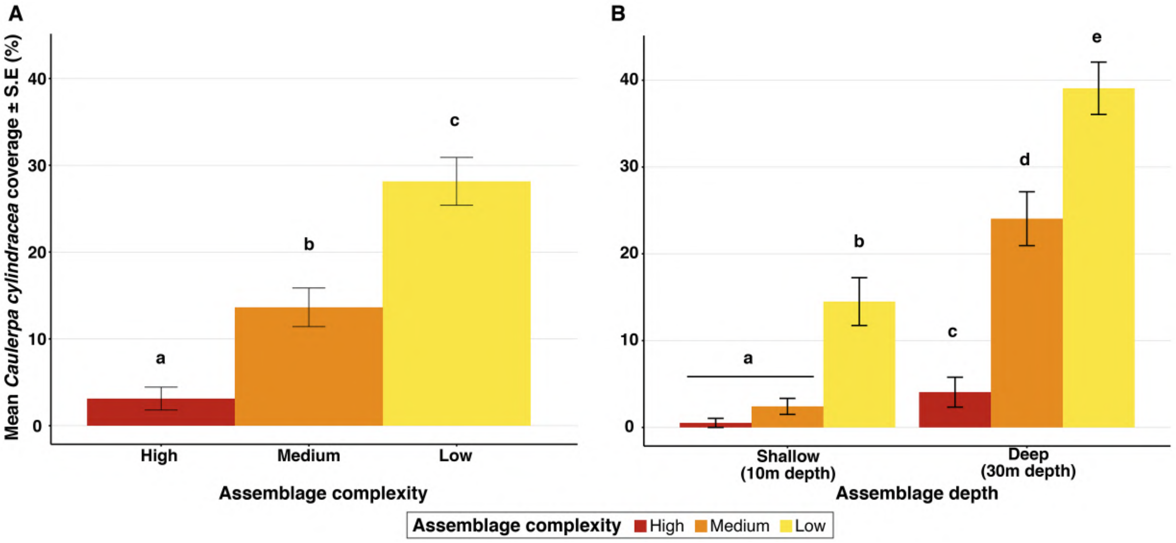

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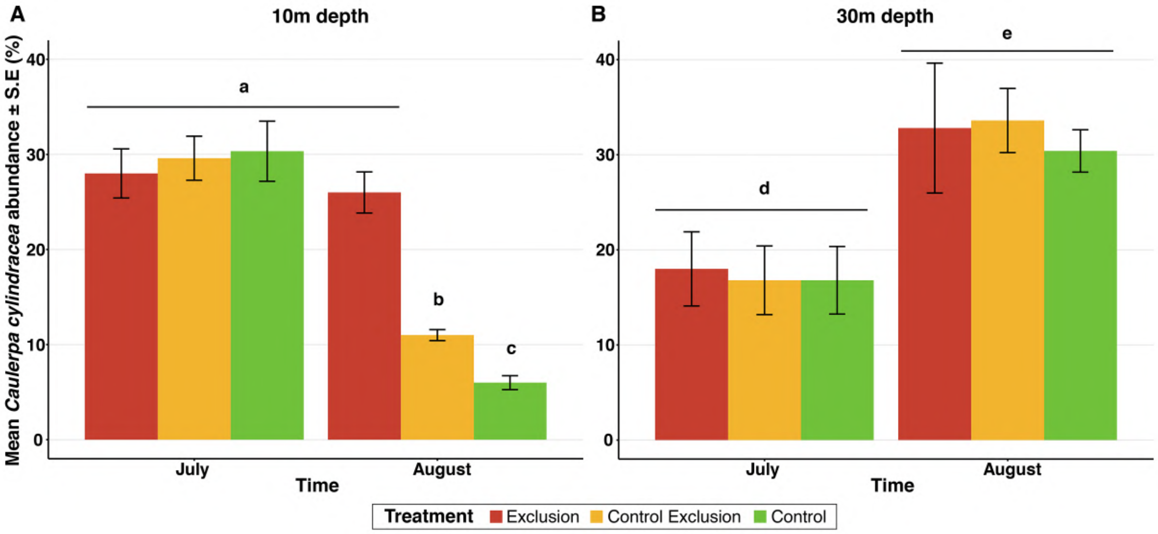

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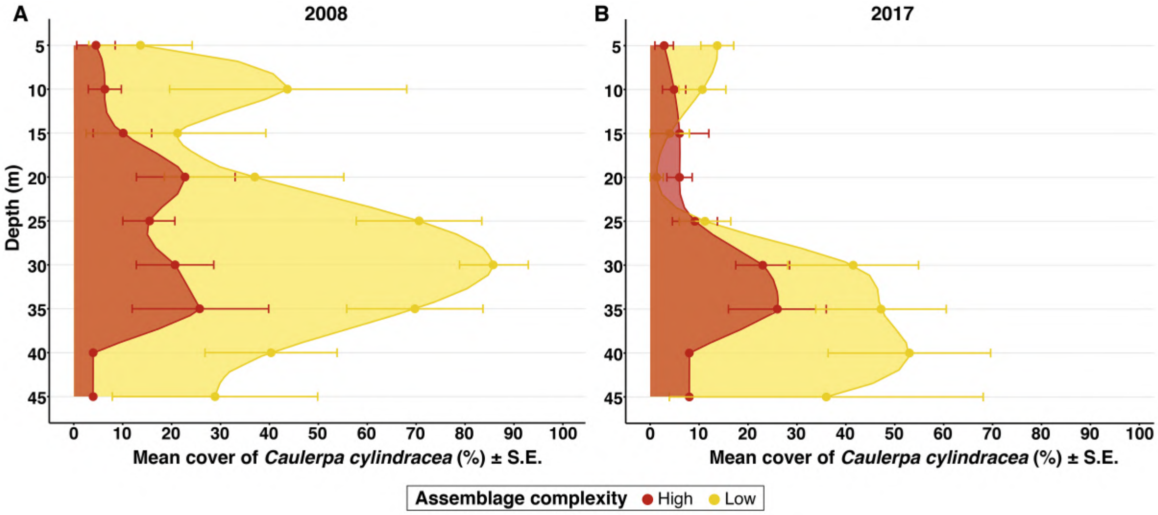

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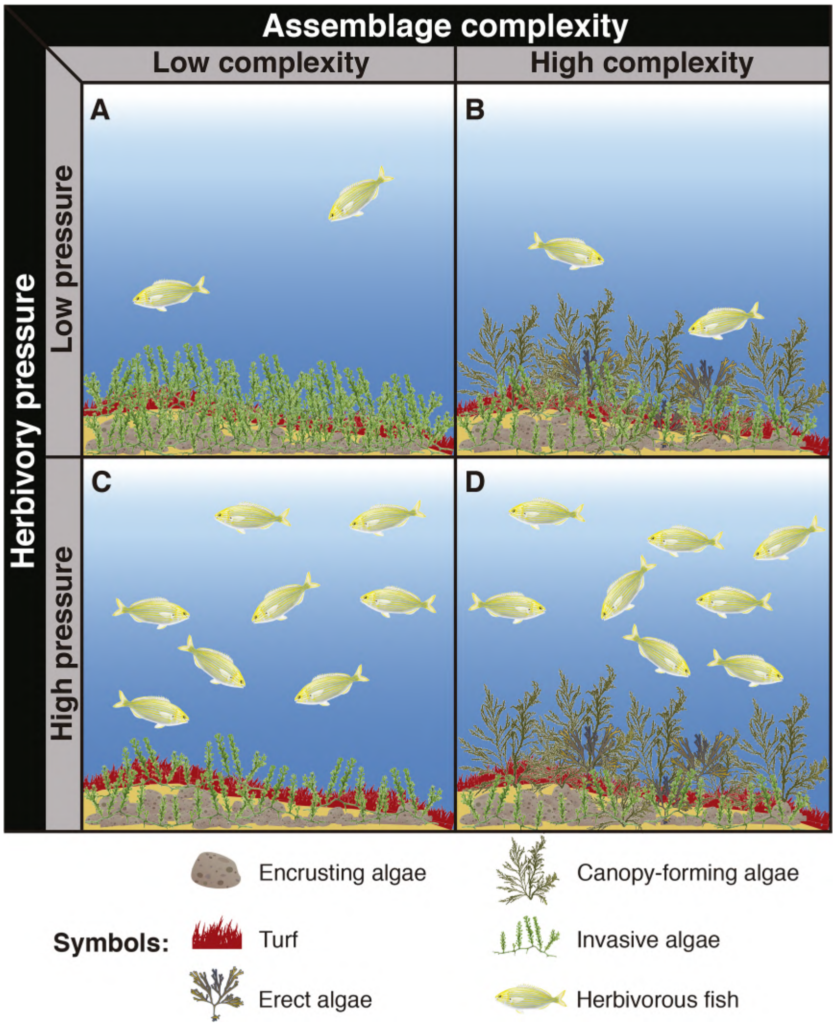

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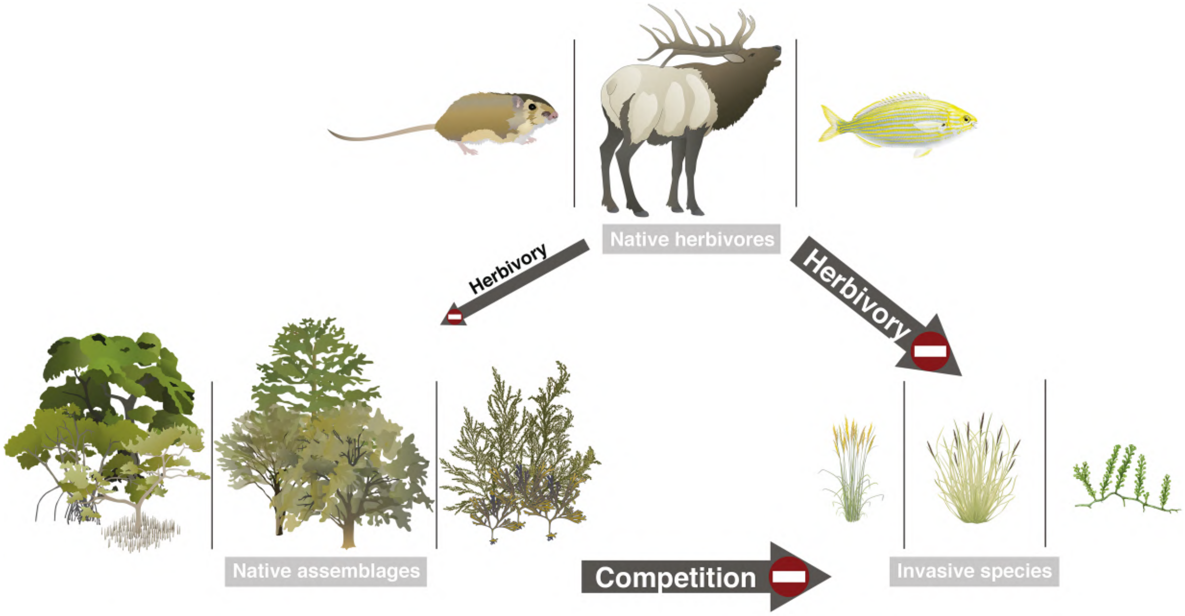

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ORIGINAL RESEARCH
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Stressful Conditions Give Rise to a Novel and Cryptic Filamentous Form of *Caulerpa cylindracea*

Jorge Santamaría[1]*, Raül Golo[1], Emma Cebrian[1,2], María García[2] and Alba Vergés[1]

[1] Marine Resources and Biodiversity Research Group (GRMAR), Institute of Aquatic Ecology, University of Girona, Girona, Spain, [2] Centre d'Estudis Avançats de Blanes, Consejo Superior de Investigaciones Científicas (CEAB-CSIC), Blanes, Spain

Morphological plasticity can enable algae to adapt to environmental change and increase their invasibility when introduced into new habitats. Nevertheless, there is still a lack of knowledge on how such plasticity can affect the invasion process of an invasive species. In this context, the high plasticity in the genus *Caulerpa* is well documented. However, after an extremely hot summer, a previously unreported filamentous morphology of *Caulerpa cylindracea* was detected; indeed, this morphology could only be confirmed taxonomically after in-depth morphological characterization and molecular analysis with the genetic marker *tuf*A. We describe an *ex situ* culture experiment which showed that stressful conditions, such as high temperatures, can trigger this morphological change. Almost all of the thalli maintained at a constant extreme temperature of 29°C died, but after being returned to optimum temperature conditions, the filamentous morphology began to develop from the surviving microscopic tissue. In contrast, thalli at a control temperature of 21°C maintained the regular morphology throughout the experiment. When *C. cylindracea* develops this filamentous morphology, it may act as a cryptic invader because it is difficult to detect in the field. Furthermore, the filaments likely improve *C. cylindracea*'s invasive capabilities with regard to resistance, persistence and dispersion and may have an important role in the re-colonization process, after a population disappears following a period of stressful conditions. Possibly, *C. cylindracea*'s ability to respond plastically to stressful conditions might explain its remarkable success as an invasive species.

Keywords: cryptic invasions, morphological plasticity, resistance form, Mediterranean Sea, *Caulerpa cylindracea*

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Anwesha Ghosh,
Indian Institute of Science Education and Research Kolkata, India
Mads Solgaard Thomsen,
University of Canterbury, New Zealand

Correspondence:
Jorge Santamaría
jorge.santamaria@udg.edu

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INTRODUCTION

Biological invasions refer to the process by which different organisms, commonly known as invasive species, can arrive and establish in a new habitat, where they disrupt the normal functioning of the system. Currently, these invasions are considered one of the main drivers of global change due to their adverse effects on biodiversity, habitat structure and native ecosystem functioning (Mack et al., 2000; Stachowicz and Byrnes, 2006; Simberloff et al., 2013; Bellard et al., 2016). Additionally, the establishment of invasive species it is often associated to great economic costs (Pimentel et al., 2001, 2005) due to their alteration of several ecosystem services (Pejchar and Mooney, 2009; Vilà et al., 2010). The impacts of invasive species are especially important and noticeable in marine ecosystems, where biological invasions are on the rise due to the increases in their main vectors of introduction, such as shipping traffic, the aquarium trade, or the opening and widening of new corridors (Katsanevakis et al., 2013; Seebens et al., 2013; Galil et al., 2017); and in the future, the establishment of non-native species is expected to continue increasing due to climate change (Stachowicz et al., 2002; Lejeusne et al., 2010).

Species invasiveness depends on the features that enable a non-native organism to invade a certain habitat (Richardson et al., 2011) with the main influence being the life-history traits of the invader (Grotkopp et al., 2002; Pyšek and Richardson, 2008; Van Kleunen et al., 2010). Several studies have suggested that phenotypic plasticity is one of the most important of such features for invasive species (Richards et al., 2006; Davidson et al., 2011). Plants and algae can adapt by modifying, among other things, photosynthetic traits (Molina-Montenegro et al., 2012; Zanolla et al., 2015), leaf-area and shoot allocation (Arenas et al., 2002; Liu and Su, 2016) and growth form (Van Kleunen and Fisher, 2001; Monro and Poore, 2009) allowing them to respond to changes in light, temperature or herbivory pressure (Lewis et al., 1987; Monro and Poore, 2005; Nicotra et al., 2010). In this sense, understanding how this phenotypic plasticity affects the success of an invasive species is crucial to our understanding of its invasion process (Schaffelke et al., 2006; Theoharides and Dukes, 2007) and might have important implications for the successful management of the species (Hobbs, 2000; Simberloff et al., 2005).

Caulerpa cylindracea is a siphonaceous green macroalga, native to Western Australia, that has become one of the most widespread non-native algae in the Mediterranean Sea (Piazzi et al., 2005; Klein and Verlaque, 2008; Montefalcone et al., 2015). Indeed, *C. cylindracea* is currently considered the most invasive species within the Mediterranean basin (Katsanevakis et al., 2016) and has also invaded areas in the Atlantic Ocean (Verlaque et al., 2004) and in Southern Australia (Klein and Verlaque, 2008). Still, it is not clear which has been the source of the primary introduction in the Mediterranean Sea, although it is quite likely that it was through the aquarium trade, with shipping traffic and fishing gear being the main sources of the secondary introductions within the basin (Verlaque et al., 2003). Ecologically, *C. cylindracea* spread causes the homogenization of native communities due to the formation of dense and continuous meadows (Klein and Verlaque, 2008), which has negative implications for the native macroalgal assemblages and the diversity of the communities (Piazzi et al., 2001; Piazzi and Ceccherelli, 2006; Klein and Verlaque, 2011). Also, this species affects the sedimentation rates, the carbon turnover, the organic matter composition and the quality of the invaded sediments (Piazzi et al., 2007; Holmer et al., 2009; Pusceddu et al., 2016; Rizzo et al., 2017). Morphologically, *C. cylindracea* is characterized by a simple morphology, formed by creeping stolons and erect shoots with grape-like ramuli (also called branchlets) that can be arranged radially or distichously (Klein and Verlaque, 2008). In addition, it has been reported that species in the genus *Caulerpa* show a high degree of morphological plasticity in response to environmental conditions (Peterson, 1972; Calvert, 1976; Coppejans and Beeckman, 1989; Collado-Vides, 2002b), allowing these species to adapt to different environments and thus increasing their invasive potential (Collado-Vides, 2002b; Raniello et al., 2004; Smith, 2009). Several factors such as temperature, light or depth, can trigger subtle morphological changes in stolon and ramuli shape (Peterson, 1972; Calvert, 1976; Ohba and Enomoto, 1987; Ohba et al., 1992), photosynthetic traits (Raniello et al., 2004, 2006) and the interspace between erect axes (Collado-Vides, 2002b; De Senerpont Domis et al., 2003). However, more acute morphological changes have been detected for the first time in *C. cylindracea* during a recent field survey (**Figure 1**). The thalli of these specimens consisted only of thin vertical filaments, which were impossible to identify as *Caulerpa* species until morphological and molecular characterization confirmed their identity. At present, the exact conditions that trigger this morphological change are unknown but, considering that the filamentous form was found in Montenegro after the extremely warm summer of 2018 (**Figure 2**), it would appear that stressful conditions brought about by high temperatures could be involved. A better understanding of the conditions that trigger this morphological shift—which allows *C. cylindracea* to become a cryptic invader—will greatly enhance our understanding of the invasive process, the collapses and the recoveries of this species.

In this study, our aim was to determine whether extreme temperature conditions can trigger the formation of a filamentous morphology in *Caulerpa cylindracea* similar to that observed in the field. To do so, the morphological plasticity of this species was studied through culture experiments at contrasting temperatures. In addition, in order to confirm the taxonomical identity of the specimens, all thalli (from both cultured and natural populations) were genetically characterized with a chloroplast molecular marker (*tuf* A), which had been used previously for the genus *Caulerpa* (Famà et al., 2002; Kazi et al., 2013; Sauvage et al., 2013).

MATERIALS AND METHODS

Study Sites and Culture

Extreme Temperature Laboratory Experiment

To study the effects of extreme temperatures on *C. cylindracea*, specimens from a population in Spain (Roses: 42° 14′ 18.26′′N; 3° 12′ 25.74′′E) were sampled in February 2019. Once in the laboratory, samples were cleaned with sterilized seawater to remove all the epiphytes and detritus (such as dead *Posidonia oceanica* rhyzomes and dead shells). For acclimation, *Caulerpa* samples were placed in aquariums (12 L) with sterile seawater and in a *Radiber AGP-360* growth chamber at 12°C and a 12:12 (L:D) cycle at 200 μmol photons m^{-2} s^{-1} to simulate natural conditions of irradiance and temperature for 1 week. After the acclimation period, algal cultures were prepared for a period of 170 days under either control conditions or extreme (i.e., very warm) conditions (**Figure 3**). Six fragments of *C. cylindracea* (≈4 cm^2 each) were randomly transferred to six plastic beakers (1 L): three control treatments and three extreme-temperature treatments, each containing 200 g of sterilized gross sand and 0.5 L of sterilized seawater to which was added 5 ml/l of K-medium (Keller et al., 1987). The temperature treatments for

FIGURE 1 | Macroscopic picture of a rock covered by a thick patch of *Womersleyella setacea* turf with the filamentous form of *Caulerpa cylindracea* growing from beneath it (black arrows).

the experiment were as follows: "control" (21°C) based on the average summer seawater temperature recorded in the sampled area and "extreme" (29°C) based on abnormally high summer seawater temperatures recorded in the Mediterranean Sea[1]. After an adaptation period of 7 days at 12°C in the growth chamber, the temperature was progressively increased (by 1°C every 2 days) in all six treatment beakers for 18 days until a temperature of 21°C was reached in the growth chamber. At this point, the beakers were split into two *Radiber AGP-360* growth chambers, one to keep the "control" beakers and the other to keep the "extreme" beakers throughout the experiment. Following this, the "control" beakers were maintained at 21°C for the remaining 152 days of the experiment in the growth chamber; whereas, in the case of the "extreme" beakers, the temperature was raised in the other growth chamber by 1°C every 5 days for the next 40 days until a temperature of 29°C was reached; this temperature was then

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[1] www.t-mednet.org

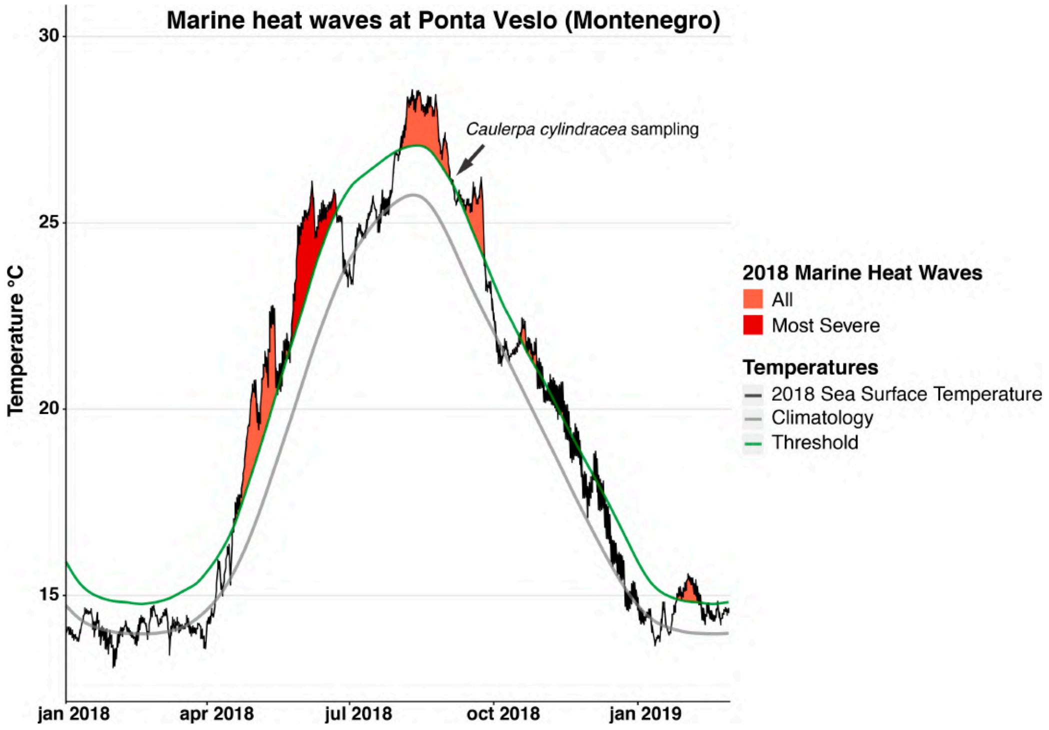

FIGURE 2 | Marine heat wave (MHW) events during 2018 at Ponta Veslo, Montenegro (42° 22′ 5.15′′N; 18° 36′ 22.50′′E), calculated with the *heatwaveR* package (Schlegel and Smit, 2018) using Reynolds Optimally Interpolated Sea Surface Temperature (OISST) data (Reynolds et al., 2007). The gray line represents the SST climatology for the last 35 years; the green line indicates the 90th percentile MHW threshold; and the black line shows the SST during 2018. The dark red filled area indicates the most severe MHW event during 2018, while the orange filled areas indicate all the other MHW events identified over the same time period. The dark gray arrow indicates the day of the year when the filamentous morphology of *Caulerpa cylindracea* was sampled from the field (5th of September).

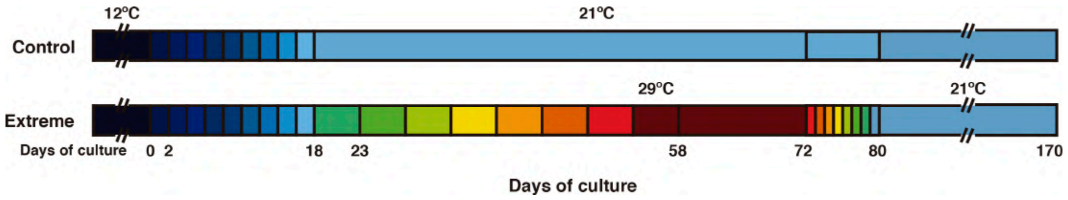

FIGURE 3 | Water temperature variation over the course of the experiment in “control” conditions (above) and “extreme” conditions (below). The numbers indicate the days of culture and each change in color represents a 1°C change in temperature.

maintained for a further 14 days and then gradually lowered by 1°C each day back to 21°C and kept at this temperature for the remaining 90 days of the experiment (**Figure 3**). Throughout the experiment, the seawater and growth medium mixture was renewed once a week.

Field Sampling for Morphological and Taxonomical Characterization

Samples of *C. cylindracea* were collected from natural populations in Spain (Roses: 42° 14′ 18.26′′N; 3° 12′ 25.74′′E); Croatia (Funtana: 45° 10′ 40.16′′N; 13° 35′ 32.31′ E and Split: 43° 30′ 28.79′′N; 16° 23′ 17.56′′E); Montenegro (Ponta Veslo: 42° 22′ 5.15′′N; 18° 36′ 22.50′E); and Albania (Kallm: 41° 19′ 27.88′′N; 19° 25′ 19.31′′E) by scuba-divers at depths of between 5 and 12 m based on previous knowledge on the presence of the invasive alga in these locations. All samples were transported in zip bags within a thermal box to maintain a constant seawater temperature until the morphological characterization was performed in the laboratory. All samples showed the typical morphology of the species (**Figure 4B**). These samples were also used for the taxonomic characterization of the populations (see “Molecular Analysis” section).

Data Collection and Analysis

Extreme Temperature Laboratory Experiment

The effects of the extreme temperatures we applied to *C. cylindracea* were assessed by measuring the macroscopic morphometric changes. The structure and area of *Caulerpa* were measured by means of macroscopic photographs taken with an

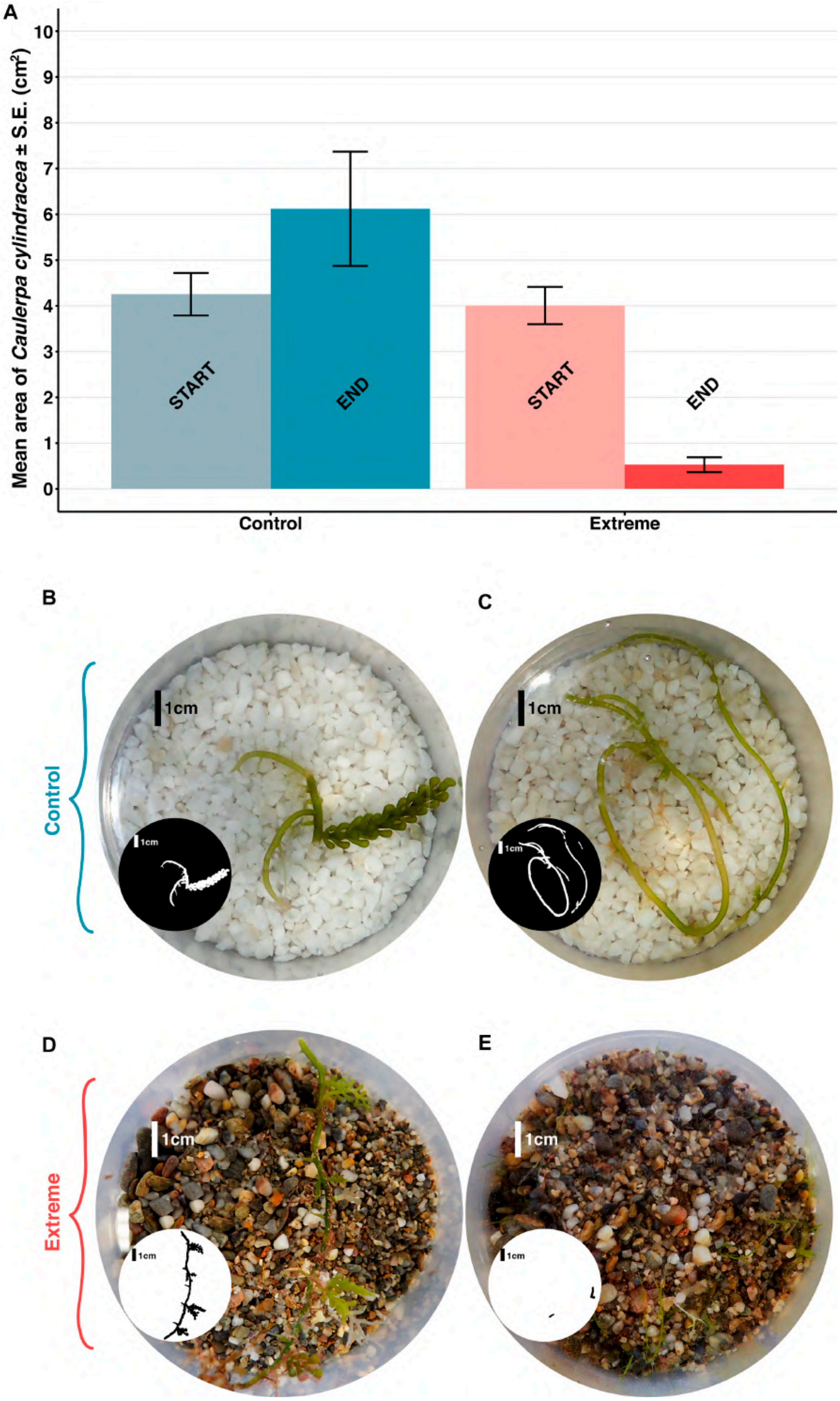

FIGURE 4 | (A) Mean area ±S.E. of *Caulerpa cylindracea* in the beakers (*n* = 6, 3 control beakers and 3 extreme temperature beakers), at the beginning of the experiment, and after 72 days. Photographs **(B,C)** show control beakers at the beginning of the treatment and after 72 days, respectively. Photographs **(D,E)** show extreme temperature beakers at the beginning of the treatment and after 72 days, respectively. The small black and white circles highlight the *C. cylindracea* present in each beaker.

Olympus TG-5 camera, which were then analyzed with Adobe Photoshop CC 2018. Living parts of *C. cylindracea* (green color) were manually selected and measured using the "analysis tool." Then, to assess whether there were differences in the area of *C. cylindracea* between treatments at the end of the experiment, a linear mixed effect model (LMM) was fitted with "*C. cylindracea* area" as the response variable and "treatment" and "time" as the explanatory variables. The interaction between both explanatory variables was included in the model and a random term for "replicate" was used to take into account the repeated measures design (Harrison et al., 2018). To fit this model, the package *lme4* (Bates et al., 2015) in the statistical environment R was used (R version 3.6.3) (R Core Team., 2018). Finally, to compare the effects between levels in the treatment factor ("control" and "extreme) at each time observation ("beginning" and "end"), Tukey *post hoc* tests were performed using the functions "pairs" and "emmeans" from the package *emmeans* (Lenth, 2018).

Morphological Characterization and Comparison

The morphological characterization of the cultured stolons and filaments was assessed by means of microscopic photographs, taken using a Zeiss AXIO Imager A.2 (Carl Zeiss, Berlin, Germany) equipped with an AxioCam MRc5 camera and a stereomicroscope Stemi 2000-C (Carl Zeiss, Berlin, Germany) equipped with an AxioCam ERc 5s camera; and the images were analyzed with Zen2011 software (Blue Edition). Also, to account for regional morphological variability, the mean stolon thickness of 10 randomly sampled stolons from each of the natural populations (Roses, Funtana, Split, Ponta Veslo, and Kallm) was compared to the thickness of filaments obtained at extreme temperatures.

To assess whether mean thickness of filaments differed from that of stolons, a linear model was fitted with "*C. cylindracea* thickness" as the response variable and "location_morphology" as the explanatory variable, in the statistical environment R. Finally, to compare between location_morphology levels ("Roses_filaments," "Roses_stolons," "Funtana_stolons," "Split_stolons," "Ponta Veslo_stolons," and "Kallm_stolons"), Tukey *post hoc* tests were performed using the functions "pairs" and "emmeans" from the package *emmeans*.

Molecular Analysis

To determine species identity, a genetic analysis was performed on all sampled and incubated thalli, including both filamentous and typical morphology. DNA extraction was performed following the DNeasy Plant Mini Kit (QiaGen) protocol in order to obtain the best DNA quality. The primer used to amplify the genetic material was *tuf* A (elongation factor A, from the chloroplast). The reactions were performed in 25 μL volumes and the master mix contained 5 mM of $MgCl_2$, 0.3 mM of each primer, 0.2 mM of dNTPs, 0.5 units of Taq DNA polymerase and 1.0 μL of the extracted DNA. The PCR reaction consisted of 40 cycles of 94°C for 1 min (denaturation), 52°C for 1 min (primer annealing) and 72°C for 2 min (extension) (Famà et al., 2002). The PCR reaction was finalized with a final 5 min step at 72°C. The PCR products were purified and sequenced by Macrogen Spain.

Sequences analyses were performed using different R (R Core Team., 2018) packages: *MUSCLE* (Edgar, 2004) to align the sequences, and *APE* (Paradis et al., 2004) and *PHANGORN* (Schliep et al., 2017) to create phylogenetic trees based on statistical analyses (Bio neighbor-joining tree, k80 distance, with 10,000 replicates).

RESULTS

Extreme Temperature Laboratory Experiment

In the model fitted to the data from the extreme temperature experiment, both "treatment" and the interaction term between "treatment" and "time" showed a significant effect on *C. cylindracea* area ($p < 0.05$, **Table 1**), being the area of *C. cylindracea* equal between treatments at the beginning of the experiment but being significantly different between "control" and "extreme" conditions at the end ($p < 0.05$, **Table 2**). Actually, after 72 days, the mean surface area of the *Caulerpa cylindracea* thalli under "control" conditions had increased by 44%, whereas that of the specimens exposed to the "extreme temperature" treatment (at 29°C for final 14 days of this 72 days period) had decreased significantly by 87% (**Figure 4A**). Furthermore, while the control specimens maintained the typical morphology of the species—characterized by thick rhizomes with some vertical vesicular fronds (**Figure 4C**)—almost all the thick parts of the specimens in the "extreme temperature treatment" had disappeared after 14 days at 29°C (**Figure 4E**).

When the cultures were returned to optimum conditions, new living parts emerged from the sediment in all the "extreme treatment" beakers, after 20 days. However, these new parts did not resemble the original *C. cylindracea*'s morphology. Instead, they presented a new type of structure characterized by erect thin filaments (**Figure 5A**), which for the next 3 months continued growing vertically and extending throughout the liquid in the beakers (**Figure 5B**).

Macroscopically, these filaments, which grow vertically from the substrate, are long (between 5 and 10 cm) and thin, and occasionally branched. Each filament has rhizoids in its basal part (which is without plastids) that serve to attach the

TABLE 1 | ANOVA summary for the LMM fitted to the extreme temperature culture data.

Dependent variable	Predictor	*F* value	Df	Pr (>F)
Caulerpa cylindracea area	Treatment	18.965	1	0.005*
	Time	1.446	1	0.275
	Treatment × Time	15.896	1	0.007*

Caulerpa cylindracea area was modeled as a function of Treatment ("control" and "extreme"), Time (start and end) and their interaction, with Replicate as a random effect to account for the repeated measures over time. The asterisk (*) denotes significant p-values.

TABLE 2 | Effect of temperature treatment on the area of *Caulerpa cylindracea*, at the beginning and end of the culture experiment.

Dependent variable	Time	Comparison	df	t ratio	*p*-value
Caulerpa cylindracea area	Start	Control—Extreme	6	0.260	0.803
	End	Control—Extreme	6	5.899	0.001*

Tukey tests were applied to the fitted mixed model to compare the area of Caulerpa cylindracea between temperature treatments at each time observation. The temperature treatments are: Control (n = 3) and Extreme (n = 3). The asterisk (*) indicates that the p-value is significant.

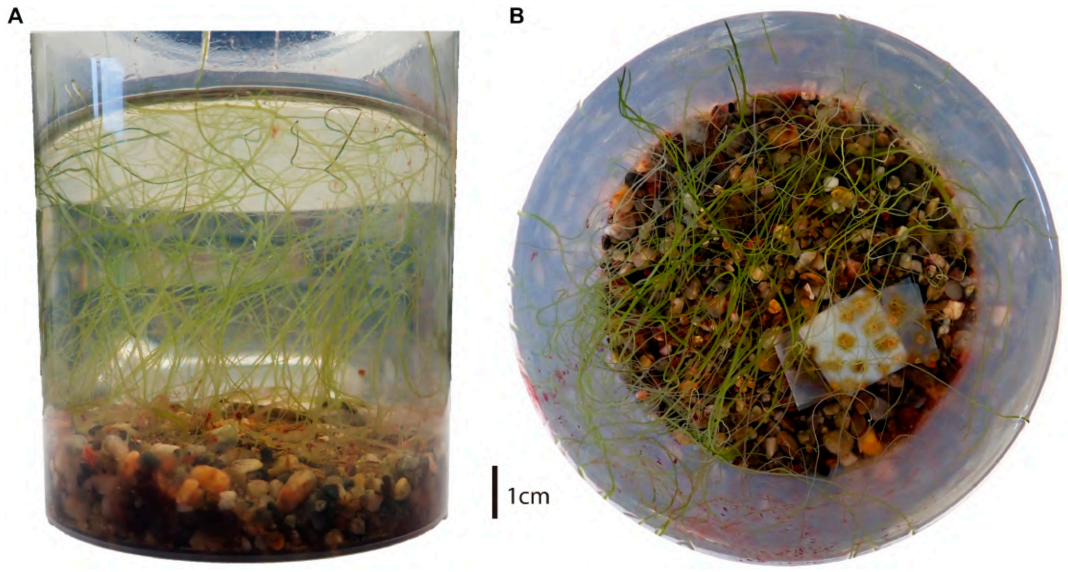

FIGURE 5 | Lateral **(A)** and vertical **(B)** view of one beaker with the filamentous morphology of *Caulerpa cylindracea*. The white square on the bottom of the beaker was added at the end of the experiment, to use it as a scale.

filament to the substrate. The upper part of the filament (with plastids) is light green and in all cases devoid of rhizoids or vesicles, which contrasts with the typical morphology of *C. cylindracea* (**Figures 4B,D**).

These filaments contrasted with the *C. cylindracea* thalli cultured under control conditions which, throughout the experiment (**Figures 4B,C**), maintained the typical morphology described for this species: the thalli were characterized by thick, prostrate stolons with interspaced rhizoids for anchoring, even where they grew unattached to the substrate. Vesicular fronds could also be observed, although these were smaller than in the field.

Morphological Comparison

In the model fitted to the morphological data, the variable "location_morphology" showed a significant effect on *C. cylindracea* thickness ($p < 0.05$, **Table 3**), with filament thickness being significantly different to stolon thickness ($p < 0.005$, **Table 4**) while stolon thickness was similar between populations (**Table 4**). Actually, microscopically, there was an almost 10-fold difference in mean thickness between the filaments (222.42 ± 9.78 μm) in the extreme temperature beakers and the stolons (2,093.38 ± 31.50 μm) sampled at different natural populations. This is amply illustrated in **Figure 6**.

TABLE 3 | ANOVA summary for the LM fitted to the *Caulerpa cylindracea* thickness data.

Dependent variable	Predictor	*F*-value	Df	Pr (>Chisq)
Caulerpa cylindracea thickness	Location_Morphology	116.84	5	<0.001*

Caulerpa cylindracea thickness was modeled as a function of Location_Morphology ("Roses_filaments," "Roses_stolons," "Funtana_stolons," "Split_stolons," "Ponta Veslo_stolons," and "Kallm_stolons"). The asterisk (*) denotes significant p-value.

TABLE 4 | Effect of location and morphology on the thickness of *Caulerpa cylindracea*.

Dependent variable	Morphological comparison	Population comparison	t ratio	*p*-values
Caulerpa cylindracea thickness	Filament vs. Stolon	Filament vs. Funtana	−18.893	< 0.001*
		Filament vs. Kallm	−18.770	< 0.001*
		Filament vs. Ponta Veslo	−20.082	< 0.001*
		Filament vs. Roses	−17.226	< 0.001*
		Filament vs. Split	−17.881	< 0.001*
	Stolon vs. Stolon	Funtana vs. Kallm	−0.123	1
		Funtana vs. Ponta Veslo	1.189	0.840
		Funtana vs. Roses	−1.667	0.559
		Funtana vs. Split	1.012	0.912
		Kallm vs. Ponta Veslo	−1.312	0.777
		Kallm vs. Roses	1.544	0.638
		Kallm vs. Split	0.889	0.948
		Ponta Veslo vs. Roses	2.856	0.064
		Ponta Veslo vs. Split	2.201	0.254
		Roses vs. Split	−0.654	0.986

Tukey tests were applied to the fitted mixed model to compare the thickness of Caulerpa cylindracea between locations and morphologies. The asterisk (*) indicates that the p-value is significant.

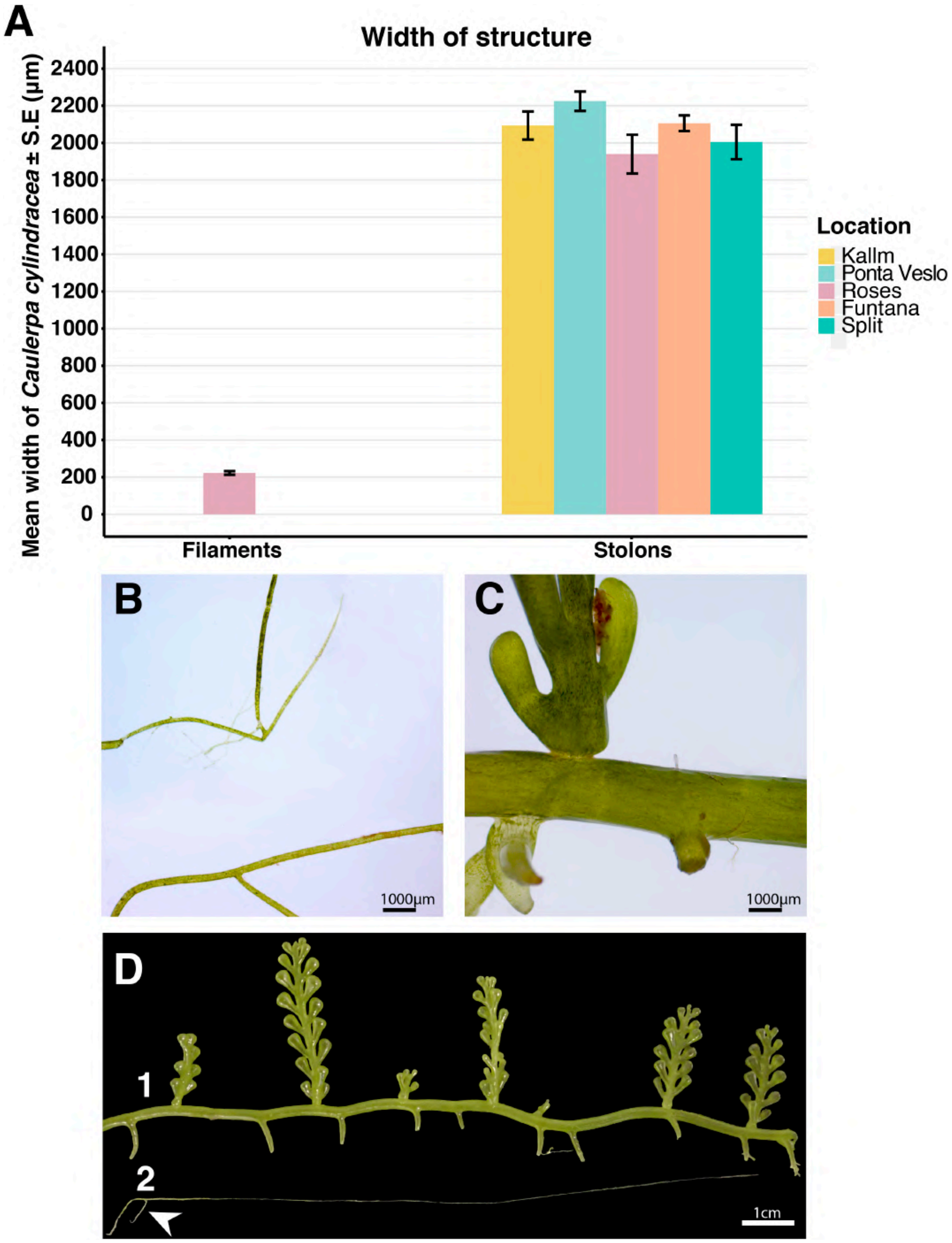

FIGURE 6 | (A) Mean width ±S.E. of *Caulerpa cylindracea* filaments (left) and stolons (right) from different populations (*n* = 10 for each population). **(B)** View of two filaments under the stereomicroscope at 1.0×. **(C)** View of a stolon with a vertical vesicular frond under the stereomicroscope at 1.0×. **(D)** (1) Macroscopic view of *C. cylindracea* with the regular morphology and (2) macroscopic view of the filamentous form of *C. cylindracea*. The white arrow shows the rhizoids that the filaments use for attaching themselves to the substrate.

Although erect filaments are rarely ascribed to *Caulerpa* genus, the presence of trabeculae—slender strands traversing the lumen of the thallus (Lamouroux, 1809; Womersley, 1984; Wynne and Bold, 1985)—confirms the identity of the thalli. The anatomical morphology of this structure in the filaments is identical to that of the regular stolons, as they traverse the interior of the filament going from one side to the other of a circular section and attach to the wall with a thickened structure that resembles a suction cup. However, the trabeculae in the filaments are much thinner and less numerous than in the stolons, which might explain why the filaments are so weak.

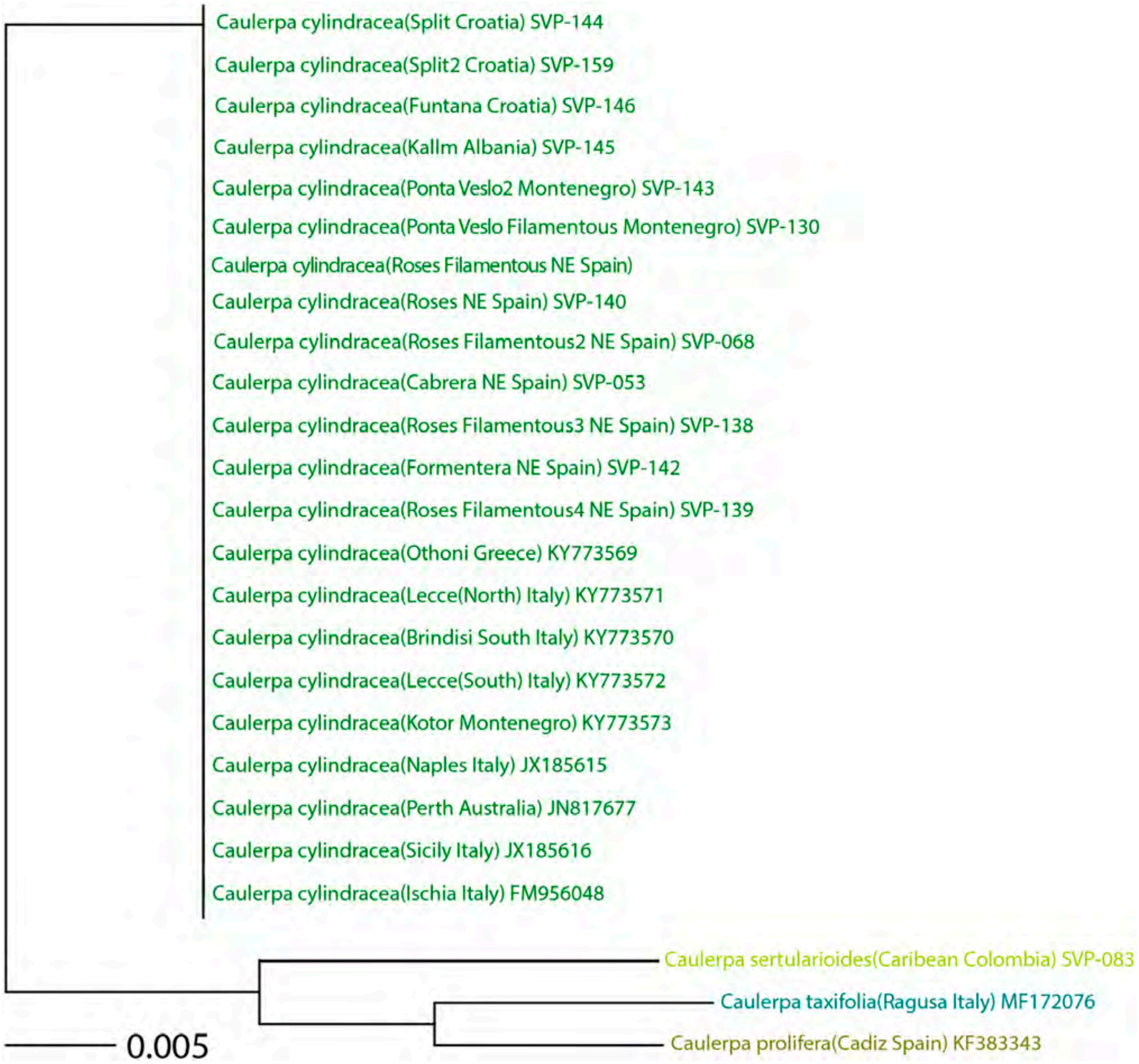

FIGURE 7 | Phylogenetic Neighbor Joining (NJ) tree for *Caulerpa cylindracea*, obtained using the *tuf*A marker. Three other species of *Caulerpa* (*C. sertularioides, C. taxifolia, and C. prolifera*) were used as closely related species to establish differences. GenBank accession numbers are given for each sequence. Values at nodes derived from the NJ. The sequences produced in the present study are highlighted in bold.

Taxonomical Characterization

For the genetic analysis, a total of 14 high quality *tuf* A sequences of up to 820 bp. were obtained and amplified from five specimens with the filamentous shape, eight specimens from the Mediterranean Sea with the common morphology of *Caulerpa cylindracea* and an additional sequence from *C. sertularioides*, which was obtained from the Caribbean Sea (**Table 5**). In addition, several *C. cylindracea* sequences were obtained from GenBank, together with sequences from *C. taxifolia* and *C. prolifera*, in order to establish differences in the phylogenetic tree. In the Neighbor Joining tree (**Figure 7**), all sequences from *C. cylindracea* formed a highly supported cluster, grouped close together, which included both filamentous and typical thalli, thus indicating that they are the same entity.

DISCUSSION

Our culture experiments showed that extreme environmental conditions, in this case high temperatures maintained for long periods of time (i.e., 14 days at 29°C), affected the survival of *Caulerpa cylindracea* and triggered the development of a new

TABLE 5 | *Caulerpa* sequences used to build the phylogenetic tree.

Species	Accession number	Herbarium code	Site
Caulerpa cylindracea	MT274435	Svp-144	Split, Croatia
Caulerpa cylindracea	MT274436	Svp-159	Split, Croatia
Caulerpa cylindracea	MT274442	Svp-146	Funtana, Croatia
Caulerpa cylindracea	MT274446	Svp-145	Kallm, Albania
Caulerpa cylindracea	MT274444	Svp-143	Ponta Veslo, Montenegro
Caulerpa cylindracea	MT274443	Svp-130	Ponta Veslo, Montenegro
Caulerpa cylindracea	MT274447	Svp-053	Cabrera, Spain
Caulerpa cylindracea	MT274445	Svp-142	Formentera, Spain
Caulerpa cylindracea	MT274440	Svp-068	Roses, Spain
Caulerpa cylindracea	MT274441	Svp-140	Roses, Spain
Caulerpa cylindracea	MT274439	Svp-138	Roses, Spain
Caulerpa cylindracea	MT274438	Svp-139	Roses, Spain
Caulerpa cylindracea	KY773569		Othoni, Greece
Caulerpa cylindracea	KY773571		Lecce, Italy
Caulerpa cylindracea	KY773570		Brindisi, Italy
Caulerpa cylindracea	KY773572		Lecce, Italy
Caulerpa cylindracea	KY773573		Kotor, Montenegro
Caulerpa cylindracea	JX185615		Naples, Italy
Caulerpa cylindracea	JX185616		Sicily, Italy
Caulerpa cylindracea	FM956048		Ischia, Italy
Caulerpa cylindracea	JN817677		Perth, Australia
Caulerpa sertularioides	MT274434	Svp-083	Colombia
Caulerpa taxifolia	MF172076		Ragusa, Italy
Caulerpa prolifera	KF383343		Cadiz, Spain

The underlined names show the sequences that were amplified in this study. The rest of the sequences were obtained from GenBank.

morphology characterized by long, thin vertical filaments from the surviving tissues. This new morphology of *C. cylindracea* has also been found in the field and is described here for the first time.

The laboratory experiment showed the remarkable capacity of *C. cylindracea* to survive under stressful culture conditions as the temperature treatment chosen (29°C) is much higher than the usual temperatures found at both the native and the invaded range (Klein and Verlaque, 2008). Additionally, *C. cylindracea* showed a great ability to adapt to environmental change, because it developed a new filamentous morphology from the damaged tissue that survived to the unfavorable culture conditions, i.e., extreme temperatures. These morphological changes during vegetative development are a common strategy in sessile organisms such as plants (Dorn et al., 2000; Puijalon et al., 2008; Nicotra et al., 2010) and algae (Kübler and Dudgeon, 1996; Garbary et al., 2004; Monro and Poore, 2005; Fowler-Walker et al., 2006) to tolerate environmental change, and to improve their competitive and survival capacity (Bradshaw, 1972; Harper et al., 1986; Price and Marshall, 1999; Collado-Vides, 2002a). Actually, more or less acute morphological changes have been previously observed and described in different algae species (e.g., some *Caulerpa* species, *Ulva prolifera*, *Chondrus crispus*, *Asparagopsis armata*, *Padina jamaicensis*, *Codium fragile,* or *Ecklonia radiata* among others) under different culture conditions (Ohba and Enomoto, 1987; Ohba et al., 1992; Kübler and Dudgeon, 1996; De Senerpont Domis et al., 2003; Garbary et al., 2004; Monro and Poore, 2005; Gao et al., 2016) and in the field (Lewis et al., 1987; Meinesz et al., 1995; Collado-Vides, 2002b; Garbary et al., 2004; Fowler-Walker et al., 2006), as a response to changes in temperature, salinity, hydrodynamism, light, or herbivory pressure.

Taking into account that *Caulerpa cylindracea* may be one of the most widely studied invasive algae species around the world, the lack of a previous description of the filamentous morphology demonstrates that when *C. cylindracea* adopts this form, it clearly goes undetected. For instance, the filamentous morphology was sampled—accidentally—in the field within a macroalgae benthic community (**Figure 1**) and was only identified as *Caulerpa* after the samples were analyzed under the microscope. Thus it is clear that *C. cylindracea* filaments also develop under natural conditions. The variables that trigger such filaments in the field may, however, be multiple and in general, remain uncertain. Nevertheless, the culture experiments and field observations described here strongly indicate that stressful conditions may induce the formation of these structures.

The phenotypic plasticity observed in *C. cylindracea* and its ability to withstand extreme conditions (high temperatures) for a long time are characteristics that clearly influence the invasiveness of this species, and improve its persistence and resistance to stress. Actually, this resistance, together with the difficulty of observing the filaments underwater, might explain why *C. cylindracea* populations sometimes seem to disappear only to bounce back after a few months (García et al., 2016). In such situations, it may be that while most of the population dies following unfavorable conditions (**Figure 8E**; Klein and Verlaque, 2008), some small remnants survive, most probably hidden and undetectable in the sediment or sand. Then, as was the case in our culture experiment, the surviving parts may produce filaments that go unnoticed by divers and researchers (**Figure 1**), and, subsequently, the regular morphology of *C. cylindracea* returns when favorable conditions allow the population to recover (**Figure 8G**). Typically, colonization of a new area by *C. cylindracea* can take approximately 3 years, but the presence of this cryptic and resistant form of the species would explain the marked reduction—from 3 years to less than 1 year—in the time it takes *C. cylindracea* to re-colonize areas in which it had disappeared (García et al., 2016), thus highlighting the importance of this morphology in the re-colonization process (**Figure 8**). Furthermore, when adopting this cryptic phase, identification is extremely difficult or simply impossible, which has further implications for the management of the species, since early detection is one of the most important requirements for the successful management of invasive species (Lodge et al., 2006; Vander Zanden et al., 2010; Giakoumi et al., 2019). This misidentification of the filaments can also affect current estimations of *C. cylindracea* in the Mediterranean Sea as this species may be present at several locations in this latent, cryptic form. Since traditional methods of direct observation may not always

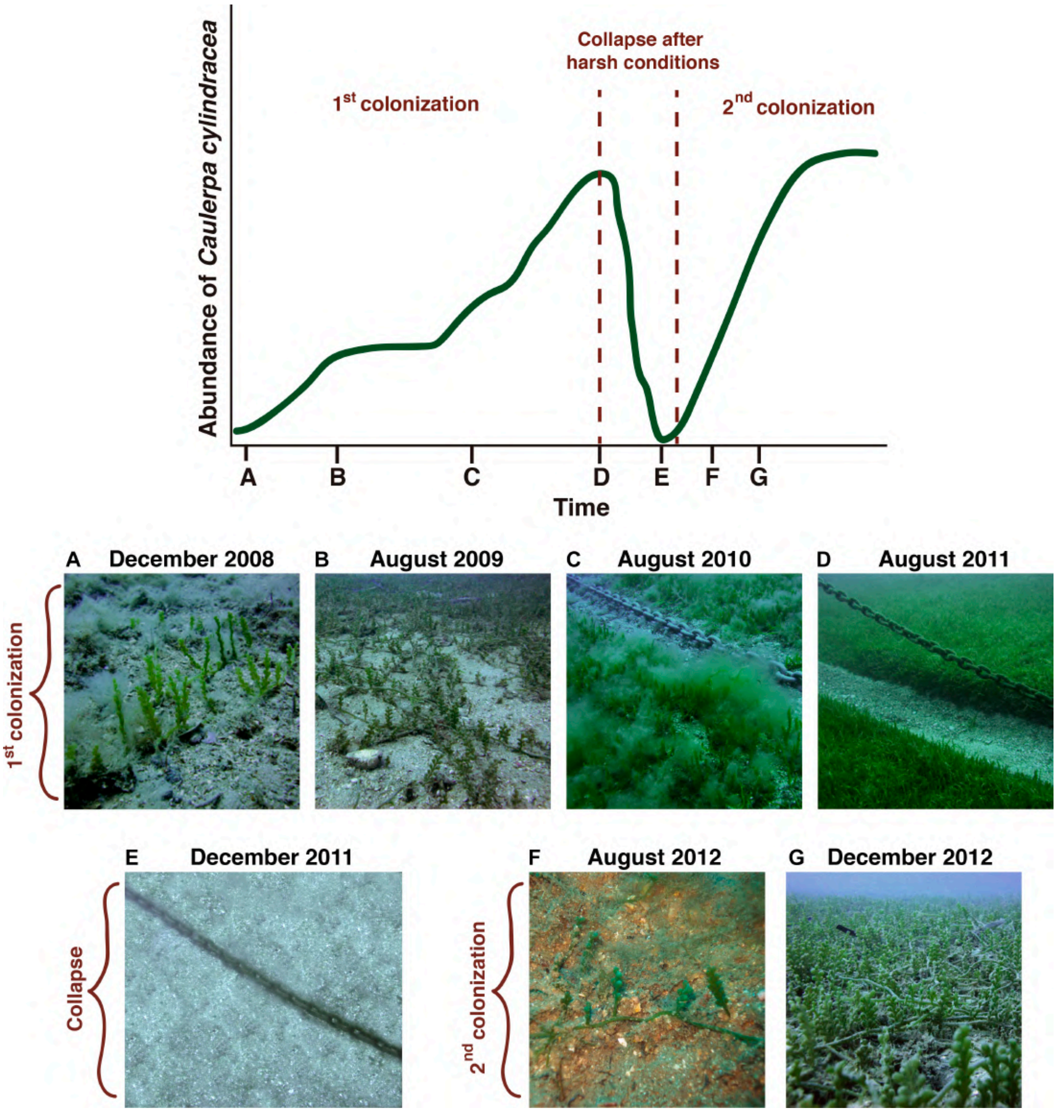

FIGURE 8 | Evolution of the abundance of *Caulerpa cylindracea* over a period of 4 years in a population under study, close to Vilanova i la Geltrú (Spain). During the first colonization, *C. cylindracea* was first detected in December 2008 **(A)**; by August 2009, *C. cylindracea* had expanded and had a patchy heterogeneous distribution **(B)**; in August 2010, *C. cylindracea* covered a larger area forming a continuous meadow **(C)**; and in August 2011, the abundance of *C. cylindracea* reached a peak, forming a dense and continuous meadow over more than 1,000 m^2 **(D)**. In December 2011, the population had collapsed after an exceptionally warm autumn and no sign of *C. cylindracea* could be found **(E)**. The first report of *C. cylindracea* in the second colonization was in August 2012 **(F)**; and just 3 months later, in December 2012, it was already forming a continuous meadow over a large area **(G)**, with greater abundance than that of August 2010 **(C)**.

be reliable in detecting filaments of *C. cylindracea*, as they can be misidentified or missed entirely, methods involving environmental DNA (Taberlet et al., 2012; Kelly et al., 2014; Thomsen and Willerslev, 2015) could be useful in detecting this species, as has been the case with the detection of other invasive species (Dejean et al., 2012; Ardura et al., 2015; Simmons et al., 2015).

Dispersion is another process that might also be favored by *C. cylindracea* filaments, because these long and weak vertical structures are more easily broken upon contact or by water movement than the regular thallus. This will release living fragments that can be transported by currents or attached to drifting objects, favoring dispersal of *C. cylindracea* and the potential for secondary introductions. Actually, in *Caulerpa* species, the fragmentation process is one of its most important reproductive strategies (Fralick and Mathieson, 1972; Ceccherelli and Cinelli, 1999; Smith and Walters, 1999; Ceccherelli and Piazzi, 2001) and allows these species to spread really fast.

To conclude, this newly identified filamentous morphology of *C. cylindracea* could act as a potential jack-of-all-trades that further improves this species' capacity as an invader. The filaments described here are involved in the persistence, resistance, and dispersion of this invasive species and have characteristics that allow this species to withstand harsh abiotic conditions and which may help to explain its successful expansion in the Mediterranean Sea.

DATA AVAILABILITY STATEMENT

The datasets generated for this study and the code to analyse the data can be found here: https://github.com/JorgeSantamaria/Filamentous-Morphology-Caulerpa. The genetic sequences amplified in this study are deposited in the GenBank repository and can be accessed with the accession numbers shown in **Table 5**.

AUTHOR CONTRIBUTIONS

JS, EC, and AV conceived the ideas and the experimental design. JS, RG, and MG collected the samples. JS and RG analyzed the data. All authors drafted the manuscript, contributed substantially to revisions, and accepted the final version before submission.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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