Ecotoxicidad y actividad antimicrobiana de las combinaciones sinérgicas de polifenoles y terpenos de origen natural con antibióticos de síntesis

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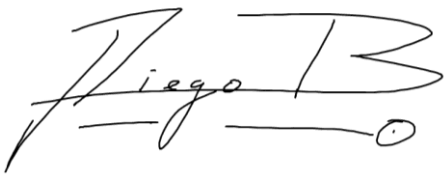

Diego Ballestero Fernández

A mi abuelo Andrés

siente devuelto el orgullo que hoy sentirías

A mis padres

ejemplo en cada vez más cosas

Y a Nat

siempre al otro lado de mi cuerda

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RESUMEN

En los casi 100 años desde su descubrimiento, los antibióticos comerciales han supuesto una mejora de la calidad y esperanza de vida a nivel global. Sin embargo, su uso masivo conlleva una serie de riesgos asociados. Entre ellos se encuentran la generación de resistencias bacterianas o su impacto medioambiental, ya que la continua descarga de antibióticos en las aguas residuales alcanza distintos ecosistemas.

Entre las distintas soluciones que la comunidad científica ha propuesto para tratar de mitigar estos problemas se encuentra la existencia de sinergias entre los antibióticos comerciales y productos de origen natural, que permitirían reducir de forma significativa las dosis de fármaco utilizado. Sin embargo, existen pocos trabajos que aborden este campo de forma sistemática, analizando no sólo la efectividad antimicrobiana de las combinaciones sinérgicas, sino el impacto que estas tienen a su vez en el medioambiente.

Para tratar de determinarlo, el presente trabajo evaluó, en primer lugar, la ecotoxicidad de 8 antibióticos ampliamente utilizados en la práctica clínica, utilizando bioindicadores estándar para ecosistemas acuáticos (*Daphnia magna* y *Aliivibrio fischeri*) o terrestres (*Eisenia fetida* y *Allium cepa*), así como en comunidades microbianas aisladas de ecosistemas fluviales y de suelos. Los resultados obtenidos reflejan que a concentraciones de 100 mg/L, estos fármacos impactan en el crecimiento microbiano y en su capacidad metabólica, especialmente en el caso del metabolismo de polímeros, carbohidratos y ácidos carboxílicos y cetónicos. Estas concentraciones, sin embargo, son elevadas y descartarían una ecotoxicidad aguda pero no efectos ante una exposición continuada y acumulativa a largo plazo.

Posteriormente, se seleccionaron 2 productos naturales, nerol y ácido tánico, con actividad microbiana reconocida. A partir de las Concentraciones Mínimas Inhibitorias (CMI) de los antibióticos y las de los productos naturales se testaron varias combinaciones a fin de detectar efectos de las posibles interacciones entre ellos, especialmente aquellos sinérgicos. Se obtuvieron 14 combinaciones sinérgicas, 10 con ácido tánico y 4 con nerol. Estas combinaciones sinérgicas, nombradas como SACTAs (*Synergic Antibiotic Combination of tannic acid*) y SACNEs (*Synergic Antibiotic Combination of nerol*), fueron evaluadas, determinando su potencial antimicrobiano y comportamiento cinético en ensayos de 7 días de duración.

A continuación, se determinó la ecotoxicidad producida por el ácido tánico y nerol, utilizando para ello los mismos bioindicadores estándar que en el caso anterior, junto con comunidades microbianas aisladas de río y suelos. Los resultados muestran, por un lado, una toxicidad aguda moderada, exceptuando el nerol para *A. fischeri* que es tóxico, siguiendo los parámetros de la ECHA (Agencia Europea de Sustancias y Mezclas Químicas) para medios acuáticos. El efecto en las comunidades microbianas muestra para nerol una inhibición del crecimiento bacteriano a partir de 100 mg/L, mientras que, para el ácido tánico, la inhibición total se da a 200 mg/L.

Por último, para evaluar la viabilidad de estas combinaciones sinérgicas como herramientas para solucionar los problemas medioambientales asociados a los antibióticos, se estudió la ecotoxicidad producida por las combinaciones sinérgicas con

ácido tánico y nerol. Para ello, se evaluó su ecotoxicidad con los bioindicadores individuales acuáticos (*D. magna* y *A. fischeri*), junto con el estudio de comunidades microbianas fluviales. Los resultados obtenidos muestran cómo algunas de las combinaciones sinérgicas consiguieron reducir la cantidad de ATB necesaria entre un 75-93,75%. Una vez evaluadas ecotoxicológicamente, las concentraciones de ATB presentes en la Concentración $Letal_{50}$ de la sinergia se encuentran entre 100 y 10000 veces inferiores a las Concentraciones $Letales_{50}$ de los antibióticos por separado evidenciando el efecto coadyuvante del producto natural sobre el antibiótico. Además, a pesar de que las sinergias presentan una mayor toxicidad intrínseca, las concentraciones de ATB en sinergia se sitúan por debajo de sus concentraciones terapéuticas, lo que garantiza un amplio margen de seguridad ambiental.

1. INTRODUCCIÓN

1.1 BLOQUE I. LOS ANTIBIÓTICOS.

1.1.1. Los antibióticos. La situación actual en cifras.

Han pasado 96 años desde que un científico escocés olvidara, durante unas vacaciones con su familia, varias placas Petri inoculadas con estafilococos apiladas en una esquina de su laboratorio. A su regreso a principios de septiembre, observaría que alguna de ellas se había contaminado de manera fortuita por un hongo. Su trabajo en los años posteriores condujo a Alexander Fleming al descubrimiento de la penicilina (PEN), el primer antibiótico (en adelante, ATB) comercial (Fleming, A, 1929; Gaynes, 2017).

A pesar de que se ha documentado que la fitoterapia tradicional ya empleaba, sin saberlo, algunas moléculas antibióticas en el tratamiento de enfermedades desde hace milenios (Cook, Molto y Anderson, 1989), la extracción y purificación que comenzó tras el descubrimiento de la PEN y su producción en masa en 1945 se considera el comienzo de la *era antibiótica* (Aminov, 2010). El número de tratamientos fue aumentando, especialmente entre 1950 y 1970, cuando se descubrieron un gran número de nuevas moléculas, la mayoría a partir de fuentes naturales (hongos, bacterias, plantas o animales) (Abdallah, 2011). A lo largo de las décadas, su uso fue extendiéndose progresivamente. Su éxito fue indudablemente uno de los mayores hitos de la historia de la humanidad, ya que permitió que enfermedades que a mediados del siglo pasado eran mortales tengan hoy prevención y tratamiento (Aminov, 2010). En la actualidad, los antimicrobianos son utilizados no sólo para el tratamiento de infecciones humanas y veterinarias; también para la prevención de daños en cultivos vegetales (Taylor y Reeder, 2020; Miller et al., 2024), como conservantes (Ross, 1958), o como coadyuvantes del crecimiento del ganado, integrado en su alimentación (Cycoń et al., 2019; Mann et al., 2021), aunque su uso en este aspecto se ha prohibido ya en zonas como la Unión Europea (UE) (Castanon, 2007).

a. Consumo a nivel mundial

Desde finales del siglo XX, se comienza a monitorizar de manera más sistemática el consumo de ATBs, observándose, en general, un aumento sostenido en el consumo, que sólo en los países más desarrollados consigue una cierta estabilización (Browne et al., 2021). Destaca, además, el contraste en la calidad de la monitorización entre los países más desarrollados y aquellos en vías de desarrollo, donde el seguimiento de los datos es más discontinuo (Bebell y Muiru, 2014; Nandi et al., 2023; Padget et al., 2016). Pese a esta frecuente dificultad, encontramos algunos estudios, como el publicado en 2021 en la revista *The Lancet Planetary Health* (Browne et al., 2021), que reflejan una tendencia de consumo que crece a niveles preocupantes, hasta un 46% entre el año 2000 y 2018, pasando de 9,8 a 14,3 DDH (Dosis Diarias Definidas por 1000 habitantes en 1 día). Este aumento (hasta un 76% en el consumo en DDH, según ese mismo artículo) se da principalmente en los países en vías de desarrollo, donde la carga de afecciones microbianas es hasta 3 veces más alta (Allegranzi et al., 2011). El estudio de Browne et al., (2021) encuentra que en la región del sur de Asia el aumento llega a ser del 116%. Otros

estudios confirman tendencias similares (Klein et al., 2021), también en países concretos, como Brasil, con zonas donde el aumento es hasta de un 85% (Neves e Castro et al., 2020). El artículo publicado en la revista *Proceedings of the National Academy of Sciences* de Estados Unidos (EE. UU.) que analiza la tendencia en 76 países entre 2000 y 2015, observa un crecimiento del consumo de un 65% en ese período. Como factor determinante en este aumento, encuentran el marcado crecimiento del consumo en países con menos recursos económicos, mientras que aquellos más prósperos, el uso de ATBs se mantiene, en líneas generales, estable (Klein et al., 2018). En esta publicación, además, se proyecta la tendencia hasta 2030, donde, de no producirse cambios sustanciales, pronostica incrementos de hasta un 200%.

El consumo de ATBs también se produce a gran escala a nivel veterinario, tanto en la ganadería, desde la aparición de las sulfonamidas en la década de 1930 (Prescott, 2017; Kirchhelle, 2018) como en la acuicultura (de Ilurdoz et al., 2022). Además de emplearse para tratar enfermedades en los animales, también se han venido utilizando a dosis subterapéuticas para fines distintos, como promotores de crecimiento, ya que permite una obtención más rápida y barata de carne (Li, 2014; Kirchhelle, 2018) aunque este uso se ha prohibido ya en algunos lugares (“Regulation (EC) No 1831/2003, 2003). La monitorización del consumo veterinario de estos fármacos, que acostumbra a ser más inexacta e irregular que aquella de uso humano, puede suponer un problema oculto de mayor gravedad (Iriti et al., 2020). Así, solamente 5 países (Tiseo et al., 2020) reportaban en el año 2000 las cifras de empleo de ATBs en ganadería, llegando sólo hasta 41 países en el año 2020 (principalmente por la ausencia de datos fiables de países en desarrollo) (Iriti et al., 2020). Estudios recientes estiman un consumo anual mundial de más de 90000 toneladas de antimicrobianos, proyectando además un aumento de en torno al 10% para el año 2030 (Tiseo et al., 2020; Mulchandani et al., 2023). Según el estudio de Tiseo et al., (2020), los 5 países que más ATBs veterinarios consumen son China (45% del volumen total), Brasil (7,9%), EE. UU. (7,0%), Tailandia (4,2%) e India (2,2%), destacando también España en séptimo lugar (1,9%). El sector ganadero asiático es, claramente, el principal consumidor, con 57000 toneladas anuales. De ellas, la mayoría corresponden a China, que reportó 30904 y 32763 toneladas en 2019 y 2020, respectivamente (Mulchandani et al., 2023). La FDA (Food and Drug Administration) estadounidense, otro de los países más consumidores, estimó un uso anual de aproximadamente 13983 toneladas de ATBs en animales de granja en 2016 (Center for Veterinary Medicine. FDA., 2017), que consiguieron reducir hasta 10449 toneladas en 2020 (Center For Veterinary Medicine. FDA., 2020).

El consumo medio en animales es frecuentemente medido en PCU (Unidad de Corrección de Población, por sus siglas en inglés, que significa los miligramos de antibióticos consumidos por kilogramo de masa animal). Analizando los datos por tipo de ganado, distintas publicaciones estiman un uso de alrededor de 45 mg/PCU para ganado bovino, 148 mg/PCU para especies aviares y 172 mg/PCU para el ganado porcino (Van Boeckel et al., 2015). Guo et al., (2016) también comprobaron cómo este último tipo de ganado recibe dosis más altas de ATBs, lo que atribuyen a posibles diferencias en la metabolización farmacológica de las diferentes especies, así como a las leyes

comunitarias más estrictas en el ganado bovino, relacionadas con la presencia de residuos farmacológicos en la leche (Conde-Cid et al., 2018).

Además de este uso veterinario, los ATBs también se han usado sobre cultivos vegetales en forma de ingredientes en aerosoles pesticidas, con el objetivo de prevenir la aparición de plagas y enfermedades microbianas. Sin embargo, los datos sobre el volumen utilizado son muy difíciles de seguir (Haynes et al., 2020). Aunque en muchos de los países ya han prohibido (salvo emergencias excepcionales) este empleo de ATBs (como la UE, Reino Unido, Brasil o China), en otros sigue estando permitido y extendido, siendo el principal consumidor EE. UU (Donley, 2019). Por este motivo, la monitorización del uso de ATBs en la agricultura es mucho más deficiente que el correspondiente a uso clínico y veterinario: sólo el 3% de los 194 países del mundo ofrecen datos al respecto (Batuman et al., 2024).

b. Consumo en la Unión Europea

En la UE, la creciente preocupación generada por el problema de las resistencias microbianas ha llevado a los organismos públicos a aumentar los esfuerzos en controlar el consumo de estos fármacos, aportando información de calidad para valorar el estado de la situación. Dentro de la UE, el Centro Europeo para la Prevención y Control de Enfermedades (ECDC, por sus siglas en inglés) publica anualmente su informe Antimicrobial consumption in the EU/EEA (ESAC-Net) - Annual Epidemiological Report for 2021. Coincidiendo con las conclusiones del estudio de The Lancet (Nandi, Pecetta y Bloom, 2023), el considerable aumento del uso de ATBs que se produjo durante la pandemia de Covid-19 (demostrando que las sucesivas olas de contagios se realizaron más prescripciones de todos los grupos de ATB, pese a no tratar ninguno la infección de este virus) no se ha reducido con éxito a niveles anteriores a ese año, por lo que muestra el informe del año 2022. El informe europeo, que analiza con detenimiento la tendencia general desde 2019, observó una muy ligera reducción, del 2,5%, en el consumo de antimicrobianos además de su portal web del ECDC donde recoge los últimos datos de consumo ATB disponibles (European Centre for Disease Prevention and Control, 2024). Todos los informes coinciden en que el consumo en Europa se mantiene estable en torno a las 20 DDH.

Respecto al ámbito veterinario en la UE, según los datos recogidos por el Instituto Nacional de Estadística (Instituto Nacional de Estadística, 2022) en la Unión se cuenta con unos 350 millones de cabezas de ganado, entre las cuales destaca el sector porcino, con 155 millones de ejemplares. La cifra estimada de consumo en el año 2020 que recoge la Agencia Europea del Medicamento (EMA, por sus siglas en inglés) para los 31 países europeos es de unas 5578 toneladas de ATBs (European Medicines Agency, 2021). La preocupación por este ámbito llevó a los organismos comunitarios a crear en 2009 el ESVAC (*European Surveillance of Veterinary Antimicrobial Consumption*) (European Medicines Agency (EMA), 2023). Asimismo, la legislación europea prohíbe desde 2006 el uso de ATBs como promotores de crecimiento en animales (European Comission, 2003).

Las cifras reflejan el impacto de cambios legislativos de esta magnitud, con el consumo total en los 27 países de la Unión reducido en un más de un 50% entre 2011 y 2022 (European Medicines Agency, 2021). Sin embargo, el uso de estos fármacos como aditivos alimentarios sigue permitido en casos preventivos, y se estima en unos 73.9 mg/PCU en los países miembros (European Medicines Agency, 2023).

c. Consumo en España

A nivel nacional, en el año 2011, la Comisión Europea solicitó a sus estados miembros un Plan de Acción sobre Resistencias Antimicrobianas (European Commission, 2011), y el Consejo de la UE, además, recomendó un año más tarde un abordaje conjunto del problema. Como respuesta, en España el Ministerio de Sanidad estableció en 2014 el Plan Nacional frente a la Resistencia a los Antibióticos (PRAN. Mapas de consumo en salud humana, 2024). Entre sus tareas principales se encuentra el monitoreo del consumo de estos fármacos, tanto en el sector comunitario como en el hospitalario. Aporta información de consumo desde el año 2015. Los datos que recoge revelan que la tendencia decreciente en el consumo de ATBs que se venía observando hasta 2019 (coincidiendo con la pandemia de COVID-19) se ha revertido, y sigue aumentando hasta la actualidad. Respecto al consumo humano, que agrupa tanto el nivel asistencial comunitario como el hospitalario, observamos que partiendo de las 28,0 DDH administradas en 2014, se consiguieron alcanzar las 19,7 DDH en 2020. Sin embargo, a partir de ese año el consumo ha continuado aumentando progresivamente, hasta volver a las 24,1 DDH en 2023.

A nivel veterinario en España, el Ministerio de Agricultura, Pesca y Alimentación ofrece el recuento de animales de granja a nivel nacional (Encuestas Ganaderas, análisis del número de animales por tipos). El ganado porcino suma un número de 33,8 millones de animales, y el bovino, ovino y caprino suman entre ellas otros 20 millones de ejemplares. A ello habría que sumar varios millones animales más en explotaciones avícolas y cunícolas. Por lo tanto, es previsible una demanda considerable de ATBs en esta industria. El consumo nacional anual se cifró en el año 2015 en 1672 toneladas, que ha conseguido reducirse hasta las 1246 toneladas en el año 2020. España sigue a la cabeza del consumo en la UE, seguida de otros países como Polonia, Italia o Alemania (European Medicines Agency, 2021).

Este mismo informe analiza los datos por sectores, reflejando que el ganado porcino, especialmente relevante en regiones como Aragón y Cataluña (Subdirección General de Producciones Ganaderas y Cinegéticas. Dirección General de Producciones y Mercados Agrarios, 2023) tiene un especial protagonismo, con un consumo de más del 60% de estos medicamentos.

d. Tipos de antibióticos más consumidos

Respecto al tipo de ATBs consumidos, el estudio de The Lancet mencionado anteriormente (Browne et al., 2021) recoge grandes variaciones en las familias más

empleadas a nivel mundial. El consumo de betalactámicos sigue siendo el más alto (aumentando particularmente en la zona asiática), el consumo de macrólidos, lincosamidas y estreptograminas y quinolonas aumentó más moderadamente, mientras que el de anfenicoles y aminoglucósidos se mantuvo estable. Los únicos grupos en los que se observa reducción en el consumo fueron las sulfonamidas y, dentro de las diaminopiridinas, la trimetoprima.

A nivel europeo, el *Annual Epidemiological Report* elaborado por el ECDC (2021) indica que los ATBs más prescritos y utilizados los últimos años son penicilinas, macrólidos, cefalosporinas y fluorquinolonas. En España, por su parte, los datos del Ministerio de Sanidad recogidos en el PRAN (PRAN. Mapas de consumo en salud humana, 2024) y el ECDC coinciden en que tanto a nivel humano comunitario y hospitalario, como a nivel veterinario, la familia de ATBs más utilizada en España sigue siendo la de los betalactámicos, especialmente amoxicilina (AMO).

En resumen, se observa que a nivel mundial el consumo de ATBs no sólo no disminuye, sino que experimenta un crecimiento considerable, que diversas publicaciones estiman que podría mantenerse (Neves e Castro et al., 2020; Tiseo et al., 2020; Browne et al., 2021; Mulchandani et al., 2023). Además, el consumo en ganadería es mayor que el humano. Solamente en algunas zonas, especialmente en países de más ingresos y con regulaciones más estrictas, se observa un consumo más estable, o con ligeras disminuciones, dependiendo del período temporal analizado.

1.1.2. Problemas ambientales asociados al uso de antibióticos. Eliminación de antibióticos en plantas depuradoras.

En consecuencia, estas cifras de consumo conllevan el riesgo de que estas sustancias terminen alcanzando el medioambiente. Los ATBs, como cualquier otro tipo de fármaco consumido por humanos y animales, sigue en sus organismos los procesos de absorción, metabolización y excreción.

Los ATBs son difícilmente absorbidos en el tracto digestivo, y la mayoría (entre el 70 y el 90%) de la dosis se excreta inalterada en orina o heces tanto en humanos como en animales (Sarmah et al., 2006), en su forma inalterada o en forma de metabolitos activos (Conde-Cid et al., 2020; de Ilurdoz et al., 2022; Massé et al., 2014). La revisión de Gao et al., (2012) en Alemania, por ejemplo, cifra en alrededor del 30% la tasa de metabolización farmacológica, infiriendo así que aproximadamente el 70% de las moléculas son excretadas a las aguas residuales en su forma molecular original. En un estudio similar, Pei et al., (2019) afirman que la tasa de ATBs que se excretan inalterados es de hasta un 85%. Una estimación en China, de hecho, cifraba en 54 toneladas de ATB excretadas en 2013, donde más del 99% se liberaba al medioambiente (Zhang et al., 2015).

Estas sustancias pasan entonces a las aguas residuales, que, al alcanzar los diferentes ecosistemas, son una causa de contaminación. A partir de la década de 1990,

la comunidad científica comenzó a dar relevancia al problema que podría presentar la diseminación de estos fármacos en la naturaleza (Sarmah et al., 2006).

La contaminación generada por las aguas residuales motivó la creación de una barrera entre estas aguas y el medioambiente: las plantas depuradoras. Son así el punto clave para monitorizar y solucionar el problema de los vertidos contaminantes al medioambiente (Michael et al., 2013; Burch et al., 2019). Sin embargo, y aunque existen técnicas especializadas en eliminar contaminantes específicos de las aguas, los procesos que implica la depuración de aguas residuales de origen urbano no están orientados al tratamiento de fármacos.

En España, como recoge el Ministerio para la Transición Ecológica y Reto Demográfico, (Vicepresidencia Tercera del Gobierno. Ministerio para la Transición Ecológica y Reto Demográfico) se conoce estas plantas como *Estación Depuradora de Aguas Residuales* (EDAR). En ellas se llevan a cabo numerosos procesos que, de forma general, se agrupan en 3 líneas principales: línea de aguas, línea de fangos, y en algunos casos, línea de gases.

En primer lugar, encontramos la línea de aguas, la parte más importante de la depuración. Se compone, a su vez, de 4 niveles: pretratamiento, tratamiento primario, tratamiento secundario y tratamiento terciario.

- Pretratamiento: los procesos que engloba consisten principalmente en la retirada de los residuos más grandes, como piedras, ramas, plásticos o arenas. También tienen una fase de retirada de grasas, y una posterior regulación de caudales y pre-aireación.

- Tratamiento primario: se ocupa del resto de sólidos en suspensión mediante decantación, seguido de tratamientos fisicoquímicos que mediante los procesos de coagulación y floculación precipitan partículas de carácter coloidal.

- Tratamiento secundario: agrupa los procesos biológicos de depuración, que tienen lugar mediante reacciones biológicas. Los microorganismos contenidos en fangos biológicos crecen y digieren la materia orgánica. Estos pueden cultivarse tanto en soportes fijos como disueltos en la matriz acuática. Es en este apartado donde más diferencias se encuentran entre distintas plantas depuradoras, ya que los tratamientos más avanzados se sitúan generalmente en las zonas más sensibles a vertidos contaminantes (agua potable, acuíferos, etc.), y no están tan extendidos. Dependiendo de la instalación se eliminará, además de la materia carbonatada, el contenido en nitrógeno o en fósforo. Por ello, es también el proceso que más puede afectar a la eventual eliminación de los fármacos contenidos en el agua residual. Una vez finalizado este proceso, se separa el agua depurada de los fangos, que pasan a ser tratados en otra línea.

- Tratamiento terciario: comprende distintos procesos de filtración, cuyo objetivo es eliminar las partículas en suspensión y distintos residuos (nutrientes) que queden presentes en el agua después de los tratamientos anteriores, mejorando la calidad del agua efluente. No es frecuente que la mayoría de las EDAR orientadas al tratamiento de aguas residuales urbanas dispongan de este último tratamiento.

En segundo lugar, encontramos la línea de fangos, resultantes de la separación tras el tratamiento primario y secundario en la línea de aguas. Se obtienen así los fangos primarios y secundarios, respectivamente.

- En primer lugar, estos lodos son espesados eliminando parte del agua que contienen mediante procesos gravitatorios y mecánicos.
- Una vez espesados, los lodos se estabilizan, destruyendo o transformando la materia orgánica que contienen. Para ello, se emplean, por ejemplo, añadidos de cal, compostajes o digestiones aerobias.
- El producto resultante continúa deshidratándose mediante procesos físicos, utilizando otros medios físicos como centrífugas, filtros o eras.
- Como último paso, el producto se seca mediante energía térmica (secadores).

Por último, algunas EDAR disponen de una tercera línea, denominada línea de gases. En ella, se recoge el gas resultante de la digestión de las aguas y fangos, que podrá utilizarse como combustible, frecuentemente para generar la energía empleada por la propia planta depuradora.

Los fármacos que llegan a las plantas se encuentran principalmente disueltos en el agua residual (o también, por ejemplo, adsorbidos en partículas fecales sólidas). Su eliminación puede darse mediante mecanismos de dilución, partición, adsorción, biodegradación o biotransformación, que tienen el objetivo de mineralizar los contaminantes o degradar la molécula original (Luo et al., 2014; Couto, et al, 2019; Tran et al., 2019).

Sin embargo, las plantas depuradoras convencionales, eficientes para eliminar materia orgánica (nitrógeno, fósforo o carbono biodegradable, por ejemplo) y partículas en suspensión (Couto et al., 2019; Luo et al., 2014), no han sido diseñadas para eliminar sustancias complejas y microcontaminantes disueltos en su matriz, como son los fármacos (Khan et al., 2020). Esta depuración es ineficiente, y en muchas ocasiones la mayoría de estas sustancias son acumuladas en los fangos o permanecen como disolución en el agua (Jelic et al., 2011), llegando de forma continua al medioambiente (aguas superficiales o subterráneas (Halling-Sørensen et al., 1998). El agua saliente de la línea de agua de estas depuradoras es posteriormente utilizada en el regadío o liberada a masas y cursos de agua, y los fangos de las EDAR son usados como abonos en la agricultura o depositados en vertederos

Son muchos los estudios y revisiones que han verificado en las últimas décadas la existencia y extensión internacional de este problema. La revisión de Michael et al., (2013), por ejemplo, que procesa más de 50 artículos de un importante número de países, concluye que la mayoría de los métodos biológicos empleados en la etapa secundaria de la depuración no son capaces de eliminar correctamente los fármacos, y los cataloga de *pseudopersistentes* en el medio, por su presencia continuada. Admiten, sin embargo, que las propiedades fisicoquímicas de las sustancias farmacéuticas, muy diferentes para cada compuesto, hacen que la capacidad de eliminarlos varíe enormemente de unos a otros. La revisión de Verlicchi et al., (2012), procesando 78 artículos de alcance internacional, llega a conclusiones similares, donde las sustancias farmacéuticas que más riesgo ambiental suponen, entre ellas los ATBs, no se eliminan en las plantas convencionales, detectándose en las aguas ya depuradas.

Batt et al., (2006), analizando las aguas entrantes y salientes de varias depuradoras en la zona de Nueva York (EE.UU), determinaron la escasa eficiencia en el eliminado de ATBs, aunque no pudo aportar información acerca de qué método conseguía mejores resultados. Otras investigaciones, también en EE.UU., han detectado numerosos fármacos en los biosólidos generados en las plantas depuradoras, que luego son utilizados en agricultura (Xia et al., 2005; Kinney et al., 2006).

La revisión de Luo et al., (2014), por su parte, procesó varios artículos de distintos países europeos y asiáticos. También comprobó la ineficiencia del proceso de depuración convencional, insistiendo en la necesidad de una mayor implementación de tratamientos más avanzados. Otros estudios revelan también que la presencia de ATBs en el agua residual puede afectar a los microorganismos encargados de la depuración (Cheng et al., 2018).

Hay otro aspecto muy destacable a considerar, y es que a veces el proceso de metabolización de los ATBs en el organismo puede dar como resultado subproductos con diferentes grados de toxicidad. Una revisión algo más reciente (Wang y Wang, 2016), alerta del riesgo añadido de que un porcentaje de estas sustancias sufran degradaciones parciales que las conviertan en otros metabolitos activos (Sarmah et al., 2006). En general, los metabolitos en los que se transforma un ATB tienden a ser menos potentes, pero en algunas ocasiones estas modificaciones pueden dotarles de tanta o más actividad que la molécula original (Boyd et al., 2005), o hacerlas más difíciles de detectar (Couto et al., 2019; Luo et al., 2014; Tran et al., 2019).

Otro problema añadido es la distribución desigual de estas plantas, que se encuentran principalmente en entornos urbanos, donde la densidad de población es mayor (ECODES, 2021). La depuración en zonas rurales, además de ser mucho más deficiente debido a razones geográficas y socioeconómicas (Liang y Yue, 2021; Li et al., 2022), tiene que hacer frente a picos estacionales debidos a movimientos de población, por ejemplo, en época estival (ECODES, 2021).

Asimismo, actividades como la agricultura o ganadería, donde el empleo de fármacos ATBs es muy considerable (ver sección 1.1.1), suponen en ocasiones un

agravamiento del problema. El sector ganadero ha seguido una tendencia progresiva de agrupación en explotaciones intensivas, con muchos animales agrupados en recintos. Esta proximidad favorece la aparición y propagación de más enfermedades, cuya prevención aumenta los requerimientos ATBs en las granjas (Doyle et al., 2006; Sarmah et al., 2006). Además, el carácter rural de esta actividad dificulta su acceso a las plantas de depuración, y sus vertidos pueden llegar a los cauces por lixiviación, escorrentía o vertido directo. Las defecaciones animales pueden ser reutilizadas como abono sin tratamiento en la agricultura (Popa et al., 2019), aumentando el número de ecosistemas expuestos a los ATBs (Massé et al., 2014).

Dentro de la ganadería, causa especial preocupación el sector porcino (ECODES, 2021; Deng et al., 2023). Además de ser la carne más consumida en el mundo (40% del volumen total) (Lahav et al., 2013), es también el principal consumidor de ATBs (Subdirección General de Producciones Ganaderas y Cinegéticas. Dirección General de Producciones y Mercados Agrarios, 2023), y su carácter intensivo y su distribución geográfica en zonas pequeñas agravan sus vertidos residuales hasta el punto de contaminar acuíferos y otros cursos de agua (Lahav et al., 2013; Cheng et al., 2020). Por otro lado, la gestión de los purines se lleva a cabo principalmente mediante su reutilización en tierras agrícolas, provocando una serie de consecuencias asociadas negativas, como contaminación y eutrofización de ecosistemas (Bayo et al., 2012).

Cabe citar también otras vías de contaminación menos estudiadas. Tal es el caso de la aún mejorable forma de reciclar los medicamentos caducados, frecuentemente eliminados como residuos comunes pese a contener más de un 90% de principio activo (de Ilurdoz et al., 2022).

1.1.3. Niveles detectados en aguas residuales, ecosistemas acuáticos y otros medios naturales

Por tanto, es un hecho comprobado que la descarga de fármacos al medioambiente, y en concreto de ATBs, se produce de forma constante en el tiempo (Figura 1). Numerosos trabajos han estudiado y evaluado la presencia de estas sustancias y el problema de su correcta eliminación en plantas depuradoras, incluyendo, además de canalizaciones urbanas y depuradoras, las distintas masas de agua (ríos, lagos, aguas costeras) donde estas plantas tienen sus puntos de descarga. ¨

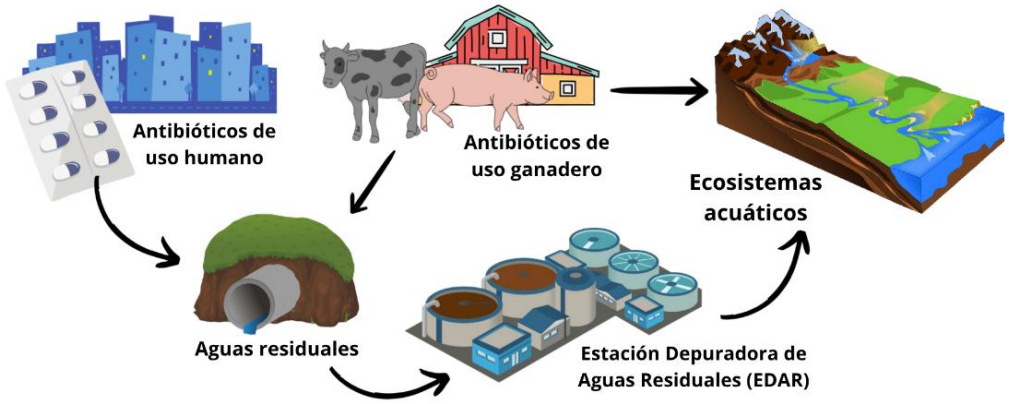

Figura 1. Rutas de llegada de los antibióticos a ecosistemas acuáticos. Fuente: elaboración propia en base a la bibliografía disponible.

a. Concentraciones detectadas en aguas residuales.

Una parte importante de la literatura disponible analiza la cantidad de ATBs en el agua residual (tabla 1). El estudio de Rodriguez-Mozaz et al., (2015), analizando las aguas residuales de hospitales en España, encontraron, entre otros, macrólidos, en concentraciones que oscilaron entre 20,1 y 241,1 ng/L. También el estudio de Watkinson et al., (2009) evaluó las aguas residuales hospitalarias en Brisbane (Australia), y su posterior destino. Las concentraciones más altas detectadas en el agua residual fueron de hasta 14500 ng/L, principalmente para los grupos de betalactámicos, quinolonas y sulfonamidas. Determinaron que, aunque las plantas depuradoras conseguían eliminar en torno al 80% de los fármacos, su presencia en las aguas salientes a la depuración llegaba hasta los 3400 ng/L (especialmente de macrólidos).

Verlicchi et al., (2012), por su parte, realizan una revisión bibliográfica de varios artículos que buscan ATBs en las aguas residuales urbanas, analizando su depuración y posterior destino, en zonas de Suiza y España. Entre sus conclusiones, destacan que los ATBs suponen un riesgo mayor que otro tipo de fármacos. En los trabajos que analizan, las concentraciones de ATBs encontradas en las aguas residuales sin tratar llegaron hasta los 3200 µg/mL detectados para quinolonas o los 10200 µg/L para macrólidos.

En canales urbanos de Vietnam, donde es posible acceder sin receta médica a fármacos ATBs, Tran et al., (2019) encontraron concentraciones de, entre otros, betalactámicos y macrólidos en concentraciones medias de 1 µg/L, y con máximos de casi 50 µg/L. Estas concentraciones, notablemente más altas, también se explican, como ya hemos mencionado, por la menor inversión de países en desarrollo en infraestructuras de depuración de aguas (Vörösmarty et al., 2010; Segura et al., 2015).

Tabla 1a. Presencia de antibióticos en aguas residuales

Antibiótico detectado	*Ámbito*	*Lugar*	*Rango de valores detectados*	*Referencia bibliográfica*
Ampicilina	Aguas residuales hospitalarias	China	140 – 670 ng/mL	Yao et al., 2021
Amoxicilina		Italia	470 – 543 ng/L	Palli et al., 2019
		China	20 –1430 µg/mL	Yao et al., 2021
		Australia	90 ng/L*	Watkinson et al., 2009
	Aguas residuales urbanas	Irán	350 – 1020 ng/L	Golchin et al., 2021
Eritromicina		Europa, América, Asia y Oceanía	10,2 µg/mL	Verlicchi et al., 2012
	Aguas residuales hospitalarias	Turquía	0,01 –101 ng/L	Aydin et al., 2019
		China	404,28 – 599 ng/L	Wang et al., 2018
		Australia	det*	Watkinson et al., 2009
	Aguas residuales urbanas	Canadá	14 – 600 ng/L	Guerra et al., 2014
Tetraciclina	Aguas residuales hospitalarias	China	1147,83 – 1598,26 ng/L	Wang et al., 2018
		Australia	N.D – 40 ng/L	Watkinson et al., 2009

rango no proporcionado (valor único). ***det: detectado, no cuantificado **N.D**: no detectado Fuente: elaboración propia.*

Tabla 1b. Presencia de antibióticos en aguas residuales (continuación)

Antibiótico detectado	*Ámbito*	*Lugar*	*Rango de valores detectados*	*Referencia bibliográfica*
Penicilina	Aguas residuales urbanas	Irán	40 ng/L	Mirzaei et al., 2019
			20 – 310 ng/L	Golchin et al., 2021
		Australia	N.D	Watkinson et al., 2009

rango no proporcionado (valor único). ***det: detectado, no cuantificado **N.D**: no detectado Fuente: elaboración propia.*

b. Niveles detectados de antibióticos estudiados en esta tesis en aguas salientes (efluentes) de Estaciones Depuradoras de Aguas Residuales (EDAR).

La bibliografía disponible nos permite comprender cuántos de estos ATBs son efectivamente retirados en el proceso de depuración. En función de las propiedades fisicoquímicas y de biodisponibilidad de las distintas moléculas, se pueden seguir diferentes rutas: pueden, por ejemplo, ser eliminados en el tratamiento secundario y pasar a formar parte de los fangos de la depuradora o bien permanecer en suspensión en el flujo de agua y salir en su forma original o como subproducto en el vertido a distintas masas de agua.

En la tabla 2 se recogen concentraciones detectadas en las aguas salientes de las EDAR de diferentes países. Como vemos, el trabajo de Rodriguez-Mozaz et al., (2020), destaca a España, junto con Portugal e Irlanda, como uno los países de la UE con niveles más altos, entre otros, de fármacos betalactámicos o tetraciclinas (TCs). Según Verlicchi et al., (2012), citado anteriormente, las tasas de eliminación, pese a variar significativamente, reflejaban una ineficiencia constante de las plantas depuradoras para eliminar los ATBs, siendo la mayoría de la tasas de eliminación menores a un 80%, llegando en algunos casos a un 4%, e incluso a valores negativos. Entre las moléculas que estimaron que suponían un mayor riesgo, citan eritromicina (ERY) (macrólido), (AMO) (betalactámico) o TC.

También la revisión de Luo et al., (2014) incluye trabajos que analizan las concentraciones de fármacos antes y después de entrar a plantas depuradoras. En concreto, varios artículos analizan las concentraciones de macrólidos y otras familias en zonas de China, España, Reino Unido y el oeste de la región balcánica. Las concentraciones en el agua residual oscilaron en los diversos estudios entre 0,14 y 10 µg/L,

y en el agua saliente de la depuradora, entre 0,02 µg/L y 2,84 µg/L. Las tasas de eliminación observadas presentaron amplias variaciones, de entre 0 y 84,5%.

Palli et al., (2019) llegaron a conclusiones similares. Tras encontrar concentraciones de hasta 2 µg/L de betalactámicos en aguas que llegaban a depuradoras en la Toscana (Italia), determinaron, analizando 3 plantas distintas, que la tasa de eliminación de algunos ATBs oscilaba en torno a un 30%. Además, este estudio apunta a un factor poco investigado: la presencia de fármacos en el agua saliente de las depuradoras variaba muy significativamente durante el año. Entre otras razones, apuntan a la variabilidad interanual en el consumo de algunos fármacos (prevaleciendo los ATBs en invierno) y la influencia de la temperatura en las bacterias responsables de biodegradar estos compuestos.

Como se cita en algunas investigaciones mencionadas (Palli et al., 2019; Verlicchi et al., 2012), las concentraciones de algunos ATBs encontradas en las aguas salientes de las plantas depuradoras pueden ser, en algunos casos, mayores que las halladas a su llegada. Hay varios factores que pueden explicar esta situación, a priori contradictoria. En primer lugar, un metabolito, de la misma forma que se degradó, puede sufrir reacciones químicas con otras sustancias disueltas en el medio que lo transformen de nuevo en la molécula original (Couto et al., 2019; Tran et al., 2019). Aunque pertenece a otra familia de fármacos (anticonvulsivos), es ilustrativo el ejemplo que ofrecen Zonja et al., (2016), analizando el metabolito inactivo lamotrigina-N2-glucurónido, generado por conjugación en el hígado humano al metabolizar el fármaco anticonvulsivo lamotrigina. Se ha demostrado que esta molécula puede retornar a su forma original (y activa) en los procesos biológicos que tienen lugar en plantas depuradoras. En segundo lugar, algunos estudios han revelado que algunos fármacos excretados mediante orina y heces pueden llegar a las aguas residuales adsorbidos en las partículas sólidas fecales, siendo liberados en disolución durante el proceso de depuración (Tran et al., 2018). Por lo tanto, si sólo se considera la fracción disuelta, se pueden estar arrojando datos erróneamente bajos, y existe la posibilidad de subestimar la cantidad de fármacos que llegan a las plantas depuradoras (Göbel et al., 2007).

En la literatura disponible aparece una gran variabilidad en las concentraciones y en las tasas de eficiencia de las plantas depuradoras, tanto entre distintas sustancias como entre diferentes EDAR. Entre los factores que pueden explicar esta variabilidad se encuentran las características fisicoquímicas de los ATBs (solubilidad, presión de vapor, lipofilia), las condiciones del medio (pH, temperatura, radiación UV, condiciones redox, etc.) y la carga bacteriana en el medio (Aydin et al., 2019; Gusmaroli et al., 2020).

Además, otros factores pueden influir en este proceso. Se ha demostrado, por ejemplo, la correlación existente entre concentraciones detectadas de ATBs y la situación socioeconómica del país, ya que en lugares con menos recursos la inversión en el tratamiento de aguas residuales es menor, y quedarán más expuestos a la contaminación del medioambiente (Vörösmarty et al., 2010; Segura et al., 2015).

Tabla 2. Presencia de los antibióticos estudiados en esta tesis en las aguas efluentes de plantas depuradoras (EDAR)

Antibióticos detectados	*País*	*Rango de valores detectados*	*Referencia bibliográfica*
Ampicilina	España	N.D. – 99,4 ng/L	Rodriguez-Mozaz et al., 2020
Amoxicilina	Italia	436 – 7692 ng/L	Palli et al., 2019
	España	<LOQ – 245 ng/L	PRAN Medioambiente Fase 2, 2024
	Australia	N.D. – 50 ng/L	Watkinson et al., 2009
Penicilina	Irán	40 ng/L	Mirzaei et al., 2019
	Australia	N.D. – 300 ng/L	Watkinson et al., 2009
		det*	
Eritromicina	España	<LOQ – 1788 ng/L	PRAN Medioambiente Fase 2, 2024
	N.E	6.3 ng/L	Verlicchi et al., 2012
Tetraciclina	España	14,5 – 231,2 ng/L	Rodriguez-Mozaz et al., 2020
	Australia	N.D. – 20 ng/L	Watkinson et al., 2009
Cloranfenicol	España	0,6 ng/L (LOQ*)	Jelic et al., 2011

LOQ: límite de cuantificación. Det*: detectado, no cuantificado* ***N.D:*** *no detectado. NE*: no especificado. Fuente: elaboración propia.*

Además de estas aguas efluentes, vertidas a masas de agua, los ATB también tienen otra vía de salida de las depuradoras, como son los fangos residuales. Es en estos fangos donde, como se ha descrito anteriormente, se acumulan numerosos ATBs durante el proceso de depuración (Li et al., 2013; Yang et al., 2021).

c. Niveles detectados en ríos y otras masas de agua

Una vez analizada el agua saliente de las EDAR, es previsible que, tras su descarga a distintas masas de agua (ríos, lagos, aguas costeras, etc.), se encuentren en ellas concentraciones de ATBs. Distintos estudios nos permiten comprobar que esta contaminación se produce de manera continuada y extendida (Tabla 3).

A nivel europeo, destaca la revisión de Carvalho y Santos, (2016) analizando la presencia de ATBs en la mayoría de los 27 países miembros. España destaca positivamente como el país con más estudios realizados al respecto. Los datos variaban en función de la clase de ATBs y el ecosistema analizado.

Entre los artículos que incluye esta revisión destacan las concentraciones de ATBs macrólidos en ríos franceses y españoles (entre 1 y 17 µg/L) (Feitosa-Felizzola y Chiron, 2009; Valcárcel et al., 2011), y su presencia en aguas subterráneas y agua potable (en concentraciones máximas de 154 y 21 ng/L, respectivamente) (Cabeza et al., 2012; López-Serna et al., 2012). Antimicrobianos del grupo de las quinolonas también se encontraron en España en el río Llobregat (2 µg/L) (López-Roldán et al., 2013), e incluso en el agua potable municipal de Barcelona (entre 13,2 y 32,9 ng/L) (López-Serna et al., 2012). También la detección de ATBs se extiende a los ecosistemas marinos europeos, cuantificándose, por ejemplo, concentraciones de betalactámicos en un rango de 5,0 – 127,8 ng/L en bahías en Grecia (Alygizakis et al., 2016) o hasta o 168 ng/L de macrólidos en aguas del Mar Menor (Murcia) (Moreno-González et al., 2015).

Diversos estudios confirman la existencia de este problema en la cuenca del río Ebro (Gros et al., 2007; Silva et al., 2011), donde la presencia de ATBs se ve agravada en las proximidades de las ciudades más grandes de la cuenca, como Zaragoza (hasta 610,7 ng/L) (García-Galán et al., 2011). También en la cuenca mediterránea, Rodriguez-Mozaz et al., (2020) encuentra concentraciones de ATBs en el río Ter hasta los 165,6 ng/L.

La contaminación por ATBs alcanza también las aguas subterráneas, como los 3,4 µg/L detectados en el sistema geotermal de La Selva (Girona) (García-Galán et al., 2010) e incluso el agua potable española (en concentraciones de varias decenas de ng/L) (Cabeza et al., 2012; M. Jesús García-Galán et al., 2010; Gros et al., 2012). En los principales ríos de Madrid se encontraron nitroimidazoles en concentraciones de entre 0,182 y 1,834 µg/L (Valcárcel et al., 2011). Fármacos del grupo de los anfenicoles fueron detectados en ríos británicos y españoles, en concentraciones de hasta 40 y 1,31 ng/L, respectivamente (Kasprzyk-Hordern et al., 2009, 2008; Osorio et al., 2012). Además, autores como Silva et al., (2011) determinaron, estudiando el río Ebro, que los ATBs de esta familia, como el cloranfenicol (CHL), tienen mucha facilidad para unirse a restos sólidos, sesgando posiblemente a la baja los datos de concentraciones en disolución.

Hirsch et al., (1999) encontraron concentraciones de macrólidos de hasta 1,70 µg/L en aguas superficiales de ríos en Alemania. También moléculas de esta familia fueron detectadas en aguas superficiales de Reino Unido, en concentraciones que llegaron a 21,10 µg/L (Boxall, 2010). Ya fuera de Europa, Watkinson et al., (2009), encontraron

concentraciones de ATBs de hasta los 2 µg/L en aguas superficiales recogidas de diferentes ríos, estuarios y ecosistemas marinos en la zona de Brisbane (Australia).

Asimismo, Tang et al., (2021), evaluando las concentraciones en el río Yangtzé (China), encontraron TCs y betalactámicos en más del 99% de las muestras, tanto en entornos urbanos como peri-urbanos). Las mediciones llegaban hasta 0,128 y 2,228 µg/L, respectivamente. También en ese mismo río, Wang y Wang, (2016) encontraron moléculas del grupo de las TCs en concentraciones medias de 0,011 y 0,056 µg/L, respectivamente. Ambos estudios advirtieron, además, variaciones de más del 200% entre la estación seca y la estación húmeda, apuntando a una mayor dilución y movimiento de agua en esa época. El estudio de Lyu et al., (2020) en varias provincias de China detectó ATBs en el 82% de muestras de ecosistemas acuáticos analizadas, siendo más alta, como es previsible, cerca de los núcleos urbanos más grandes. El estudio detectó en aguas superficiales TCs, sulfonamidas, quinolonas y macrólidos en concentraciones medias de 0,482 µg/L, 0,466 µg/L, 0,184 µg/L y 0,102 µg/L, respectivamente. En aguas costeras también se detectaron quinolonas y sulfonamidas en concentraciones medias de 0,331 y 0,0715 µg/L.

En ríos subtropicales en Brasil, por ejemplo, Böger et al., (2021) encontraron concentraciones de ATBs que variaron entre 0,13 y 4,63 µg/L, siendo la concentración más alta para AMO. Salvo algunas excepciones, la presencia de betalactámicos no es común, ya que se degradan fácilmente en el medio (Kovalakova et al., 2020).

Tabla 3a. Presencia de los antibióticos estudiados en esta tesis en distintas aguas medioambientales

Antibióticos detectados	*Ámbito*	*Lugar*	*Rango de valores detectados*	*Referencia bibliográfica*
Penicilina	Aguas fluviales	Australia	N.D. – 250 ng/L	Watkinson et al., 2009
Amoxicilina		Australia	N.D. – 200 ng/L	
		Reino Unido	<10 – 622 ng/L	Kasprzyk-Hordern et al., 2008
	Aguas marítimas costeras	Grecia	127,8 ng/L	Alygizakis et al., 2016
	Aguas fluviales	Brasil	180 – 4630 ng/mL	Böger et al., 2021
Ampicilina		China	16,9 – 68,7 ng/L	Tang et al., 2021

concentración máxima detectada. ***det: detectado, no cuantificado. **N.D**: no detectado. Fuente: elaboración propia*

Tabla 3b. Presencia de los antibióticos estudiados en esta tesis en distintas aguas medioambientales (continuación)

Antibióticos detectados	*Ámbito*	*Lugar*	*Rango de valores detectados*	*Referencia bibliográfica*
Eritromicina	Aguas subterráneas (delta fluvial)	España	154,33 ng/L	Cabeza et al., 2012
	Aguas fluviales	España	0 – 362,5 ng/L	Osorio et al., 2012
		España	N.D – 71 ng/L	Gros et al., 2007
		España	N.D. – 42,4 ng/L	Silva et al., 2011
	Laguna litoral	España	107,6 ng/L	Moreno-González et al., 2015
	Aguas fluviales	España	<1 – 603 ng/L	Valcárcel et al., 2011
	Aguas marítimas costeras	Grecia	6,7 ng/L*	Alygizakis et al., 2016
	Aguas fluviales	Reino Unido	0,5 – 351 ng/L	Kasprzyk-Hordern et al., 2008
		China	65,8 ng/L	Lyu et al., 2020
Tetraciclina		Australia	N.D. – 80 ng/mL	Watkinson et al., 2009
		España	<23 ng/L	Valcárcel et al., 2011
		China	30,5 – 128 ng/L	Tang et al., 2021
		China	92,2 ng/L	Lyu et al., 2020

concentración máxima detectada. ***det: detectado, no cuantificado. **N.D**: no detectado. Fuente: elaboración propia*

Tabla 3c. Presencia de los antibióticos estudiados en esta tesis en distintas aguas medioambientales (continuación)

Antibióticos detectados	*Ámbito*	*Lugar*	*Rango de valores detectados*	*Referencia bibliográfica*
Cloranfenicol	Aguas fluviales	España	N.D. – 11 ng/L	López-Serna et al., 2012
		Reino Unido	2 – 40 ng/L	Kasprzyk-Hordern et al., 2008

concentración máxima detectada. ***det: detectado, no cuantificado. **N.D**: no detectado. Fuente: elaboración propia*

d. Contaminación en suelos

Una de las rutas alternativas es la llegada de los ATBs a aguas superficiales y subterráneas de los purines y estiércoles acumulados en las granjas, que contienen estos fármacos (Conde-Cid et al., 2018; Hou et al., 2015; Kay et al., 2005; Martínez-Carballo et al., 2007). Además, el uso de estos residuos biológicos (y también con los fangos procedentes de depuradoras (Barreiro et al., 2022) como abono en la agricultura contamina los suelos agrarios (Chen et al., 2014; Gao et al., 2015; Kairigo et al., 2020; Lyu et al., 2020; Lyu et al., 2020; Sarmah et al., 2006; Wei et al., 2019). También los ATBs utilizados en la acuicultura alcanzan el medioambiente (Doyle et al., 2006).

Además, la contaminación de los suelos con ATBs puede tener una consecuencia previsible: aquellos vegetales cultivados en ellos absorben parte de esta contaminación, como comprobaron Conde-Cid et al., (2018). Un estudio más reciente de este mismo equipo demostró en Galicia en 2018 la presencia de ATBs tanto en un 17% de muestras de suelo agrícola como en un 44% de muestras de cultivos (Conde-Cid et al., 2020). También Azanu et al., (2018) encontraron concentraciones significativas de ATBs en tejidos de lechugas en Ghana.

Sin embargo, la bibliografía que recopila los rangos a los que se detectan estos ATBs en distintos suelos es todavía escasa y varía entre ng/kg hasta mg/kg. En la revisión que proporcionan Cycoń et al., (2019), por ejemplo, se documenta que la concentración máxima detectada en suelos agrícolas para ERY fue de 7.2 µg/kg, y TC, 2,683 µg/kg, y CHL, en rangos de entre 315 y 554 µg/kg en suelos fertilizados con lodos.

El suelo agrícola se define como un recurso no renovable, por lo que la acumulación de ATBs en este medio puede causar alteraciones importantes a nivel fisicoquímico y microbiológico (Xie et al., 2022).

1.1.4. Ecotoxicidad asociada a antibióticos en organismos no diana.

Como hemos visto, existe una extensa bibliografía documentando la presencia de ATBs en ecosistemas acuáticos y terrestres.

Estos fármacos, diseñados para ejercer acciones biológicas, presentan un riesgo para los organismos en los diferentes ecosistemas que pueden alcanzar (Kemper, 2008). Pueden matar, inhibir o afectar de distintas maneras a organismos no diana, tanto unicelulares como pluricelulares, que habiten en esos hábitats (Jjemba, 2006; Yoshimura et al., 2005), alterando el equilibrio de los ecosistemas (Conde-Cid et al., 2020).

Para intentar evaluar este riesgo, nace ya a finales del siglo XX el concepto *biomonitoring* (biomonitorización). Este consiste, en síntesis, en la observación del impacto que ejercen factores externos sobre ecosistemas y su desarrollo en el tiempo (Markert, 1999). Para ello, se utilizan ensayos estandarizados de toxicidad, tanto aguda como crónica, sobre organismos de diversos niveles tróficos, como bacterias, algas, invertebrados, plantas o peces (Carvalho y Santos, 2016). Estos organismos, denominados bioindicadores, aportarán información sobre la calidad del medioambiente y, entre otras, reúnen una serie de características (Markert , 2002):

- Solidez taxonómica (especies reconocibles incluso por no especialistas)
- Distribución amplia en el medioambiente, siendo muchos de ellos especies claves de los ecosistemas estudiados
- Escasas particularidades locales dentro de la especie
- Abundancia numérica de los organismos
- Idoneidad para experimentos en laboratorio (por ejemplo, facilidad de cuantificación y estandarización)
- Características ecológicas conocidas
- Sensibilidad a agentes estresantes

Estos organismos bioindicadores pueden ser clasificados en función del medio en el que se encuentran, distinguiendo así, por ejemplo, organismos indicadores de ecosistemas acuáticos o de suelos.

a. Ecotoxicidad en organismos acuáticos

Entre los organismos bioindicadores utilizados para evaluar la sensibilidad de ecosistemas acuáticos a contaminantes, encontramos crustáceos planctónicos (*Daphnia magna*), bacterias bioluminiscentes marinas (*Aliivibrio fischeri*), distintas clases de cianobacterias (*Microcystis aeruginosa*, *Anabaena flos-aquae),* o algas verdes (*Pseudokirchnella subcapitata*).

Un número considerable de publicaciones han evaluado la toxicidad provocada por los ATBs utilizados en esta tesis a estos bioindicadores, algunos de ellos recogidos en la tabla 4.

Tabla 4a. Ecotoxicidad en bioindicadores acuáticos frente a los antibióticos estudiados en esta tesis

Bioindicador	*Antibiótico*	*Valor detectado*	*Referencia bibliográfica*	*Protocolo*
Daphnia magna	Amoxicilina	CE_{50}: 2,391 mg/L (48h)	Yisa et al., 2023	OECD 202
	Estreptomicina	CE_{50}: 408 mg/L (48h)	Wollenberger et al., 2000	ISO 1989b
	Gentamicina	CE_{50}: 875,5 mg/L (24h)	Lomba et al., 2020	OECD 202
	Tetraciclina	NOEC 340 mg/L (48h)	Wollenberger et al., 2000	ISO 1989b
		CE_{50}: 8,16 mg/L (48h)	Havelkova et al., 2016	OECD 202
	Penicilina G	CE_{50} 1496,9 mg/L (48h)		
	Eritromicina	CE_{50}: 22,45 mg/L (24h)	Isidori et al., 2005	ISO 6341
		NOEC: 0,001 mg/L (6d)	Flaherty y Dodson, 2005	N.E
	Eritromicina	NOEC 100 mg/L (30 min)	Isidori et al., 2005	Microtox® Manual 2232
	Ampicilina	CE_{50} : 2627 mg/L (15 min)	Park y Choi, 2008	N.E
	Amoxicilina	CE_{50}: 3597 mg/L (15 min)		
	Tetraciclina	CE_{50}: 6,70 mg/L (30 min)	Havelkova et al., 2016	ISO 11348
	Gentamicina	CE_{50}: > 10000 mg/L (30 min)	Lomba et al., 2020	ISO 11348
Aliivibrio fischeri	Ampicilina	CE_{50}: 163 mg/L (24 h)	Backhaus y Grimme, 1999	N.E.
		CE_{50} : 163,00 mg/L (30 min)	Ioele et al., 2015	Microtox® Manual 2232
	Amoxicilina	CE_{50} : 150,02 mg/L (30 min)		
	Cloranfenicol	CE_{50} : 10,03 mg/L (30 min)		
	Estreptomicina	CE_{50} : 8,21 mg/L (30 min)		
	Eritromicina	NOEC: 82,03 mg/L (30 min). Efecto hormético.		
		CE_{50}: 560 (430 – 690) mg/L (15 min)	Christensen et al., 2006	ISO 11348-3
		CE_{50}: 136,88 mg/L (15 min)	Ukić et al., 2019	ISO 11348-3:2007
		CE_{50} >100 mg/L (30 min)	Isidori et al., 2005	Microtox® Manual 2232
	Gentamicina	CE_{50} > 10,000 mg/L	Lomba et al., 2020	ISO 11348-3
	Tetraciclina	CE_{50}: 0,0251 mg/L	Backhaus y Grimme, 1999	N.E.
		CE_{50}: 104,9 (97,73 – 113,0) mg/L (15 min)	Tong et al., 2015	ISO 11348-1

***CE_{50}**: Concentración Efectiva, que produce un 50% de los efectos observados. **NOEC**: Concentración a la que no se observa ningún efecto. **N.E**: no especificado. Además de los valores (CE_{50}, generalmente) se indica entre paréntesis la duración del test de ecotoxicidad, en días (d) u horas(h). Fuente: elaboración propia*

Tabla 4b. Ecotoxicidad en bioindicadores acuáticos frente a los antibióticos estudiados en esta tesis (continuación)

Bioindicador	*Antibiótico*	*Valor detectado*	*Referencia bibliográfica*	*Protocolo*
Microcystis aeruginosa	Amoxicilina	CE_{50}: 0,004 mg/L (7d)	Holten Lützhøft et al., 1999	ISO 8692
	Penicilina G	CE_{50}: 0,006 mg/L (7d)	Halling-Sørensen, 2000	ISO 8692
	Estreptomicina	CE_{50}: 0,007 mg/L (7d)		
		CE_{50}: 0,034 mg/L (24h)	van der Grinten et al., 2010	N.E
	Tetraciclina	CE_{50}: 0,09 mg/L (7d)	Halling-Sørensen, 2000	ISO 8692
Anabaena flos-aquae	Eritromicina	CE_{50}: 0,022 mg/L (72h)	González-Pleiter et al., 2013	USEPA 2002
	Tetraciclina	CE_{50}: 6,2 mg/L (72h)		
	Amoxicilina	CE_{50}: 56,3 mg/L (72h)		
	Eritromicina	CE_{50}: 5,62 mg/L (7d)	Pomati et al., 2004	U.S Environmental Protection Agency (1996)
	Tetraciclina	CE_{50}: 1,06 mg/L (7d)		
Pseudokirchnella subcapitata	Penicilina G	CE_{50}: 7114,3 mg/L (72h)	Havelkova et al., 2016	ISO 8692
	Tetraciclina	CE_{50}: 3,3 mg/L (72h)	González-Pleiter et al., 2013	OECD 201
	Amoxicilina	CE_{50}: >1500 mg/L (72h)		
	Eritromicina	CE_{50}: 0,35 mg/L (72h)		
		CE_{50}: 0,02 mg/L (3d)	Robinson et al., 2005	N.E
	Estreptomicina	CE_{50}: 1,5 mg/L (24h)	van der Grinten et al., 2010	N.E

***CE_{50}**: Concentración Efectiva, que produce un 50% de los efectos observados. **NOEC**: Concentración a la que no se observa ningún efecto. **N.E**: no especificado. Además de los valores (CE_{50}, generalmente) se indica entre paréntesis la duración del test de ecotoxicidad, en días (d) u horas(h). Fuente: elaboración propia*

La toxicidad encontrada en la literatura para *D. magna* de estos ATBs oscila entre los 0,001 mg/L de ERY (Flaherty y Dodson, 2005) y los 3594 mg/L (Park y Choi, 2008) para AMO, aunque con ensayos de 15 min y 6 días, respectivamente. Para *A. fischeri*, por su parte, los valores son similares, oscilando entre los 8,21 mg/L para estreptomicina (STM) en el estudio de Ioele et al., (2015) y los 560 mg/L para ERY (Christensen et al., 2006). En ambos casos, los valores varían notablemente, tanto entre ATBs como entre diferentes estudios de una misma molécula.

Las cianobacterias *M. aeruginosa y A. flos-aquae* parecen ser notablemente más sensibles a estos fármacos. Mientras que los valores de CE_{50} para *A. flos-aquae* oscilan entre 0,022 mg/L para ERY y los 56,3 mg/L para AMO (González-Pleiter et al., 2013), para *M. aeruginosa* el valor más alto fue de 0,09 mg/L para TC (Halling-Sørensen, 2000). En el caso del alga *P. subcapitata* los valores presentan la mayor variación, ya que el valor de CE_{50} más alto encontrado en la literatura es de 7114,3 mg/L para PEN (Havelkova et al., 2016), y el más bajo, 0,02 mg/L para ERY (Robinson et al., 2005).

En los anteriores estudios se observa cómo los valores en que los ATBs ejercen toxicidad sobre estos organismos son, en muchas ocasiones, inferiores a 0,1 mg/L. Este orden de magnitud equivale a 100 ng/L, y retrocediendo de nuevo a los apartados anteriores (1.1.3), vemos es superado de forma frecuente en las concentraciones de ATBs encontradas en aguas residuales y medioambiente. Por tanto, cabe esperar que estos fármacos estén causando efectos tóxicos a los distintos organismos no diana de aquellos ecosistemas que alcancen, con un efecto impredecible debido a la presencia simultánea de distintos ATBs unido a la presencia de otras sustancias y a los efectos de todas las posibles interacciones que se puedan producir entre ellos.

b. Ecotoxicidad en organismos edáficos

A diferencia de los ecosistemas acuáticos, donde los contaminantes se encuentran en disolución, los suelos y medios terrestres (edáficos) presentan una mayor complejidad, pudiendo mitigar los efectos de la contaminación antibiótica actuando como un medio tampón, pero pudiendo acumularlos más en zonas concretas debido a la menor movilidad del medio (Dong et al., 2012). A ello se añade que la bibliografía disponible en organismos edáficos, propios de estos ecosistemas, es más escasa y dispar que en el caso de los ecosistemas acuáticos (Pino-Otín et al., 2015, 2024).

Uno de los principales organismos bioindicadores utilizados para evaluar la calidad de suelos es la lombriz de tierra común, *Eisenia fetida* (Organisation for Economic Co-operation and Development (OECD), 1984; Cortez y Bouche, 1992). Este invertebrado cumple un rol clave en la mejora del suelo mediante la digestión de materia orgánica, transformándola en *vermicompost* rico en nutrientes y microorganismos beneficiosos. Sus excreciones actúan como fertilizante natural, mejorando la disponibilidad de nitrógeno, fósforo y otros elementos esenciales. Además, al excavar, airea y estructura el suelo, favoreciendo la oxigenación y la retención de agua. Esto estimula el crecimiento vegetal y reduce la necesidad de insumos químicos, promoviendo una agricultura más sostenible. Esta gran vinculación con el medio, y otras características (como su epidermis fina y permeable) hacen que sea sensible a la contaminación de los suelos (Fourie et al., 2007; Vasseur y Bonnard, 2014), siendo afectado por la presencia, por ejemplo, de biocidas usados en la agricultura o actividades industriales (Calisi et al., 2011). En resumen, estas características lo hacen idóneo como bioindicador de los ecosistemas de suelo (Paoletti, 1999).

Tabla 5. Ecotoxicidad de antibióticos estudiados en esta tesis en bioindicadores de suelo

Animales

Antibiótico	*Bioindicador*	*Valor detectado*	*Referencia bibliográfica*	*Protocolo*
Penicilina G	*Eisenia fetida*	CE_{50}: 348 mg/kg (56d)	Havelkova et al., 2016	ISO 11268
Tetraciclina		CE_{50}: 2735 mg/kg (56d)		
		NOEC: 2000 mg/kg (14d)	Pino-Otín et al., 2015	OECD 207
		NOEC: 1000 mg/kg (14d)	Rutkoski et al., 2024	OECD 207

Plantas

Antibiótico	*Bioindicador*	*Valor detectado*	*Referencia bibliográfica*	*Protocolo*
Tetraciclina	*Daucus carota*	CE_{50} > 1000 µg/L (28d)	Hillis et al., 2008	N.E
		CE_{50}: 10,3 mg/L	Pan y Chu, 2016	N.E
	Lactuca sativa	CE_{50}: 14,4 mg/L		
	Lycopersicon esculentum	CE_{50}: 11,6 mg/L		
	Cucumis sativus	CE_{50}: 34,8 mg/L		
		CE_{50}: 69 mg/L	Liu et al., 2009	OECD, 1984.
	Oryza sativa	CE_{50}: 57 mg/L		
	Cichaorium endivia	CE_{50}: 203 mg/L		
Eritromicina	*Lactuca sativa*	CE_{50}: 69 mg/L	Pan y Chu, 2016	N.E
	Daucus carota	CE_{50} > 300 mg/L		
	Cucumis sativus	CE_{50} > 300 mg/L		
	Lycopersicon esculentum	CE_{50} > 300 mg/L		
Cloranfenicol	*Lactuca sativa*	CE_{50}: 204 mg/L		
	Daucus carota	CE_{50} > 300 mg/L		
	Cucumis sativus	CE_{50} > 300 mg/L		
	Lycopersicon esculentum	CE_{50} > 300 mg/L		

***CE_{50}**: Concentración Efectiva que produce un 50% de los efectos observados. **NOEC**: Concentración a la que no se observa ningún efecto. **IC:** Concentración inhibitoria del crecimiento radicular. **N.E**: no especificado. Además de los valores (CE_{50}, generalmente) se indica entre paréntesis, si se proporciona, la duración del test de ecotoxicidad, en **días (d**) u **horas (h).** Fuente: elaboración propia.*

Como se aprecia en los valores recogidos, en general las concentraciones ensayadas de ATBs no causaron efectos ecotóxicos observables en estas lombrices, o los causaron a concentraciones muy altas (Havelkova et al., 2016). Sin embargo, otros autores indican que, aunque no se refleje en la supervivencia de los organismos, sí puede provocar

cambios poblacionales (Zhao et al., 2022), afectando otros parámetros, como su sistema inmune, viabilidad celular, microbiota intestinal o mucopolisacáridos en la epidermis (Rutkoski et al., 2024).

En el caso de las plantas, las concentraciones a las que se ha encontrado toxicidad son, como vemos en la tabla 5, valores bastante altos. El estudio de Wei et al., (2024), por ejemplo, demostró que los ATBs pueden tener efectos tóxicos en diversos procesos fisiológicos de algunas plantas, pero a mayores concentraciones que las encontradas en el medioambiente, comprobando así la compleja relación entre los vegetales y ATBs. En este sentido, Migliore et al., (2003) demostraron que el enrofloxacino provocó el efecto de hormesis (es decir, estimulación a bajas dosis, pero inhibición a concentraciones más altas) en diversos cultivos como lechuga (*Lactuca sativa*), pepino (*Cucumis sativus*), judías (*Phaseolus vulgaris*) y rábanos (*Raphanus sativus*).

Debido a la aplicación de abonos de forma repetida en un mismo suelo, aunque las concentraciones de ATB no sean muy altas, es necesario conocer los efectos que puede tener la presencia de ATBs a muy largo plazo (Kuppusamy et al., 2018).

c. Ecotoxicidad en microorganismos

Como vemos, hay un creciente interés por comprender la ecotoxicidad en organismos no diana, aunque sigue siendo necesaria una mayor profundización. La gran mayoría de los estudios analizan la ecotoxicidad aguda con bioindicadores individuales, y el uso de esta metodología para la evaluación de riesgos medioambientales ha sido cuestionada por su excesiva simplicidad (Ferrari et al., 2004). Pocos estudios examinan los efectos que la exposición a largo plazo a estos fármacos puede tener sobre comunidades complejas (Minguez et al., 2016; Bielen et al., 2017; Kovalakova et al., 2020), más semejantes al medioambiente real y más adecuadas, por tanto, para un mejor conocimiento del problema (Kergoat et al., 2021).

Entre estas comunidades, destacan aquellas formadas por los microorganismos presentes en un determinado ecosistema. Al formar la base de la cadena trófica y del proceso de descomposición de materia orgánica en todos los ecosistemas (como suelos o masas de agua) (Perujo et al., 2020), estos organismos son un eslabón fundamental en los intercambios de energía y degradación de los contaminantes (Schoffelen et al., 2019; Zhenglu Wang et al., 2020). Cualquier impacto en estas comunidades, por tanto, puede provocar repercusiones más extensas en sus ecosistemas (Christensen et al., 2006; M. Zhang et al., 2022). Estas características los convierten en un bioindicador adecuado para estudiar el impacto de contaminantes, como los ATBs, en entornos fluviales y obtener una representación más real del impacto de los contaminantes (Clements y Rohr, 2009; Geiszinger et al., 2009; Liu et al., 2021a).

Existen diferentes técnicas para evaluar estos bioindicadores. Una de ellas es la identificación de las diferentes especies microbianas presentes en una muestra de cualquier ecosistema. Esta identificación se realiza mediante secuenciación genética (16S

ARNr) un gen ribosomal con regiones variables específicas para cada grupo bacteriano, lo que permite conocer la diversidad bacteriana, incluyendo la abundancia de cada grupo, lo que nos da una idea de la diversidad. Su empleo, por ejemplo, permite conocer la estructura microbiana en dos puntos de un mismo ecosistema, permitiendo conocer el impacto de diferentes focos de contaminación y evaluar cambios en la composición taxonómica de la microbiota (Mackenzie et al., 2011).

Otra de las técnicas más utilizadas es el *Community Level Physiologycal Profiling* (CLPP), o Perfil Fisiológico a Nivel de Comunidad, que permite conocer la diversidad funcional o la actividad metabólica de dichas comunidades microbianas. El término fue originalmente acuñado para describir la caracterización y clasificación de comunidades microbianas heterótrofas en función de sus patrones de utilización de una única fuente de carbono (CSUPs) (Lehman et al., 1995). Aunque en principio hacía referencia a diferentes tipos de estudios y ensayos, actualmente se utiliza casi exclusivamente como indicador ecológico en referencia los experimentos con placas de BIOLOG Ecoplate™ (Goberna et al., 2005; Perujo et al., 2020; Weber y Legge, 2009).

Las placas BIOLOG Ecoplate™, introducidas a finales de la década de 1980, son placas de 96 pocillos que contienen diferentes fuentes de carbono utilizadas por los microorganismos y un colorante redox denominado violeta de tetrazolio. En cada pocillo se inoculan microorganismos aislados del ecosistema a estudiar que, a medida que crecen, pueden metabolizar la fuente de carbono que contiene.

Esta metabolización de la fuente de carbono provoca el cambio de coloración del violeta de tetrazolio, y este cambio de color puede determinarse mediante la lectura de la densidad óptica a 590 nm (DO_{590}). Este procedimiento permite un análisis individualizado del comportamiento metabólico de los microorganismos, ya que el patrón de utilización del sustrato de carbono (CSUP) resultante se utiliza para desarrollar el CLPP (Garland y Mills, 1991).

Las placas BIOLOG Ecoplate™ han demostrado ser un método fiable, rápido y barato para estimar el efecto de distintos contaminantes en las comunidades microbianas naturales (Schultz y Ducklow, 2000; Christian y Lind, 2006; Németh et al., 2021). Sin embargo, aunque la utilización de estas placas para el CLPP de muestras de suelos está bien documentada (Balser et al., 2002; Classen et al., 2003; Goberna et al., 2005; Smalla et al., 1998), su uso para el estudio de comunidades microbianas de ecosistemas acuáticos (tanto fluviales como marinos) es aún novedosa (Schultz y Ducklow, 2000; Goberna et al., 2005; Németh et al., 2021).

Pese a que la utilización de las placas de BIOLOG Ecoplate™ es sencilla, el análisis de los datos de CLPP presenta una mayor complejidad. Al igual que en otras metodologías, la interpretación de los resultados se realiza en base a determinados parámetros resultantes del procesamiento de los datos obtenidos, denominados *endpoints*. En este caso, los parámetros más utilizados son AWCD (Average Well Color Development, la media del desarrollo del color de los pocillos) y AUC (Área Bajo la Curva) (Boteva et al., 2024; Németh et al., 2021; Tam et al., 2003).

1.1.5. El problema añadido de las resistencias a los antimicrobianos

Los ATBs, actúan con el objetivo de eliminar bacterias (bactericidas) o, en todo caso, evitar su propagación o crecimiento (bacteriostáticos). Su extracción y purificación, frecuentemente a partir de otros seres vivos que emplean estas moléculas como ventaja competitiva en la naturaleza, supuso un avance histórico frente a infecciones que, de forma inevitable, tenían un alto coste en vidas humanas. Pocos años después del descubrimiento de la PEN, ya con su uso extendido, el mismo A. Fleming llamó la atención sobre una consecuencia perniciosa inherente al uso de estos fármacos: siguiendo las leyes de selección natural, los ATBs ejercen una presión selectiva hacia las bacterias. De este modo, advirtió que un uso incorrecto de estas sustancias puede facilitar el desarrollo de resistencias a los antimicrobianos (RAM) (Abdallah et al., 2023).

Además de la selección natural (donde la presión selectiva sobre una población facilita la selección de mutaciones resistentes con el paso de las generaciones), las bacterias disponen de distintos mecanismos para acelerar la adquisición de resistencias a los ATBs (Henschel et al., 1997; Foster, 2017; Kovalakova et al., 2020; Jia et al., 2023). Aunque se escapan al objeto de esta investigación, cabe citar la existencia de genes de resistencia intrínsecos (tanto como cromosómicos como en plásmidos) en numerosas especies, así como la capacidad de éstas de transferencia horizontal (dentro de la misma generación) de genes de resistencia (transducción, conjugación, transformación). Estos mecanismos consiguen dotar a la población bacteriana de resistencia a los ATBs en pocas horas tras la exposición (Hermsen et al., 2012). En este estudio se ha trabajado con ATBs de amplio consumo, pertenecientes a diferentes familias con diferentes mecanismos de acción, que se encuentran entre los más detectados en ecosistemas fluviales: PEN, AMO, ampicilina (AMP), gentamicina (GTM), TC, ERY, STM y CHL.

La OMS (Organización Mundial de la Salud), en particular, clasifica las bacterias en función de su capacidad de generar resistencia, alertando sobre las que suponen un mayor riesgo para la salud (WHO, 2024b). Las especies bacterianas seleccionadas para este estudio tienen cepas pertenecientes a este grupo, y se encuentran además entre las principales causas de muertes por infecciones bacterianas a nivel global (GBD 2021 Antimicrobial Resistance Collaborators, 2022). Las bacterias incluidas en este estudio son *Bacillus subtilis, Enterococcus faecalis, Listeria monocytogenes, Streptococcus agalactiae, Staphylococcus aureus, Acinetobacter baumannii, Escherichia coli, Klebsiella aerogenes, Klebsiella pneumoniae, Pasteurella aerogenes, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enterica sv.* Typhimurium y *Serratia marcescens*.

1.2. BLOQUE II. SOLUCIONES Y ALTERNATIVAS A LOS ANTIBIÓTICOS

1.2.1. Nuevas estrategias en la búsqueda de alternativas a los antibióticos comerciales:

Como hemos visto, el elevado consumo de ATBs a nivel mundial ha generado una serie de problemas, tanto medioambientales como a nivel de resistencias bacterianas. Por ello, la comunidad científica ha trabajado durante las últimas décadas en la búsqueda de posibles soluciones, y los gobiernos de países occidentales van desarrollando ya objetivos de reducción en su consumo de ATBs (D'Atri et al., 2019), así como en la búsqueda de nuevas alternativas.

a) Fagos

La terapia con fagos se caracteriza por emplear virus capaces de infectar específicamente bacterias y destruirlas (lisis), pudiendo usarse como alternativa o complemento de los ATBs. Este enfoque ofrece ventajas como especificidad (disminuye el daño sobre microbiota benéfica), capacidad de atacar bacterias en biofilms donde los ATBs clásicos tienen menor penetración, y la posibilidad de ingeniería genética de fagos o uso de proteínas líticas derivadas de ellos (Kapoor et al., 2024).

b) Péptidos antimicrobianos

Los péptidos antimicrobianos son moléculas cortas, generalmente con carga eléctrica positiva, y con capacidad de interactuar con membranas bacterianas, provocando su permeabilización o destrucción, aunque muchos también tienen mecanismos intracelulares u otros no líticos (Ma et al., 2024; Zhang et al., 2025). Además, su combinación con ATBs ha mostrado reducir las dosis de fármaco necesarias, aumentar eficacia frente a cepas resistentes y mejorar la eliminación de biofilms. Un estudio reciente, de hecho, mostró cómo combinaciones de péptidos con ATB frente a cepas de *Acinetobacter baumannii* extremadamente resistentes resultan sinérgicas tanto en células libres como en biofilms (Meng et al., 2022).

c) Antimicrobianos de origen vegetal

Los compuestos vegetales (fitocompuestos), incluyendo extractos completos, aceites esenciales y productos naturales (en adelante, PN) específicos provenientes de distintas familias bioquímicas, muestran actividad antimicrobiana directa contra patógenos, tanto sensibles como resistentes a los ATB (Singh et al., 2018). También pueden modular mecanismos de resistencia, como inhibición de bombas de eflujo, daño oxidativo, alteración de la integridad de la pared o membrana celular (Khameneh et al., 2019)

d) Sinergias entre diferentes agentes antimicrobianos.

La utilización de distintas combinaciones de ATBs puede aumentar la eficacia frente a cepas resistentes, reducir la probabilidad de aparición de resistencia, y permitir dosis menores de cada uno, disminuyendo la probabilidad de aparición de efectos adversos asociados.

Además, otras técnicas, como el *re-purposing* (cambio de indicación farmacéutica) de fármacos originalmente desarrollados para otros propósitos (no ATBs) ha mostrado que algunos tienen efecto antimicrobiano o actúan como adyuvantes que potencian ATBs, entre otras, al inhibir mecanismos de resistencia (Liu et al., 2021c). Por ejemplo, la combinación de polimixinas (péptidos antimicrobianos) con ATB como carbapenemes o con azitromicina mostró sinergia contra cepas resistentes de *A. baumannii* (Meng et al., 2022).

e) Sinergias entre PN y ATB comerciales.

Esta estrategia, en la que se basa la presente Tesis Doctoral y se detalla posteriormente, consiste en la potenciación de la acción de los ATB comerciales con distintos PN, permitiendo reducir su CMI (Concentración Mínima Inhibitoria). Es una estrategia que no descarta, por tanto, el uso de los ATBs ya comercializados, pero sí reduce su consumo y, por tanto, previsiblemente también sus efectos adversos de ecotoxicidad y generación de resistencias.

1.2.2. Antimicrobianos de origen vegetal

La naturaleza es la mayor reserva conocida de moléculas bioquímicas y farmacéuticas que conocemos (Abdallah, 2011), ya que la presión evolutiva ha guiado a los organismos a generar una gran diversidad de PN para multitud de funciones biológicas (Saleem, 2014). Revisiones recientes demuestran que actualmente nuevas moléculas y estructuras moleculares están siendo descubiertas y usadas en la actualidad (Dai et al., 2020). Estas sustancias de origen natural (Seow et al., 2014), han sido moldeadas por la evolución durante millones de años (Peláez, 2006), y gracias a ello presentan un amplio abanico de propiedades (antimicrobianas, antioxidantes, antiinflamatorias, antialergénicas, anticancerígenas, antifúngicas o insecticidas, por ejemplo) (Lee et al., 2011; Svoboda et al., 2006). De hecho, los ATBs comerciales proceden también en su mayoría de fuentes naturales (Betoni et al., 2006; Coutinho et al., 2009; Petrovska, 2012). De las 9 familias antibióticas conocidas, 6 (betalactámicos, aminoglucósidos, TCs, macrólidos, glicopéptidos y lincosamidas) fueron descubiertas a partir de moléculas naturales (Rossiter et al., 2017).

1.2.2.1. Aceites esenciales y su problemática

La mayor parte de los estudios de la actividad antimicrobiana de PNs de origen vegetal comenzaron con aceites esenciales (AE), mezclas de sustancias aromáticas, volátiles y lipídicas presentes en distintas partes de la planta, como frutos, corteza, semillas, raíces, o tallo (Ríos, 2016). Estas mezclas de sustancias se forman en el citoplasma y existen generalmente como gotículas situadas entre las células.

Los AE están compuestos principalmente por varios metabolitos secundarios (Hyldgaard et al., 2012), que son aquellos producidos por el metabolismo que no es esencial para su crecimiento y desarrollo de las plantas (Zhi-Lin et al., 2007), y son responsables de la relación del organismo vegetal con el medio (atracción de polinizadores, protección frente a infecciones, defensa frente a insectos, color y olor, etc.) (Demain, 2009).

Los AE son extraídos por diferentes métodos, como arrastre de vapor, shoxhlet, hidrodestilación o prensado (en casos como cortezas de cítricos) (Seow et al., 2014).

Ya desde finales del siglo XX se comenzó a investigar con más detalle la actividad frente a microorganismos de los AE extraídos de diversas plantas (Ismaiel y Pierson, 1990; Bayoumi, 1992), alcanzando hoy en día una bibliografía considerable. Extractos de cilantro, clavo y orégano han demostrado actividad frente a varias bacterias (Vergis et al., 2015). El AE de orégano (*Rosmarinus officinalis* L.), utilizado ya como conservante en la industria alimentaria, también es activo incluso frente a esporas de *Clostridium botulinum*, a concentraciones de 100-200 µg/mL (Ismaiel y Pierson, 1990), o *Escherichia coli* O157:H7 (Skandamis y Nychas, 2000). El AE de menta (*mentha piperita*) resultó ser eficaz frente a cepas de *S. enterica* a concentraciones también bajas 50 - 200 mg/L (Bayoumi, 1992). El AE extraído de la flor de *Cinnamomum zeylanicum* fue muy eficaz contra *Pseudomonas sp*., con valores de CMI muy bajos (Stan et al., 2021).

Numerosas revisiones sistemáticas han recopilado la mayor parte de la bibliografía disponible: Lang y Buchbauer, (2012) remarcan la amplitud del espectro de acción de los AE, poniendo como ejemplo destacado el AE de *Melaleuca alternifolia* frente a biofilms de *Staphilococcus aureus* multirresistente o dermatofitos, pese a que algunos bacilos gram negativos, como *Pseudomonas aeruginosa*, presentan menos sensibilidad. Los AE de especies de lamiáceas como *Thymus sp*., *Salvia sp*. y *Origanum sp*. tienen una actividad particularmente alta. Seow et al., (2014), concluyen también que los microorganismos gram positivos son notablemente más sensibles a los AE. Aun así, hay evidencias sólidas de que los AE son efectivos frente a bacterias como *Bacillus subtilis, E. coli, Listeria monocytogenes, Proteus mirabilis, P. aeruginosa, S. enterica* o *S. aureus*. Por su parte, la revisión de Reyes-Jurado et al., (2015) nos ofrece también algunos datos de concentraciones inhibitorias, que tienden a concordar en concentraciones moderadamente bajas. Por ejemplo, el AE de *Origanum glandulosum* fue efectivo frente a o *S. aureus, E. coli* y *L. monocytogenes* a concentraciones de entre 79,25 y 58 µg/mL, y el AE *de Cinnamonmum osmophloeum* fue efectivo contra *S. enterica, E. coli*, y *S. aureus* a concentraciones de entre 250 y 500 µg/mL. Encontramos cifras parecidas con la mayoría de más de 500 µg/mL, con alguna excepción de hasta 1 µg/mL, en estudio de Iseppi et al.,

(2021), trabajando con diversos AE (*Citrus aurantium*, *Citrus x limon, Eucaliptus globulus* o *M. alternifolia* (Iseppi et al., 2021).

Este estudio, además, demostró la existencia de interacciones sinérgicas tanto entre ATBs como entre distintos AE, frente a bacterias multirresistentes. La mayoría de CMIs que encontraron fueron mayores de 500 µg/mL, salvo casos como el de *M. alternifolia*, con valores muy bajos, de hasta 1 µg/mL) (Iseppi et al., 2021).

En global, destaca la gran variabilidad de resultados que ofrece la literatura, en el caso de las concentraciones efectivas contra microorganismos concretos. Hay varias causas que podrían explicar este fenómeno:

En primer lugar, la variabilidad en la composición de los AE es cambiante entre especímenes, localizaciones e incluso entre estaciones. Rathore et al., (2022), por ejemplo, publicó en la revista Nature un estudio sobre la variabilidad estacional del AE del orégano (*Rosmarinus officinalis L*), comprobando las diferencias en algunos de sus componentes entre las estaciones húmedas y secas. Barra A., (2009), por su parte, hace una recopilación de los diferentes factores que pueden afectar a la composición de un AE, tanto exógenos (luz, irrigación, pH del suelo, altitud, latitud, etc.) como endógenos (partes de la planta con diferentes concentraciones, edad del organismo, o particularidades genéticas). Esta variabilidad dificulta la práctica clínica y la replicabilidad de resultados. La revisión sistemática de Lang y Buchbauer, (2012), sobre la efectividad antimicrobiana de AEs, por ejemplo, llama la atención en sus conclusiones sobre el problema de la homogeneidad y necesidad de estandarización de la composición de los AE.

En segundo lugar, la complejidad en composición de estos extractos supone la interacción simultánea y poco predecible de varios PNs. Además de aditivas, estas interacciones pueden ser de carácter sinérgico (Radulović et al., 2007), o también antagónicos. El estudio de Lambert et al., (2001) pone como ejemplo la combinación de timol y carvacrol, la cual es responsable del efecto antimicrobiano y en combinación con ATBs del AE de orégano (*Origanum vulgaris*). Su estudio concluye que esta combinación tiene más actividad de forma aislada que en presencia del resto de componentes del AE donde se encuentran, sugiriendo así que actúan en ocasiones como inhibidores. Lang et al., (2012) también llaman la atención acerca del desconocimiento de las complejas interacciones que se pueden dar en productos con tantas sustancias actuando simultáneamente (Lang y Buchbauer, 2012).

Por tanto, esta variabilidad en su composición impide hacer un estudio sistemático, comparable y cuantificable acerca de sus interacciones y efectos combinados con ATBs comerciales.

1.2.2.2. Productos naturales y sus diferencias con los antibióticos comerciales

Debido a ello, numerosos autores prefieren trabajar con los PN predominantes en los AE, pero aislados de forma individual (Tsai, 1997; Editorial Board, 2007; Mir et al., 2023) que presenten bioactividad. La utilización de PN aislados permitiría estandarizar la cantidad de principio activo, garantizando así unos resultados comparables.

Se estima que el número de metabolitos secundarios individuales existentes en la naturaleza se cuentan por centenares de miles (Mendelsohn y Balick, 1995; Bérdy, 2005). Aunque existen numerosas familias (alcaloides, lectinas, etc.), las más interesantes desde el punto de vista microbiológico son los terpenos y los polifenoles (Zacchino et al., 2017; Borges et al., 2024):

1. Terpenos

Los terpenos son la familia de PN más grande (más de 30000 compuestos conocidos) (Gupta y Birdi, 2017) y que más atención ha recibido en los últimos años, gracias a una actividad antimicrobiana intrínseca considerable (Dias et al., 2022). Entre los terpenos con más actividad investigada en la literatura destacan algunos como timol, carvacrol, eugenol, limoneno. La literatura disponible, además, parece apuntar a que presentan una mayor efectividad sobre microorganismos grampositivos, como *S. aureus, S. mutans, S. agalactiae*, o *E. faecalis*, incluso multirresistentes (dos Santos Barbosa et al., 2021; Nourbakhsh et al., 2022), aunque algunas cepas gramnegativas también parecen ser sensibles (Dias et al., 2022).

2. Polifenoles

Los polifenoles son metabolitos secundarios encontrados en la mayoría de las plantas superiores. Tienen roles importantes en la defensa del vegetal y su respuesta a estímulos estresantes. En humanos, tienen numerosas propiedades, como antioxidantes, antiinflamatorias, anticancerígenas o antimicrobianas (Daglia, 2012). Existe una amplia literatura demostrando una actividad antibacteriana interesante. La revisión bibliográfica de Cushnie y Lamb, (2005), por ejemplo, recopila más de 50 artículos, confirmando la actividad frente a diversas bacterias de moléculas de esta familia, como flavonas, quercetinas, naringenina, rutina o kaepmferol. Biharee et al., (2020) demostraron, más recientemente, que un número importante de compuestos flavonoides como artonina, licoflavona o astragalina demostraron actividad antimicrobiana frente a bacterias potencialmente resistentes, *como E. coli, S. aureus, B. subtilis, E. faecalis* o *S. enterica* serovar Typhy. Entre sus mecanismos de acción encontraron la inhibición de factores de virulencia, bombas de eflujo, formación de biofilms, síntesis de la pared celular, síntesis de ácidos nucleicos o movilidad bacteriana.

Otras familias, sin embargo, no disponen de actividad antibacteriana notable. Es el caso, por ejemplo, de los alcaloides, cuyos metabolitos secundarios tienen conocidas capacidades analgésicas o citostáticas, pero son frecuentemente antimicrobianos muy moderados (Moreno-Cárdenas y Çiçek, 2023).

Para todas las familias de PN, la mayoría de los datos bibliográficos concuerdan en unos valores de Concentraciones Mínimas Inhibitorias (CMI) que se mantienen, por lo general, en más de 50 –100 µg/mL. En la revisión realizada por Nourbakhsh et al., (2022), por ejemplo, sólo el lignano (2 – 24 µg/mL) y citronelol (5 µg/mL) tenían unos valores tan bajos. El resto de los PN presentaron unos valores de entre 100 y 300 µg/mL, que en ocasiones llegaban hasta 2500 µg/mL, como la vainillina y el estragol. La revisión de Moreno-Cárdenas y Çiçek, (2023), sin embargo, encontró que la mayoría de CMIs de los 223 PN que analizaron fueron bastante más bajas, de entre 10 y 50 µg/mL.

En el presente trabajo, se seleccionaron dos PN de interés. En primer lugar, el ácido tánico (AT). Es el miembro más representativo de la familia de los taninos naturales, un tipo de polifenoles (García-Ballesteros et al., 2016), cuya actividad antimicrobiana, entre otras, es conocida en la literatura (Akiyama et al., 2001; H. Shen et al., 2017). Se puede encontrar en una gran variedad de plantas y frutos (White, 1957).

En segundo lugar, el nerol (NE), un alcohol monoterpenoide encontrado de forma natural en frutos como la naranja amarga, plantas u hongos (Mukarram et al., 2021; Li et al., 2024). Sus propiedades antimicrobianas, entre otras, también han sido discutidas en la bibliografía disponible (Jirovetz et al., 2007; Coêlho et al., 2022).

Estos PN, pese a que presenten actividad antimicrobiana intrínseca, tienen diferencias fundamentales con los ATBs comerciales:

a) Diferencias en los mecanismos de acción

Los ATBs funcionan empleando distintas dianas bacterianas específicas, como pueden ser la pared bacteriana (PEN, AMO o AMP), la membrana celular (polimixina), la síntesis de proteínas (ERY, CHL, GTM, STM o TC), o inhibiendo otras rutas metabólicas esenciales.

Los PN, sin embargo, atacan simultáneamente varias dianas de forma más inespecífica. Uno de sus mecanismos de acción más comunes es la afectación de la estabilidad de la bicapa lipídica que forma la membrana celular bacteriana. Esto se da especialmente en el caso de los terpenos, altamente lipofílicos, que interaccionan con los fosfolípidos de la membrana y comprometen su permeabilidad e integridad (Nourbakhsh et al., 2022).

Otros PN actúan inhibiendo las bombas de eflujo, que son, a su vez, de los mecanismos más utilizados por las bacterias para adquirir resistencia a los antimicrobianos (dos Santos Barbosa et al., 2021; Kongkham et al., 2020; Kumawat et al.,

2023), y la gran mayoría de las familias de ATBs se ven afectadas por ello: betalactámicos, TCs, macrólidos, estreptograminas, fluorquinolonas, anfenicoles, etc. (Elmaidomy et al., 2022). La inhibición de las bombas de eflujo, además, evita otro de los mecanismos de resistencia, el *quorum sensing*, así como la formación de biofilms (Prasch y Bucar, 2015). Son numerosos los casos de PNs con esta actividad, como el caso de la artonina (Farooq et al., 2014). También en la familia de los terpenos se observan disruptores de las bombas de eflujo, tanto en bacterias grampositivas como gramnegativas (Dias et al., 2022).

b) Diferencias en la adquisición de resistencias

Diferentes estudios parecen apuntar a que, como resultado de ello, la adquisición de resistencias sería más complicada y menos probable (Lewis et al., 2024; Matei y Visan, 2025). Magi et al., (2015) también sugieren que no parece probable la adquisición de resistencias frente a carvacrol, un PN semejante al nerol, tras investigar su exposición en aislados faríngeos multirresistentes.

La evidencia parece apuntar a que la generación de resistencias también se ve dificultada, en el caso de los aceites esenciales, cuando varios compuestos actúan de forma simultánea (Calvo y Martínez-Martínez, 2009).

c) Diferencias en actividad antimicrobiana

Sin embargo, debido a estos mecanismos de acción más inespecíficos, los PN tienen en general una actividad antimicrobiana más moderada que las moléculas de origen microbiano, como los ATBs comerciales (Yamada, 1991) y requieren por tanto de concentraciones más elevadas para conseguir un efecto semejante (Pauli, 2001), con CMIs por encima de 100 µg/mL frente a los rangos de 2 y 32 µg/mL de los ATBs comerciales (Giske et al., 2022). Esto, unido a la falta de referencias clínicas sobre su efecto en humanos, aleja la posibilidad de emplearlos como sustitución directa de los ATBs en la práctica clínica (Kon y Rai, 2013).

d) Diferencias en efectos adversos

Es muy escasa la literatura disponible acerca de los efectos adversos de PNs. Según el estudio realizado en Italia por Cuzzolin et al., (2006), resulta complicado determinar la seguridad de las terapias naturales por la menor regulación que soportan. Sin embargo, autores como Lin et al., (2005) concluyen que la toxicidad de los PNs como los flavonoides no puede ser muy alta, ya que la ingesta diaria a través de frutas y verduras resulta inocua. Algunos PN, de hecho, se utilizan como fragancias o saborizantes alimentarios, avalado por organismos como la FDA o la FEMA (Agencia Federal de Gestión de Emergencias) de EE.UU. (Adams et al., 2011). Sin embargo, su aplicación clínica frente a infecciones exigiría ensayos clínicos aleatorizados y controlados que confirmen eficacia y seguridad (Gopalakrishnan et al., 2022)

e) Diferencias en ecotoxicidad

En los últimos años ha crecido el interés por la ecotoxicidad de AEs y extractos vegetales para los organismos no diana. Destaca en este campo la revisión de Ferraz et al., (2022), que observa cómo muchos de los AEs y extractos estudiados no tienen efectos tóxicos para los organismos seleccionados (o sus efectos sólo eran observables a altas concentraciones). Otros, no obstante, sí han demostrado cierta toxicidad a concentraciones bajas.

Sin embargo, los efectos en el medioambiente de los PNs aislados es un campo con notables lagunas bibliográficas, que sólo recientemente se ha empezado a encontrar en la literatura disponible.

La EPA (Environmental Protection Agency), la agencia de protección medioambiental del gobierno de EE. UU., considera que el riesgo para el medioambiente de PNs como linalool o timol es despreciable (US EPA Office of Pesticide Programs, 1993). Pero trabajos recientes, como el de Pino-Otín et al., (2021) y Gan et al., (2024) observan que, pese a que no se espera toxicidad aguda a las concentraciones ambientales por parte de PNs como citronelol, carvacrol o timol, estos productos sí causan toxicidad en organismos no diana (como *D. magna* o *E. fetida*) a concentraciones de entre 0,5 a 8 mg/L, y su exposición crónica podría ser perjudicial para el medioambiente.

Otros trabajos, sin embargo, observan ausencia de toxicidad en organismos no diana (en su caso, especies de escarabajo, *A. cicatricosus*) de timol, carvacrol y cinamaldehído, destacando su valor como antihelmínticos menos ecotóxicos que los tradicionales, como la ivermectina (Verdú et al., 2023). En la misma línea, otros PNs, como limoneno o eugenol, han demostrado su capacidad insecticida y están siendo investigados como biopesticidas (Liu et al., 2021b) con un impacto en el medioambiente mucho más reducido que los compuestos tradicionales (Sarma y Khanikor, 2025).

No obstante, pese a que puedan producir ecotoxicidad en algún organismo no diana, un factor para tener en cuenta es el tiempo en el que estos PN se degradan en los ecosistemas.

Los terpenos, por ejemplo, se caracterizan por una alta volatilidad y reactividad, lo que los hace susceptibles a una rápida degradación en contacto con agentes oxidantes. Su vida media en la atmósfera, por tanto, varía desde minutos hasta unas pocas horas (Berg et al., 2024; Patra et al., 2025). También en ecosistemas acuáticos su degradación es rápida, aunque depende en mayor medida de los factores predominantes, como son la irradiación UV o la temperatura (Yang et al., 2007), y puede ser una de las razones por las cuales sus concentraciones en el medioambiente son relativamente bajas (Goldberg et al., 1992).

También los compuestos fenólicos son susceptibles a estas condiciones, aunque en menor medida que los terpenos. Se ha demostrado, por ejemplo, la alta inestabilidad de polifenoles como el cinamaldehído frente a procesos de oxidación (Yu et al., 2020), y sus vidas medias en la atmósfera son frecuentemente inferiores a 1 día (Atkinson y Arey, 1994).

En entornos acuáticos, al igual que los terpenos, el proceso es más complejo, y factores como la fotólisis (UV) o la temperatura son los principales responsables de su degradación. Sus vidas medias, en este caso, son inferiores a 7 días (Zhang et al., 2009; Zhao et al., 2000).

Por último, estos productos probablemente son más biodegradables que los ATBs comerciales como se deduce de experimentos con comunidades microbianas de aguas y suelos que son más resilientes a la exposición de PN que los organismos individuales no diana (Ferrando et al., 2024a, 2024b; Gan et al., 2024; Pino-Otín et al., 2021; Valenzuela et al., 2025).

1.3. BLOQUE III. SINERGIAS DE ANTIBIÓTICOS CON PRODUCTOS NATURALES.

1.3.1. Combinación de productos naturales y antibióticos.

Gracias a las diferencias en los mecanismos de acción de los dos tipos de productos, se abre la posibilidad de que un ATB que haya perdido efectividad debido a las resistencias bacterianas pudiera ser combinado con un PN, permitiendo así una recuperación de, por lo menos, parte de su actividad (Sadeer y Mahomoodally, 2020), ampliando su espectro, y permitiendo una disminución de las dosis clínicas administradas de estos fármacos (Kongkham et al., 2020). Algunos PN han demostrado, además de su actividad antimicrobiana intrínseca, un fenómeno interesante: la reversión de resistencias y la reducción de factores de virulencia, es decir, aquellos otros factores necesarios para agravar la patogénesis (*quorum-sensing*, producción de toxinas o formación de biofilms, por ejemplo) (Johny et al., 2010; Kongkham et al., 2020; Wright, 2017).

Este efecto se puede explicar, entre otras, por la actuación en distintas dianas celulares simultáneamente o por cambios fisicoquímicos y farmacocinéticos (como solubilidad, biodisponibilidad, etc.) (Daglia, 2012). El efecto sería análogo al que presentan sustancias coadyuvantes como el ácido clavulánico, administrado junto a los betalactámicos (Hemaiswarya et al., 2008), ya que la combinación de varios mecanismos de acción simultáneos disminuye la capacidad de los microorganismos de enfrentarse a su exposición (Kon y Rai, 2013).

Como apuntan algunos autores, la actuación de varias sustancias de forma simultánea puede presentar una mayor dificultad para generar resistencias (Calvo y Martínez-Martínez, 2009). Pese a ello, no existe todavía ninguna combinación de un ATB con un PN, que haya llegado a la práctica clínica. Las únicas interacciones existentes son combinaciones ATB – ATB o ATB – coadyuvante (Worthington y Melander, 2013). Esto se debe, entre otras razones, al desconocimiento de los efectos tóxicos de muchas sustancias naturales y la ausencia de ensayos clínicos, así como la dificultad de comprobar las dosis correctas de cada producto (Ayaz et al., 2017).

Este tipo de combinaciones, en la que se consigue mejor respuesta de la combinación de sustancias (PN + ATB) que ambos productos por separado, se conoce como interacción sinérgica. Su principal ventaja, y consecuencia directa de la reducción de la dosis necesaria tanto de ATB como de PN, es la disminución de la toxicidad y los efectos adversos esperables asociados a ambas sustancias. Existen numerosas publicaciones que recopilan PN capaces de interaccionar sinérgicamente con ATBs:

a) Sinergias con aceites esenciales

Extractos vegetales procedentes del té, así como PN activos aislados (epigalocatequin-galato, de la familia de los taninos naturales), han demostrado actividad sinérgica junto a diversos ATBs (TC, ERY, PEN, AMP o GTM) especialmente frente a *S. aureus* (incluido SARM, *S. aureus* Resistente a Meticilina) (Hemaiswarya et al., 2008). El estudio de Iseppi et al., (2021), trabajando con AE de diversas plantas (*Citrus aurantium*, *Citrus x limon*, *Eucaliptus globulus* o *Malaleuca alternifolia*), demostró la existencia de interacciones sinérgicas, tanto con ATBs como entre distintos AE, frente a bacterias multirresistentes. La mayoría de CMIs que encontraron fueron mayores a 500 µg/mL, salvo casos como el de *Malaleuca alternifolia*, con CMIs muy bajas, de hasta 1 µg/mL.

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b) Sinergias con terpenos

El número de publicaciones relativas a sinergias de estos PN con ATBs ha ido aumentando en la literatura científica en los últimos años, reconociéndose las sinergias con PNs de origen natural como una de las líneas más prometedoras frente al problema de las RAM (Sadeer y Mahomoodally, 2020). Su trabajo recopila productos como ácido tánico o carvacrol, capaces de reducir hasta 4 veces las CMIs de ATBs comerciales. De hecho, muchos terpenos como timol, carvacrol, eugenol o limoneno disponen ya de varios artículos corroborando su capacidad sinérgica: Palaniappan y Holley, (2010) cuantifican las sinergias que producen con ATBs como AMO, PEN, TC, AMP o ERY frente a bacterias resistentes, como *E.coli*, *S. aureus* o *S. enterica sv*. Typhimurium.

Dos Santos Barbosa et al., (2021) verifican que estos PN, entre otros, pueden incrementar la actividad de ATBs mediante la inhibición de bombas de eflujo detallada anteriormente. Gan et al., (2023) también identifican la interacción sinérgica de timol con CHL y STM. Recientemente, el trabajo de Ferrando et al., (2024a) caracterizó hasta 15 sinergias producidas entre cinamaldehído, otro terpenoide, y ATBs como CHL, STM, AMO, y ERY frente a cepas de *Serratia marcescens*, *S. aureus*, *Pasteurella aerogenes* y *Salmonella enterica*, con reducciones de CMIs de hasta un 98%. Las sinergias con cinamaldehído, que es un componente mayoritario de la canela, también se certifican en el trabajo de Palaniappan y Holley, (2010).

c) Sinergias con polifenoles

También otras familias, como flavonoides (Aelenei et al., 2020; Meenu et al., 2021) y otros polifenoles (Zuo et al., 2014; Bocquet et al., 2019) han demostrado interaccionar de forma sinérgica con diversos ATBs de uso común. Los polifenoles hidrolizables también producen sinergias con ATBs, además de su actividad antibacteriana, antiviral y antifúngica intrínseca (Ekambaram et al., 2016). Shiota et al., (2004, 2000) demostraron la capacidad de generar estas interacciones con numerosos betalactámicos o TCs frente a cepas de *SARM,* y se apunta como posible mecanismo de acción la inhibición de las betalactamasas bacterianas (Shimizu et al., 2001). El ácido elágico y el ácido gálico, también taninos hidrolizables, disminuyen las dosis de TC y sulfametoxazol frente a diversas cepas de *P. aeruginosa* (Jayaraman et al., 2010). El miembro más conocido de esta familia es el ácido tánico. El estudio de Akiyama et al., (2001) concluyó que un ATB betalactámico, oxacilina, vio aumentada su actividad frente a *S. aureus* en presencia del ácido tánico.

En resumen, hay evidencia bibliográfica que confirma el efecto sinérgico que algunos PN, en especial terpenos y polifenoles, pueden tener con los ATBs. Esta potencial disminución de las dosis clínicas necesarias para enfrentar a los microorganismos multirresistentes puede ser una herramienta para reducir el consumo global de ATBs, así como los problemas ecotoxicológicos derivados de este uso.

Sin embargo, para avanzar de cara al uso clínico de estas combinaciones, es necesario conocerlas con más exactitud (Hemaiswarya et al., 2008), ya que la literatura disponible acerca de la ecotoxicidad de los PN es escasa, y más aún acerca de las combinaciones sinérgicas.

2. HIPÓTESIS

Los antibióticos comerciales, pese a sus beneficios para la salud humana, plantean problemas de ecotoxicidad y resistencia bacteriana. La combinación de antibióticos comerciales junto con productos naturales antimicrobianos podría reducir sus CMI, permitiendo disminuir la cantidad de antibióticos que llega al medio ambiente sin comprometer su eficacia. Como es previsible que los productos naturales presenten menor ecotoxicidad que los antibióticos, se reducirá el impacto ambiental ayudando a combatir el problema de la generación de resistencias.

3. OBJETIVOS

Para probar la hipótesis planteada, se han marcado los siguientes objetivos:

Objetivo Principal:

Identificar sinergias entre antibióticos comerciales y 2 productos naturales (ácido tánico y nerol) que puedan suponer una alternativa para disminuir el consumo de estos fármacos, y reducir su ecotoxicidad.

Objetivos secundarios:

- OBJETIVO 1. Determinar la ecotoxicidad de 8 antibióticos de uso común, seleccionados como base para la búsqueda de sinergias con productos naturales, utilizando comunidades microbianas no diana como bioindicadores.

- OBJETIVO 2. Caracterizar la eficacia y la cinética de las combinaciones sinérgicas entre los 8 antibióticos estudiados y los 2 productos naturales seleccionados (ácido tánico y nerol).

- OBJETIVO 3. Evaluar la ecotoxicidad del ácido tánico en organismos y comunidades microbianas no diana.

- OBJETIVO 4. Evaluar la ecotoxicidad del nerol en organismos y comunidades microbianas no diana.

- OBJETIVO 5. Evaluar la ecotoxicidad de las combinaciones sinérgicas identificadas entre los antibióticos y los productos naturales (ácido tánico y nerol) en comunidades microbianas no diana.

4. MATERIAL Y MÉTODOS

4.1. Antibióticos y productos naturales seleccionados

4.1.1. Antibióticos seleccionados

Para desarrollar el presente estudio, se seleccionaron 8 de los ATBs más empleados en uso clínico (Ripoll et al., 2002), pertenecientes a familias como betalactámicos, aminoglucósidos, TCs y anfenicoles.

a) Penicilina G (PEN)

Este ATB, el primero descubierto, es el referente dentro de la familia de los betalactámicos, cuyo mecanismo de acción es la inhibición de la síntesis de la pared celular bacteriana, principalmente a través del proceso de síntesis de peptidoglicano (Yocum et al., 1979). Su espectro es estrecho, siendo activa principalmente frente a cocos grampositivos (como estreptococos) y alguno gramnegativo, (como *P. aerogenes*) (DrugBank Online, 2024b). No cubre a la mayoría de los estafilococos y enterococos, ni a bacterias productoras de betalactamasas, capaces de degradarla (Asociación Española de Pediatría, 2025). A lo largo de las décadas, el creciente número de resistencias a las que se enfrenta ha provocado un descenso de su utilización.

b) Amoxicilina (AMO)

Pertenece también a la familia de los betalactámicos, y por tanto su mecanismo de acción es también la inhibición de la síntesis de peptidoglicano, bloqueando la inhibición de pared celular bacteriana. Al igual que la PEN, su espectro cubre las bacterias grampositivas, principalmente (DrugBank Online, 2024a), pero es efectiva frente a más bacterias gramnegativas *Salmonella sp.,* entre otras (Asociación Española de Pediatría, 2024a). AMO es uno de los ATBs más prescritos en España, frecuentemente acompañada de clavulánico, un inhibidor de las betalactamasas bacterianas (Ripoll et al., 2002).

c) Ampicilina (AMP)

Es un derivado de PEN, y por tanto también pertenece a la familia de los betalactámicos. Actúa del mismo modo, inhibiendo la síntesis de la pared celular bacteriana, y su amplio espectro cubre una variedad de grampositivas y gramnegativas, entre ellas *E. coli*, *P. mirabilis*, *Salmonella sp.*, estafilococos y enterococos (Asociación Española de Pediatría, 2024b). Sin embargo, numerosas cepas han logrado adquirir resistencia a este ATB (DrugBank Online, 2024b).

d) Estreptomicina (STM)

Fue el primer ATB del grupo de los aminoglucósidos, de administración exclusivamente parenteral. Ejerce su actividad uniéndose a la subunidad 30S de los ribosomas bacterianos, alterando la translocación ribosomal y la lectura del ARN (impidiendo así la formación de proteínas esenciales) (Demirci et al., 2013). Es activo, entre otras, frente a estreptococos*, E. coli, Yersinia sp., Proteus sp.* o *Brucella sp*. (Asociación

Española de Pediatría, 2024c). Por su amplio espectro de acción, es utilizado en terapias combinadas en casos de multirresistencia (DrugBank Online, 2024f).

e) Gentamicina (GEN)

También pertenece al grupo de los aminoglucósidos, por lo que su mecanismo de acción también es la alteración del proceso de lectura del ARNm y la translocación ribosomal mediante la unión a la subunidad 30S de los ribosomas (Shoji et al., 2009). Su espectro cubre numerosas bacterias, tanto grampositivas (*S. aureus, S. epidermidis o L. monocytogenes*) como gramnegativas (*E. coli, Proteus sp., Serratia sp*., y especialmente, *P. aeruginosa*) (DrugBank Online, 2024a). Su mecanismo de acción es la inhibición de la síntesis proteica, y sus efectos sinérgicos con betalactámicos son el motivo de su uso en terapias combinada (Asociación Española de Pediatría, 2024f).

f) Eritromicina (ERY)

Pertenece al grupo de los macrólidos. Es un ATB bacteriostático (Asociación Española de Pediatría, 2024d), y su mecanismo de acción es la unión a la subunidad 50S del ribosoma bacteriano, (específicamente al gen 23S ARNr), bloqueando la síntesis de las cadenas proteicas y la translocación ribosoma (Krawczyk et al., 2024). Es especialmente activa frente a estreptococos, y también frente a otros grampositivos aerobios y anaerobios gramnegativos (DrugBank Online, 2024c).

g) Tetraciclina (TC)

Pertenece a la familia del mismo nombre, y también es un ATB bacteriostático. Ejerce su actividad uniéndose a la subunidad 30S de los ribosomas bacterianos, bloqueando la unión ARNm – ribosoma, deteniendo la elongación (y por tanto la síntesis) de proteínas (Schlünzen et al., 2001). Su espectro antimicrobiano es amplio, cubriendo desde cocos grampositivos hasta bacterias gramnegativas (como *E. coli*, *Pasteurella* o *Klebsiella*) (Asociación Española de Pediatría, 2024e).

h) Cloranfenicol (CHL)

Este ATB bacteriostático pertenece a la familia de los anfenicoles, y su mecanismo de acción consiste en el bloqueo de la síntesis proteica mediante la unión a la subunidad 50S de los ribosomas bacterianos(Lin et al., 2018). Fue el primer miembro de esta familia, y cubre a bacterias grampositivas y negativas, incluyendo anaerobios y espiroquetas (Asociación Española de Pediatría, 2024c). Sin embargo, su alta toxicidad hematológica lo relega de la primera línea de tratamientos (DrugBank Online, 2024c).

4.1.2 Productos naturales seleccionados

Para el desarrollo de esta investigación, se escogieron 2 PN, nerol y ácido tánico:

a) Nerol

El nerol (NE), o *(Z)-3,7-dimetilocta-2,6-dien-1-ol* (figura 2), es un terpenoide volátil, aislado de fuentes naturales diversas, como el naranjo amargo (*Citrus aurantium* subsp. *amara* o *Bigaradia*), limonaria (*Cymbopogon citratus*), el lúpulo (*Humulus lupulus*) o levaduras como *Saccharomyces cerevisiae*. Según Lapczynski et al., (2008), cada año se emplean industrialmente entre 100 y 1000 toneladas métricas. Debido a sus propiedades aromáticas, estos usos comprenden, entre otros, la industria alimentaria, la perfumería, o productos domésticos de limpieza. Burdock, (2004), estima que la ingesta anual promedio por persona es baja, cuantificándola en unos 10 mg anuales. Según la FDA, el NE se encuentra en la categoría GRAS (*Generally Regarded as Safe*), y algunos autores descartan una toxicidad significativa (Lapczynski et al., 2008).

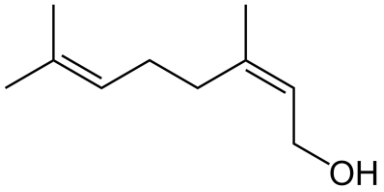

Figura 2. Estructura química de nerol. Fuente: Sigma-aldrich

A nivel medioambiental, sin embargo, no existe literatura suficiente para determinar su ecotoxicidad, tanto a corto como a largo plazo, ni su presencia en los ecosistemas, ya que únicamente un estudio lo ha detectado (en cantidades no cuantificables) en aguas del río Danubio (Milic et al., 2014). Sus características químicas y estructurales podrían apuntar a que la degradación del NE puede ser relativamente rápida, sensible al de pH y a la temperatura (Fagundes et al., 2022), aunque no mediante hidrólisis en el medio natural (Lyman et al., 1990). Así, en la atmósfera su degradación parece ocurrir sólo en minutos, pero en medios acuáticos podría retrasarse hasta 28 días (Lyman et al., 1990). Su coeficiente de reparto (K_{OW}) es moderado (3,47), por lo que, según algunos autores, su riesgo de bioacumulación, aunque existe, es bajo (Gimeno et al., 2024).

Al igual que otros terpenos, las propiedades antimicrobianas de este PN han sido objeto de investigación en las últimas décadas. Varios estudios confirman así su actividad antibacteriana intrínseca contra un amplio espectro de bacterias. Wang et al., (2019) determinaron que la CMI de NE *para E. coli*, *P. aeruginosa* y *S. aureus* fue de 41 µg/mL. Jirovetz et al., (2007), por su parte, establecen que las CMI para esas 3 mismas bacterias y *K. pneumoniae* oscilaron entre 60 y 600 µg/mL. El estudio de Kotan et al., (2007), haciendo un screening de la actividad de varios terpenos en una extensa batería de bacterias, demostró la actividad de NE frente a la gran mayoría de ellas, entre las que se encuentran *P. aeruginosa* (8 mm de inhibición en disco), *E. faecalis* (7 mm*), A. baumanii* (10 mm), *K. pneumoniae* (9 mm) o *S. enterica* sv. Typhimiurium (11 mm). Otros autores, además, señalan que el NE puede tener propiedades sinérgicas junto a ATBs: en su trabajo, Coêlho et al., (2016) determinan que la combinación de NE con norfloxacino es de carácter sinérgico frente a la bacteria grampositiva *S. aureus*.

b) Ácido tánico

El ácido tánico (AT) (*2,3-dihidroxi-5-[[(2R,3R,4S,5R,6S)-3,4,5,6-tetrakis[[3,4-dihidroxi-5-(3,4,5-trihidroxibenzoil)oxibenzoil]oxi]oxan-2-yl]metoxicarbonil]phenil] 3,4,5-trihidroxibenzoato*), por su parte, es una molécula compleja que pertenece a la familia de los polifenoles (figura 3), y es el miembro principal de la familia de los taninos hidrosolubles (Akiyama et al., 2001; García-Ballesteros et al., 2016; Ghosh y Bhadury, 2022; Y. Zhang et al., 2022). Este metabolito secundario se encuentra de forma natural en prácticamente todos los tejidos vegetales aéreos y frutos (Wrisez y Lambert, 2001; Zhang et al., 2022) y es la más conocida de las sustancias curtientes, es decir, aquellas usadas desde la antigüedad en el proceso de transformación de pieles en cuero (Falcão y Araújo, 2018; Maharani et al., 2022). A diferencia de otros PN, como los terpenos, el AT es hidrosoluble.

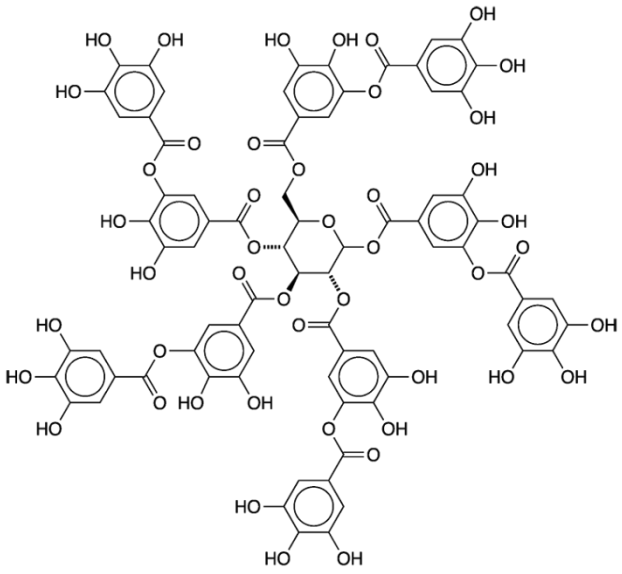

Figura 3. Estructura química del ácido tánico. Fuente: Sigma-aldrich

Son escasos los estudios que evalúen la presencia de AT en el medioambiente. Es bien conocido el papel de las industrias de curtido de cuero, ya que las pieles suelen curtirse con extractos vegetales ricos en taninos (Falcão y Araújo, 2018; Maharani et al., 2022), así como la industria del corcho. Se estima, por ejemplo, que la industria del corcho emplea más de 350000 toneladas de taninos de origen vegetal (Kanth et al., 2009). Sus aguas residuales son, en consecuencia, ricas en taninos. Bernardo et al., (2011), por ejemplo, cuantificaron entre 360 y 410 mg/L de ácido tánico en este tipo de aguas. En el medioambiente, se han cuantificado concentraciones más bajas, de hasta 6,4 µg/mL en el río Suwannee, en Florida (EE. UU.) (Crandall et al., 1999).

Entre las numerosas funciones que esta sustancia ejerce en la naturaleza y que explican su amplia distribución, el TA actúa como protector de los tejidos vegetales frente a infecciones (Farha et al., 2020). Numerosos extractos vegetales, donde el AT es uno de los componentes principales, han demostrado su actividad antibacteriana frente a bacterias como *S. aureus, E. coli, E. faecalis* o *P. aeruginosa* (Çolak et al., 2010; Kaczmarek, 2020), incluso frente a cepas resistentes a ATBs, como SARM (ver sección 2.3.4) (Maharani et al., 2022). El AT es capaz de inhibir mecanismos de señalización de *S. enterica sv.*

Typhimurium a concentraciones de 400 µg/mL (Sivasankar et al., 2020). Reyes et al., (2017) demostraron su actividad antimicrobiana también frente a *S. enterica*, tanto *in vitro* (inhibiendo el crecimiento bacteriano a concentraciones de 40 µg/ml) como *in vivo*, a concentraciones de 80 µg/mL, en ratones inoculados con la bacteria (Reyes et al., 2017). El estudio de Dong et al., (2018) determinó que las CMI de este PN frente a 100 cepas de *S. aureus*, tanto SARM como SASM (*S. aureus* sensible a meticilina), oscilaban entre 40 y 160 µg/mL, remarcando además posibilidad de reducir la formación de biofilms a concentraciones por debajo de la CMI. Çolak et al., (2010) determinaron que la concentración más efectiva de AT es de un 3%.

En lo referido a la posible interacción sinérgica entre el AT y ATBs, el estudio de Kırmusaoğlu, (2019) demuestra que la exposición de cepas resistentes de *S. aureus* a este PN revierte su resistencia adquirida a ATBs betalactámicos. Tintino et al., (2016) también comprobó la existencia de este tipo de interacción con la misma bacteria frente a norfloxacino y AT, apuntando además a la inhibición de las bombas de eflujo como posible mecanismo de acción.

El AT llega al medioambiente de forma significativa, principalmente a través de la industria del cuero.

La presencia de taninos en el agua de desecho de esta industria provoca efectos de ecotoxicidad para organismos acuáticos tan diversos como dafnias, algas o peces (De Nicola et al., 2007; Tigini et al., 2011; Chagas et al., 2019; China et al., 2020).

Sin embargo, son pocos los estudios que analizan la toxicidad de este PN de forma aislada. El estudio de De Nicola et al., (2004), por ejemplo, determinó que la CE_{50} que presentó una solución de taninos hidrosolubles fue de 248 µg/mL y que inhibía el crecimiento de varias especies de algas en concentraciones de entre 3 y 30 µg/mL. En cualquier caso, aún no se ha realizado ningún estudio ecotoxicológico completo que permita extraer conclusiones definitivas.

En la tabla 6 se detallan algunas características químicas de interés, junto con el número CAS y el proveedor de cada uno de ellos.

Las disoluciones stock de cada compuesto se prepararon en agua destilada (SIEMENS Ultra Clear™), añadiendo en el caso de los PN un 5% de DMSO (CAS: 67-68-5) (Fischer Bioreagents, Madrid), con una pureza del 99,7%. Esta concentración se determinó en ensayos de toxicidad, verificando que no afectaba al crecimiento de ninguno de los microorganismos utilizados (Ferrando et al., 2024a) (ver Anexo 11.4)

Tabla 6. Antibióticos y productos naturales seleccionados

Antibióticos

Nombre	*Abrev.*	*Familia*	*Número CAS*	*Proveedor*	*Masa molecular (g/mol)*	*Solubilidad en agua (mg/mL)*	*pKa*		*log Ko/w*
							pKa1	*pKa2*	
Amoxicilina[a]	AMO	Beta-lactámicos	26787-78-0	Sigma Aldrich	365,4	1,0	2,9	11,7	0,9
Ampicilina[b]	AMP		69-53-4		349,4	10,0	2,6	7,2	1,4
Cloranfenicol[c]	CHL	Anfenicoles	56-75-7	Acofarma	323,1	2,5	7,5	-2,8	1
Eritromicina[d]	ERY	Macrólidos	114-07-8		733,9	2,0	8,9	8,9	-3
Estreptomicina (sulfato)[e]	STM	Amino-glucósidos	3810-74-0	Sigma Aldrich	581,6	75,0	10,9	12	-5,2
Gentamicina (sulfato)[h]	GTM		1405-41-0		447,6	50,0	12,6	10,1	-2,4
Penicilina G (sal sódica)[i]	PEN	Beta-lactámicos	69-57-8		356,4	75,0	3,5	-2,8	1,2
Tetraciclina (clorhidrato)[j]	TC	Tetraciclinas	64-75-5	Acofarma	444,4	75,0	3,3	9,2	-2

Productos naturales

Nombre	*Abrev.*	*Familia*	*Número CAS*	*Proveedor*	*Masa molecular (g/mol)*	*Solubilidad en agua (mg/mL)*	*pKa*	*log Ko/w*
Nerol	NE	Terpenos	106-25-2	Sigma Aldrich	154,25	531 mg/L	14,45 ± 0,10 (predicción)	3,47
Ácido Tánico	AT	Polifenoles	72401-53-7	Sigma Aldrich	1701,2	250 g/L	2,5 - 8,5	–0,19

a - j: National Center for Biotechnology Information, 2025a-j

4.2 Microorganismos seleccionados

Para este estudio se seleccionaron 14 especies bacterianas. Todas ellas presentan cepas incluidas, como se detalla anteriormente, en la Lista de Patógenos Bacterianos Prioritarios de la OMS (Abdallah et al., 2023; WHO, 2024b), que se encuentran entre las principales causas de muerte por infecciones bacterianas a nivel global (GBD 2021 Antimicrobial Resistance Collaborators, 2022).

a) Bacillus subtilis

B. subtilis (actualmente denominado *Bacillus spizizenii*) es un bacilo grampositivo aerobio facultativo, cuya principal característica es su capacidad de formar biofilms y endosporas protectoras especialmente resistentes (Setlow, 2006; Harwood et al., 2013). Es uno de los microorganismos más estudiados (Stülke et al., 2023). Aunque no se

considera un frecuente patógeno humano, puede causar infecciones oportunistas, como bacteriemia asociada a catéteres, endocarditis, queratitis tras traumas oculares e infecciones de heridas, especialmente en pacientes con dispositivos, traumas o inmunosupresión (Weber, D. J. et al.,1989; Sliman, R. et al., 1987).

Es una de las bacterias más encontradas en las aguas y fangos de las depuradoras (Hlordzi et al., 2020), donde se ha demostrado su capacidad para adquirir resistencias antimicrobianas (Ul Haq et al., 2022).

b) Enterococcus faecalis

E. faecalis es una bacteria grampositiva anaerobia facultativa, perteneciente al género de los enterococos. Es, junto con *E. faecium*, una de las dos especies de enterococos comensales en el ser humano y presentes en su intestino (Fisher y Phillips, 2009). En España y Reino Unido, ambas bacterias (mayoritariamente *E. faecalis*) son las más comúnmente aisladas, tanto de muestras clínicas como medioambientales (Kühn et al., 2003).

A pesar de que fue considerada inocua durante años, *E. faecalis* es uno de los más comunes y peligrosos patógenos nosocomiales (Arias y Murray, 2012; Pöntinen et al., 2021) (es decir, aquellos que se transmiten en centros sanitarios), y está asociado a una alta morbilidad y mortalidad (Fisher y Phillips, 2009). Entre sus cepas más virulentas, destacan aquellas que han desarrollado resistencia a sus tratamientos habituales (Pelletier, 1996), como AMP o vancomicina (Fisher y Phillips, 2009; Brinkwirth et al., 2021).

c) Listeria monocytogenes

L. monocytogenes es una bacteria grampositiva y anaerobia facultativa, ampliamente distribuida en el medioambiente. Es una de las causas de contaminación alimentaria más conocida, y es capaz de reproducirse incluso a temperaturas de refrigeración (Agencia Española de Seguridad Alimentaria y Nutrición (AESAN), 2024). Una vez en el organismo puede producir listeriosis, una infección que por su virulencia y mortalidad sitúan a esta bacteria como uno de los patógenos alimentarios más peligrosos (Instituto Nacional de Seguridad y Salud en el Trabajo. Ministerio de Trabajo, 2024). Además, *L. monocytogenes* genera una creciente preocupación por el aumento en el número de cepas aisladas resistentes a varios ATBs (Rostamian et al., 2022).

d) Strepcococcus agalactiae

Se trata de un coco grampositivo anaerobio facultativo, y también es conocido como *Estreptococo Grupo B*. Es un patógeno habitual del ser humano, capaz de provocar infecciones graves, especialmente en recién nacidos (van Kassel et al., 2021). Numerosas cepas de *S. agalactiae* resistentes a penicilinas o macrólidos han ido siendo aisladas de

forma creciente en diversas partes del mundo (Sabroske et al., 2022), y en algunos lugares, como Japón, se han detectado cepas multirresistentes (es decir, resistentes a 3 o más tipos de antimicrobianos) (C. Li et al., 2020). Los estreptococos del grupo B resistentes a PEN están incluidos en la lista de patógenos prioritarios de la OMS en un nivel de prioridad medio.

e) Staphylococcus aureus

S. aureus es un coco grampositivo, anaerobio facultativo, y un patógeno oportunista, distribuido como comensal en gran parte de la población (Lee et al., 2018). Entre otras, se asocia comúnmente a infecciones agudas y crónicas en la piel y en los tejidos blandos (Keim y Horswill, 2023), presentando una muy elevada variabilidad entre sus cepas, patología y factores de virulencia (Piewngam y Otto, 2024). *S. aureus* es la bacteria que más muertes causa a nivel mundial, estimándose en más de un millón anualmente (GBD 2021 Antimicrobial Resistance Collaborators, 2022).

Estas altas tasas de mortalidad y morbilidad se deben, en parte, a la extraordinaria capacidad de esta bacteria para adquirir resistencia a los ATBs (Lee et al., 2018; Turner et al., 2019), habiéndose detectado cepas resistentes a PEN en la primera mitad del siglo XX (Foster, 2017). Desde entonces, estas cepas resistentes se han extendido, siendo especialmente conocidas el SARM, o la más reciente *SARV* (*S. aureus* resistente a Vancomicina), aún más peligrosa, ya que la vancomicina es uno de sus tratamientos de último recurso (Melo-Cristino et al., 2013; Foster, 2017; Shariati et al., 2020). Las cepas de SARM también están incluidas en la lista de patógenos prioritarios de la OMS, e incluidas en el grupo de riesgo alto (WHO, 2024a).

f) Acinetobacter baumanii

A. baumanii es una bacteria gramnegativa aerobia estricta. Es un patógeno habitual del ser humano, conocido por provocar numerosas infecciones oportunistas nosocomiales (es decir, aquellas adquiridas en centros sanitarios por pacientes inmunocomprometidos) (Hong et al., 2021).

Esta bacteria ha adquirido resistencias a numerosos ATBs, como betalactámicos, fluorquinolonas o aminoglucósidos, llegando a representar un porcentaje muy significativo de todas las infecciones en algunas zonas, como en el este de Europa (Kyriakidis et al., 2021). En consecuencia, algunas de sus cepas resistentes (como aquellas a carbapenemes) se encuentran en el tercer lugar en la lista de patógenos prioritarios de la OMS (WHO, 2024b) y en el nivel “crítico”, en el que se recogen aquellas cepas patógenas que suponen el mayor riesgo para la salud, por sus opciones limitadas de tratamiento, alta mortalidad y crecientes resistencias adquiridas.

g) Escherichia coli

E. coli es un bacilo gramnegativo anaerobio facultativo, que pertenece a la familia de las enterobacterias. Se encuentra habitualmente en el intestino distal de mamíferos, incluido el ser humano. Aunque en condiciones normales es parte de la flora intestinal, varias cepas son patogénicas, y pueden causar infecciones localizadas (como la gastroenteritis) o sistémicas, tanto en individuos sanos como inmunocomprometidos (Kaper et al., 2004). Algunas de ellas son especialmente virulentas, como las cepas *enteropatogénicas* (EPEC), *enterohemorrágicas* (EHEC), productoras de toxina Shiga, o *enteroinvasivas* (EIEC), entre otras (Gomes et al., 2016). Es la segunda bacteria que más mortalidad causa a nivel mundial, provocando cerca de un millón de decesos anualmente (GBD 2021 Antimicrobial Resistance Collaborators, 2022).

Además, un número preocupante de cepas de *E. coli* han desarrollado multirresistencias a ATBs, incluyendo algunas resistentes a carbapenemes y productoras de betalactamasas. Tanto es así que las cepas de *E. coli* resistentes a cefalosporinas de tercera generación ocupan el segundo puesto de la Lista de Patógenos Prioritarios de la OMS, actualizada en enero de 2024 (WHO, 2024a), y están incluidas, al igual que *A. baumanii*, en el grupo de prioridad crítica.

h) Klebsiella aerogenes

Conocida hasta fechas recientes como *Enterobacter aerogenes*, es un bacilo gramnegativo. Es un patógeno humano nosocomial y virulento y, con tasas de mortalidad de entre el 13,5%–28%, según reportan algunos estudios (Feng et al., 2024), frecuentemente asociadas a neumonías y tracto urinario. Es considerado como un patógeno emergente, con numerosos casos reportados de brotes de cepas resistentes a carbapenemes, o multirresistentes (Morgado et al., 2024).

i) Klebsiella pneumoniae

K. pneumoniae es un bacilo gramnegativo anaerobio facultativo perteneciente, al igual que *K. aerogenes*, a la familia de las enterobacterias (Yang et al., 2023). Es un patógeno muy diverso, y supone un porcentaje significativo de las muertes relacionadas con multirresistencias bacterianas (GBD 2021 Antimicrobial Resistance Collaborators, 2022; Kochan et al., 2023).

Algunas de sus variedades son especialmente virulentas (Dong et al., 2022) y causan infecciones complicadas y difíciles de tratar, especialmente pulmonares (neumonía) (Patil et al., 2023), con una elevada mortalidad a nivel mundial. Las cepas con resistencia adquirida a carbapenemes y cefalosporinas, de hecho, han escalado hasta el primer y sexto puesto, respectivamente, de la Lista de Patógenos Prioritarios de la OMS (WHO, 2024b).

j) Pasteurella aerogenes

P. aerogenes es un cocobacilo gramnegativo. Está presente (como comensal o patógeno) fundamentalmente en animales, por lo que puede causar infecciones de piel y partes blandas, especialmente tras heridas o mordeduras en este tipo de entornos (Sathe et al., 2023). Algunas de sus cepas han adquirido resistencias a los antimicrobianos: se han detectado cepas resistentes a ATBs como las TCs, por ejemplo, en el tracto intestinal de ganado bovino (Kehrenberg y Schwarz, 2001).

k) Proteus mirabilis

P. mirabilis es una bacteria gramnegativa, anaerobia facultativa y extremadamente móvil, gracias a sus flagelos y secreciones de polisacáridos (Jamil et al., 2023). Es conocida por ser la causante más habitual de infecciones urinarias asociadas al empleo de catéteres (Armbruster y Mobley, 2012; Schaffer y Pearson, 2015). Esta bacteria ha ido adquiriendo multirresistencias causantes de preocupación (Alqurashi et al., 2022), estando incluidas algunas ellas en la Lista de Patógenos Prioritarios de la OMS.

l) Pseudomonas aeruginosa

P. aeruginosa es un bacilo gramnegativo aeróbico, con una alta complejidad genética, y especialmente versátil (Sathe et al., 2023). Esta bacteria es una de las principales causantes de infecciones nosocomiales, tanto agudas como crónicas, y se asocia a altas tasas de morbilidad, mortalidad y resistencia a antimicrobianos (Sastre-Femenia et al., 2023), siendo una de las 5 bacterias causantes de más muertes por infecciones a nivel global (GBD 2021 Antimicrobial Resistance Collaborators, 2022).

Algunas de las cepas de *P. aeruginosa*, como aquellas resistentes a carbapenemes, están incluidas en la Lista de Patógenos Prioritarios de la OMS, y en el grupo de prioridad crítico (WHO, 2024a). Estas cepas productoras de carbapenemasas están aumentando considerablemente, como atestiguan estudios realizados en España (Sastre-Femenia et al., 2023) o EE.UU. (Tenover et al., 2022), hasta llegar a más del 20% de los aislados clínicos.

m) Salmonella enterica sv. Typhimurium

S. enterica es un bacilo gramnegativo anaerobios facultativos, perteneciente, como *E. coli* o *K. pneumoniae*, a la familia de las enterobacterias, y por tanto presente en el intestino de mamíferos, como los humanos (Yang et al., 2023). Debido a las notables diferencias entre algunos grupos, esta especie se subdivide en 5 serovariantes (*sv*), siendo una de ellas *S. enterica sv. enterica* (Andino y Hanning, 2015). Dentro de esta subespecie existen, a su vez, multitud de serovariantes, que habitualmente se clasifican por su capacidad infectiva en el ser humano. Debido a que una de las condiciones que puede causar se denomina *fiebre entérica* o *fiebre tifoidea*, la subespecie causante de esta infección se conoce como *S. enterica* Typhi (Gal-Mor et al., 2014). Al igual que otras, esta

bacteria también ha conseguido adquirir resistencia a varios de sus tratamientos, como AMP, CHL o la combinación de sulfametoxazol con trimetoprim, entre otros (Kavai et al., 2024). A pesar de que la existencia de vacunas ha mejorado la situación, sigue siendo una de las bacterias que más mortalidad causa a nivel mundial (Gal-Mor et al., 2014; GBD 2021 Antimicrobial Resistance Collaborators, 2022). Las cepas de *S. enterica* Typhy resistentes a fluorquinolonas están incluidas en el séptimo lugar de Lista de Patógenos Prioritarios de la OMS, y en el grupo de prioridad crítico (WHO, 2024a).

n) Serratia marcescens

S. marcescens es un bacilo gramnegativo y patógeno humano, perteneciente también a la familia de las enterobacterias. Es conocido desde principios del siglo XIX, y presenta la capacidad de producir, en algunas de sus cepas, una característica pigmentación roja (Zivkovic Zaric et al., 2023). Es un patógeno principalmente nosocomial, y en humanos puede causar, entre otras, infecciones urinarias, respiratorias, endocarditis o infecciones de heridas (Acar, J. F., 2015).

Se trata de una especie con una alta variabilidad genómica, y muchas de sus cepas tienen una elevada capacidad de generación de RAMs (Iguchi et al., 2014). Se han observado brotes nosocomiales multirresistentes en varias zonas de Europa (Moradigaravand et al., 2016).

Las distintas cepas seleccionadas se recogen en la tabla 7, junto con su temperatura y medio de cultivo indicadas en su guía de producto ATCC (siglas de la empresa *American Type Culture Collection*):

Tabla 7. Bacterias seleccionadas y condiciones de cultivo

Gram	Bacteria	Cepa	Temperatura de cultivo	Medio de cultivo
+	*Bacillus subtilis*	*ATCC 6633*	*30°C*	*BHI*
	Enterococcus faecalis	*ATCC 19433*	*37°C*	
	Listeria monocytogenes	*ATCC 7644*		
	Streptococcus agalactiae	*ATCC 12386*		
	Staphylococcus aureus	*ATCC 9144*		*TSA*
–	*Acinetobacter baumannii*	*ATCC 19606*	*37°C*	*CN*
	Escherichia coli	*ATCC 25922*		*TSA*
	Klebsiella aerogenes	*ATCC 13048*	*30°C*	*CN*
	Klebsiella pneumoniae	*C6*		
	Pasteurella aerogenes	*ATCC 27883*	*37°C*	*TSA*
	Proteus mirabilis	*ATCC 35659*		
	Pseudomonas aeruginosa	*ATCC 27853*		
	Salmonella enterica sv. typhimurium	*ATCC13311*		*CN*
	Serratia marcescens	*ATCC 13880*	*26°C*	

***TSA**: Tryptic soy agar. **BHI**: Brain-Heart Infusion. **CN** (Nutrient broth). Fuente: elaboración propia.*

Todos los microorganismos se adquirieron como liofilizados (Culti-loops™) al proveedor Thermo Scientific (Datford, Reino Unido), para posteriormente ser rehidratados y congelados a -80°C en crioviales (Deltalab S.L, Barcelona) hasta el comienzo de cada ensayo. Los procesos de rehidratación, cultivo y ensayos microbiológicos se llevaron a cabo siguiendo las instrucciones detalladas en las fichas técnicas facilitadas por Thermo Scientific y ATCC ("ATCC: The Global Bioresource Center," 2024) y en condiciones de esterilidad.

Los medios de cultivo utilizados fueron TSA (Tryptic soya broth, BHI (Brain-Heart Infusion) y CN (caldo nutritivo).

4.3 Concentraciones Mínimas Inhibitorias y Concentraciones Mínimas Bactericidas

La actividad antibacteriana de cada compuesto se determinó mediante ensayos de CMI. Para ello, se utilizó el método de *broth-microdilution* (basado en diluciones en serie) en placas de 96 pocillos de fondo redondo (Deltalab), según las directrices detalladas por el ISO 207776-1 (2019) y el Laboratory Standards Institute (CLSI, M07-A9 2018) (Figura 4). Todos los ensayos se llevaron a cabo en una campana extractora (Modelo MSC Advantage) en condiciones de esterilidad.

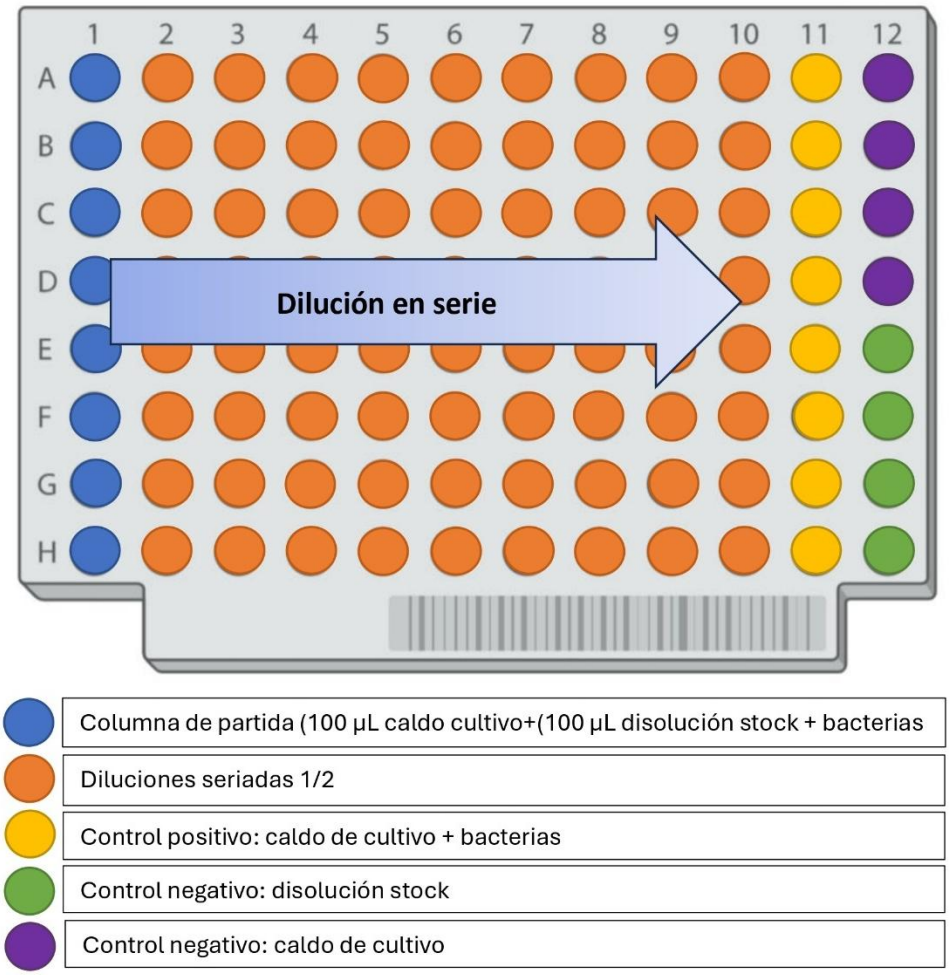

Figura 4. Representación de una placa de 96 pocillos y realización del ensayo de CMI

En cada pocillo de la placa se introdujeron primeramente 100 µL del medio de cultivo adecuado para cada bacteria, según las instrucciones de producto ATCC. Posteriormente, se introdujeron otros 100 µL de sustancia a testar (ATB o PN), aplicando entonces el método de diluciones seriadas a la mitad desde la columna 1 a la 10, que dejan un volumen final de cada pocillo de 100 µL. Las columnas 11 y 12 se reservaron para un control positivo (para medir el crecimiento bacteriano) y un control negativo (para comprobar las condiciones de esterilidad), respectivamente.

Finalmente, a todas las columnas salvo la correspondiente al control negativo (columna 12) se añadieron 10 µL de inóculo bacteriano. Dicho inóculo se obtuvo 24h después de haber incubado los crioviales en caldo de cultivo, este inóculo fue estandarizado según el método McFarland (Sayed y Tzanis, 2025), con el objetivo de conseguir una concentración inicial de bacterias de aproximadamente 2.5×10^5 unidades formadoras de colonias (UFC)/mL (Clinical and Laboratory Standards Institute, 2018). Para ello, se utilizó el lector de placas Hybrid Multimode BioTekTM Synergy H1 (625 nm).

Una vez completadas e inoculadas, las placas se incubaron 24 horas a la temperatura adecuada para cada bacteria (incubador Incuterm, Trade Raypa®). Los resultados finales se obtuvieron en el mismo lector de placas en el que se efectuó el protocolo McFarland. La definición de CMI empleada fue la descrita por la guía CLSI M07-A9 (2018). Según esta definición, la CMI se establece como la concentración más baja que inhiba visiblemente el crecimiento bacteriano. Además, se midió la absorbancia de cada pocillo (625 mm) para una medición precisa de los resultados.

Tras identificar los pocillos correspondientes a la CMI y a diluciones superiores (concentraciones menores), se tomó el contenido de varios de estos pocillos replicados y se sembró en placas de Petri con medio de cultivo fresco. Posteriormente, las placas se incubaron durante 24 horas. Si tras la incubación se observó crecimiento de colonias, el efecto del ATB se consideró bacteriostático (inhibición del crecimiento, pero sin eliminación completa de la población bacteriana). Si no se observó crecimiento de colonias, el efecto se consideró bactericida. De esta forma, la CMB se definió como la concentración más baja del agente antimicrobiano en la que no se detectó crecimiento bacteriano en las placas de cultivo.

Una vez obtenidos los valores CMI y CMB se calculó el cociente CMI/CMB, obteniendo así una estimación de la capacidad bacteriostática o bactericida de cada compuesto. Si este cociente es ≤4, se considera que la sustancia tiene una actividad bactericida (Adrar et al., 2016; Tokam-Kuaté et al., 2021).

4.4. Combinaciones sinérgicas

Tras procesar y analizar los datos obtenidos en los ensayos de CMI y CMB para ATBs y PNs, se ensayaron las distintas combinaciones ATB + PN + bacteria para determinar posibles interacciones sinérgicas. En estos cruces se seleccionaron aquellas combinaciones con una CMI antibiótica moderadamente baja, y concentraciones de AT y NE que permitan solubilizar el PN (no mayores a 4000 µg//mL).

Los cruces resultantes se testaron de forma similar al anterior ensayo, en placas de 96 pocillos de fondo redondo. Sin embargo, en este caso se ensayan dos productos, por lo que uno (el ATB) se diluye en dirección vertical, desde la fila A hasta la G, y otro (el PN) se diluye en serie en dirección horizontal, desde la columna 1 a la 7. Este método, llamado tablero de ajedrez (*checkboard)*, permite obtener combinaciones diferentes en cada pocillo (Figura 5). Las disoluciones stock de cada producto fueron 4 veces más altas que su CMI para cada bacteria, para garantizar una lectura adecuada de los resultados en las columnas centrales de la placa.

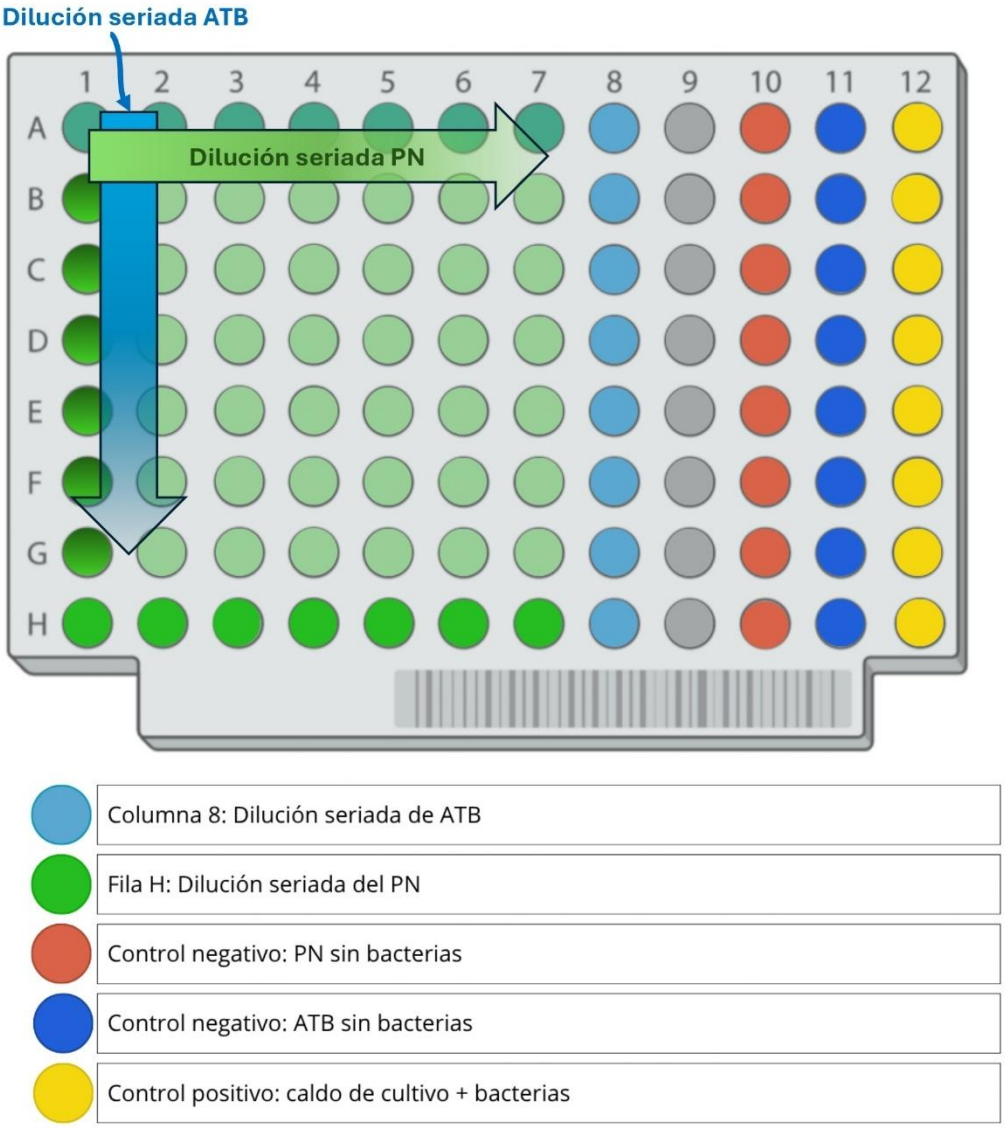

Figura 5. Representación de una placa de 96 pocillos y realización del ensayo checkboard *para la identificación de combinaciones sinérgicas*

Una vez completadas, las placas fueron inoculadas con 10 µL de suspensión bacteriana, preparada de la misma manera que en la sección 4.3. Como se detalló, las placas se incubaron durante 24h a la temperatura adecuada para cada cepa (Tabla 7), para leer posteriormente los valores de absorbancia (625 nm) que determinan el crecimiento bacteriano.

Los resultados de las posibles sinergias se interpretaron calculado el índice FIC_I, (Ecuación 1) que define el tipo de interacción producida entre dos sustancias (Jayaraman et al., 2010):

$$FIC_I = FIC_A + FIC_B = \frac{CMI_{A+B}}{CMI_A} + \frac{CMI_{B+A}}{CMI_B} \quad (Ec.\,1)$$

En la ecuación anterior, el compuesto A es un PN (NE o TA), y B es un ATB comercial ATB_X. FIC es el Factor de Interacción Combinada. El FIC_A es, por tanto, el cociente entre la CMI del compuesto A en presencia del compuesto B (CMI_{A+B}) entre la CMI del compuesto A sólo (CMI_A). FIC_B, por su parte, es el cociente entre la CMI del compuesto B en presencia del compuesto A (CMI_{B+A}) y la CMI del compuesto B sólo (CMI_B).

Siguiendo las directrices del *European Committee on antimicrobial susceptibility testing ("Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents," 2000)*, se define como sinergia toda aquella interacción con un cociente $FIC_I \leq 0{,}5$. Los valores entre 0,5 y 1 corresponden a interacciones de carácter aditivo, y los valores ≥2 indican efectos antagónicos (Hu et al., 2002; Novy et al., 2013).

Partiendo de las 10 combinaciones sinérgicas obtenidas, se decidió evaluar la ecotoxicidad de 6 de ellas, atendiendo en especial a aquellas en las que se produjo la mayor reducción del ATB, con el fin de alcanzar el objetivo general de la Tesis y valorar la ecotoxicidad de estas sinergias en comparación con su ATB a su CMI.

Las sinergias seleccionadas para el estudio ecotoxicológico se nombraron, para facilitar la lectura de resultados, como *Synergic Antibiotic Combination* (SAC). Para las combinaciones sinérgicas de AT, que fueron las siguientes:

- SACTA 1: AT 46,87 + CHL 3,91
- SACTA 2: AT 46,87 + AMP 7,81
- SACTA 3: AT 40,62 + CHL 1

Por su parte, para las combinaciones sinérgicas de NE:

- SACNE 1: NE 125 + STM 1,17
- SACNE 2: NE 125 + GTM 0,78
- SACNE 3: NE 250 + STM 31,25

4.5. Representación en isobologramas

Para visualizar de manera más clara las interacciones entre los ATB y los PN, los resultados obtenidos en los ensayos *checkerboard* se representaron mediante isobologramas (figura 6) (Garrido-Suárez et al., 2021; Ndip et al., 2023). Las combinaciones mostradas fueron aquellas con un coeficiente de interacción (FIC) ≤ 0,5, considerado indicativo de sinergia.

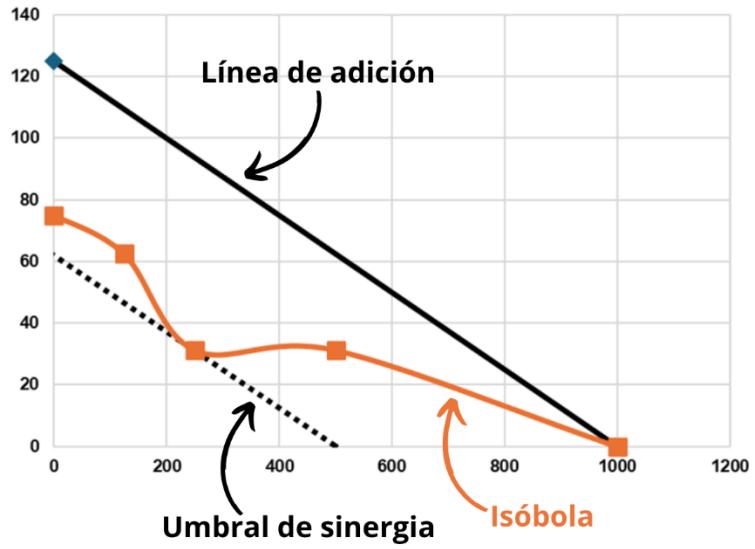

Figura 6. Ejemplo de representación de un isobolograma. Fuente: elaboración propia

En la representación gráfica, el eje de abscisas (X) corresponde a la CMI del PN, mientras que el eje de ordenadas (Y) representa la CMI del ATB. Cada punto del gráfico refleja una combinación específica de concentraciones que produjo un mismo efecto biológico, en este caso la inhibición del crecimiento bacteriano. Al unir los puntos que generan igual nivel de inhibición, se obtiene una curva denominada isóbola.

Como se observa en la figura 6, la línea continua, conocida como línea de adición, señala el comportamiento teórico esperado si la combinación presenta un efecto puramente aditivo. Las desviaciones respecto a esta línea permiten interpretar el tipo de interacción: una isóbola cóncava situada por debajo de la línea de adición indica sinergia, mientras que una isóbola convexa, por encima de ella, indica antagonismo. Finalmente, se incluye una línea discontinua que representa el umbral clásico de sinergia; los puntos localizados sobre o por debajo de esta línea reflejan los distintos grados de interacción sinérgica entre ambos agentes.

4.6. Estudio cinético de las combinaciones sinérgicas

Para comprender la evolución en el tiempo de las interacciones sinérgicas (es decir, aquellas combinaciones en las que se observó un $FIC_I \leq 0{,}5$), se llevaron a cabo estudios cinéticos de crecimiento bacteriano.

Al igual que en los ensayos detallados en las secciones 4.3 y 4.4, los cultivos bacterianos se estandarizaron mediante el protocolo McFarland. Posteriormente, siguiendo la información obtenida del ensayo *checkboard* (sección 3.4), se prepararon nuevas placas de 96 pocillos, exponiendo los microorganismos a concentraciones de CMI y sub CMI de ATBs, PNs y sus combinaciones. Las placas preparadas se introdujeron en el lector de absorbancias, que permite tomar lecturas de absorbancia cada hora durante

el proceso de incubación. Los datos resultantes, representados como absorbancia frente a tiempo, son representados en curvas crecimiento/tiempo. Cada lectura de absorbancia consiste en la lectura de 4 replicados (pocillos) iguales. Las curvas cinéticas fueron ajustadas a un modelo logístico (ecuación 2) para crecimiento microbiano sigmoidal:

$$Absorbancia\ (Abs) = \frac{Cmax}{1 + e^{b-rt}} \qquad (Ec.2)$$

C_{max} corresponde al crecimiento microbiano máximo alcanzado durante el experimento, r es la ratio de crecimiento bacteriano, y b es el parámetro de ajuste.

La significancia estadística de las diferencias de estas curvas cinéticas, en comparación con el control, fue determinada utilizando el test ANOVA, realizado con el software SPSS (versión 28.0.1.0, 142).

4.7. Perfil fisiológico de comunidades microbianas utilizando Ensayos con BIOLOG EcoPlates™

Con el objetivo de detectar cambios en el perfil fisiológico de los microorganismos expuestos a diferentes concentraciones de las diferentes sustancias objeto de ensayo, se utilizaron las placas BIOLOG EcoPlate™ (Biolog Inc., Hayward, CA, EE.UU). Estas placas contienen en sus pocillos, por triplicado, 31 fuentes diferentes de carbono además de un control que pueden agruparse en 5 clases metabólicas: aminas/amidas, aminoácidos, ácidos cetónicos y carboxílicos, polímeros y carbohidratos.

Cuando se agrega un inóculo microbiano a cada pocillo, la utilización del sustrato se indica mediante la reducción de un tinte de tetrazolio, lo que provoca un cambio de color proporcional a la actividad metabólica. Estos ensayos se realizaron tanto para los ATB, los PN y 6 de las combinaciones sinérgicas. Previamente, las comunidades bacterianas fueron secuenciadas utilizando el gen 16S para conocer su diversidad. Se aplicó esta técnica tanto a comunidades microbianas de suelo como fluviales.

Para los ensayos con microorganismos de río, se recogieron muestras en un punto del Río Gállego cercano a la ciudad de Zaragoza en 3 fechas diferentes: junio de 2019, mayo de 2021 y octubre de 2022.

Las muestras de agua fueron recogidas en recipientes estériles de 1,5 L (Anaclin S.L, Móstoles, España), y se comprobaron in situ los siguientes parámetros:

- Temperatura, utilizando un termómetro Nahita (ICT S. L., España)
- pH, medido utilizando un pHmetro PanReac AppliChem A011435
- Conductividad, medida utilizando un conductímetro Hanna HI8733 (Merck, Spain).

Ya en el laboratorio, y trabajando siempre en campana de flujo laminar (Modelo MSC Advantage 1.2), el agua muestreada se filtró utilizando filtros de 70 mm de poro (BD

Falcon®, Thermo Fisher Scientific, Waltham, MA, EE.UU) para eliminar los residuos sólidos de mayor tamaño

Para los ensayos con microorganismos de suelo, por su parte, se obtuvieron muestras de un campo de cultivo desprovisto de pesticidas u otros contaminantes de composición conocida. El procedimiento seguido (Pino-Otín et al., 2021) se inició con el tamizado de la muestra de suelo con tamiz de 2 mm (Becton Dickinson, Zaragoza). A 10 g de ese tamizado se le añadieron 95 mL de agua estéril, y se agitó la muestra en un matraz Erlenmeyer durante 30 minutos, dejando un tiempo de reposo posterior de 1 hora. A continuación se recogieron los primeros 10 mL del sobrenadante (es decir, la porción líquida no precipitada), y se centrifugaron a 1000 × g durante 10 minutos. Este proceso se repitió 5 veces. Finalmente, el sobrenadante resultante fue pasado por un filtro de 70 µm de poro (Becton Dickinson, Zaragoza) para eliminar posibles restos aún en suspensión.

Los ensayos con BIOLOG EcoPlate™, independientemente del origen de la muestra, se realizaron de la misma manera. Con una pipeta multicanal, se colocaron 75 µL de la muestra de ecosistema en cada pocillo, a los cuales se añadieron 75 µL de la disolución a testar (PN o ATB comercial). El pH final de las disoluciones fue medido con un pH-metro (Hach, Sension+ pH3) para asegurar que se encontraba en un rango de neutralidad. Cada concentración se probó por triplicado. Como control, se utilizó agua de río filtrada del mismo modo, pero sin añadir disolución a testar.

En el caso de los ATBs, las concentraciones ensayadas fueron 0,1, 100 y 1000 mg/L. En el caso de NE, las concentraciones testadas fueron 0,1, 10 100 y 1000 mg/L, y para AT fueron 0,2, 2, 20 y 200 mg/L. Para los ensayos de ecotoxicidad de las concentraciones sinérgicas, por su parte, se expuso a las comunidades microbianas a la concentración sinérgica y a sus componentes por separado (tanto a la concentración a la que se encuentran en la combinación sinérgica como a sus respectivas CMI).

Para la lectura de resultados, se midió la densidad óptica (DO) de cada pocillo a 590 mm utilizando el lector de placas y el software de análisis de datos Gen5™. Tras la primera lectura, contabilizada como *tiempo 0*, las placas se incubaron a 25ºC (incubador J.P Selecta, Barcelona) durante 144 horas (7 días), y se procedió a medir de nuevo su DO cada 24 h durante ese tiempo, obteniendo de esta forma 8 medidas finales.

Una vez obtenidos los datos de DO, se calculó el parámetro *Desarrollo Medio del Color de los Pocillos* (AWCD, por sus siglas en inglés), como se describe en bibliografía previa (Garland y Mills, 1991; Gu et al., 2014) (Ecuación 3):

$$AWCD = \sum_{i=0}^{i=7}(Abs_{t=xi} - Abs_{t=x0}) \quad (Ec.3)$$

$Abs_{t=xi}$ corresponde al valor de la DO de un pocillo en un momento dado, y $Abs_{t=x0}$ es la DO del mismo pocillo al principio del experimento.

Las curvas de crecimiento AWCD se modelaron utilizando una función logística (ecuación 4) para describir el crecimiento microbiano sigmoidal . El ajuste de curvas se realizó con el complemento Solver de Excel (Microsoft 365):

$$AWCD = \frac{Cmax}{1 + e^{b-rt}} \qquad (Ec. 4)$$

4.8. Análisis genético de las muestras de agua y suelo

Para comprender mejor el impacto de las distintas sustancias y combinaciones en el crecimiento y metabolismo de los microorganismos, se analizó mediante secuenciación genética la composición taxonómica y los taxones predominantes de estas comunidades. Esta secuenciación genética de los microorganismos fluviales y de suelo, fue realizada por la Unidad de Genómica del Parque de las Ciencias de Cantoblanco (Madrid).

En el caso de las muestras obtenidas del Río Gállego, parte del volumen de agua se procesó además con un equipo de ultrafiltración (0,22 m de diámetro de poro, Sterifix B. Braun Medical S. A. U, Rubí, Barcelona) con el objetivo de retener las bacterias. Una vez saturados los filtros, se lavaron con PBS (solución salina tamponada con fosfato, pH = 7,5) y las suspensiones bacterianas se recogieron en placas de Petri. Las suspensiones bacterianas se distribuyeron entonces en 6 tubos Falcon de 10 mL y se centrifugaron a 5000 g durante 10 minutos a temperatura ambiente (centrifugadora modelo Heraeus Biofuge Primo R, Thermo Fisher Scientific, Waltham, MA EE.UU.). Tras el centrifugado, se retiró el sobrenadante con pipetas Pasteur y se añadieron 10 mL de agua estéril. Este proceso se repitió 5 veces y el producto final se almacenó a -80 C (congelador Froilabo Trust, Collégien, Francia) para su posterior secuenciación genética.

Para las muestras de suelo, primeramente, se mezclaron en 100 mL de agua estéril, agitándose durante 30 minutos, y se dejó reposar 1 hora. A continuación, la muestra se separó en tubos Falcon de 10 mL, se sometió a ultrasonidos durante 1 minuto y se centrifugó a 1000 × g durante otros 10 minutos. En ese momento se recogió el sobrenadante, y siguiendo el mismo proceso descrito para las muestras de río, fue filtrado y conservado de la misma forma.

En ambos casos, el ADN genómico bacteriano de las muestras previamente homogeneizadas en PBS se extrajo mediante columnas G-spin a partir de alícuotas de 200 µL (INTRON Biotechnology, Corea del Sur). La concentración de ADN se evaluó utilizando el reactivo Quant-IT PicoGreen de Thermo Fischer. Las muestras de ADN se utilizaron para amplificar la región V3-V4 del gen del ARN ribosómico 16S (ARNr) (Caporaso et al., 2011, 2012; Pino-Otín et al., 2021).

Las bibliotecas de amplicones individuales se analizaron en un Agilent Bioanalyzer 2100, y la concentración se determinó mediante PCR en tiempo real (Kapa Biosystems). Las muestras de ADN se secuenciaron utilizando un instrumento Illumina MiSeq de acuerdo con un procedimiento 2 x 300. Utilizando las aplicaciones actuales de Base Space, las lecturas se filtraron por calidad utilizando los valores estándar de Illumina, se demultiplexaron y los archivos fastq se asignaron a la base de datos GreenGenes (16S Metagenomics, Illumina).

4.9. Ecotoxicidad en organismos bioindicadores

Las comunidades microbianas, que fundamentan muchos de los procesos metabólicos en ecosistemas fluviales y terrestres, son excelentes indicadores, reflejan de manera más realista el impacto de un compuesto sobre comunidades completas, su estudio presenta información más integradora y ecológicamente relevante. Sin embargo, el análisis de indicadores individuales estándar permite obtener datos de ecotoxicidad más estandarizados, cuantitativos y comparables entre estudios y laboratorios. Estos organismos, al ser ampliamente utilizados y validados, facilitan establecer niveles de toxicidad específicos y garantizar la reproducibilidad de los resultados. Por ello, la combinación de ambos enfoques es fundamental para una evaluación comprensiva y robusta del riesgo ecotoxicológico en ambientes naturales. Por ello se seleccionaron indicadores estándar clave de ecosistemas acuáticos y terrestres como: *Daphnia magna* y *Aliivibrio fischeri* y algas del perifiton, para ecosistemas acuáticos. Para ecosistemas terrestres, se utilizó tanto la lombriz común (*Eisenia fetida*) como la planta de cebolla (*Allium cepa*).

4.9.1 Ensayos de inmovilización de *Daphnia magna*

D. magna es un crustáceo planctónico de pequeño tamaño, y es ampliamente utilizado como bioindicador en ecosistemas acuáticos (OECD, 2004; Shahnawaz, 2023), es un crustáceo planctónico. Es característico por su condición de organismo filtrador, facilitando la posible acumulación de contaminantes.

Los ensayos llevados a cabo con esta especie, adquirida a la empresa española Vidrafoc (ref. DM121219) se llevaron a cabo siguiendo las instrucciones operativas contenidas en el Daphtoxkit® FTM magna (1996), y las directrices OECD 202 (2004) (OECD, 2004).

Las epifias (huevos de este crustáceo) de *D. magna* fueron almacenadas a 5ºC hasta el comienzo de los ensayos. Como primer paso del experimento, estas epifias se introdujeron en placas Petri con medio el medio de cultivo incluido en el Daphtokit®, y fueron incubadas a 20–22 °C y luz de 6000 lx durante 72 horas (incubador TOXKIT modelo CH-0120D-AC/DC, suministrado por ECOTEST, Valencia), cambiando el medio cada 24 h. Pasadas 72 h a estas placas Petri se le añadió el alga espirulina como alimento, también incluida en el Daphtokit®, dos horas antes de exponer los especímenes a la sustancia a testar.

Las sustancias fueron disueltas en agua esterilizada (ISO 6341, 2012), y diluidas hasta obtener las diversas concentraciones de ensayo. Esta agua esterilizada fue la única sustancia en contacto con los crustáceos en el control negativo. El pH se ajustó a 7 – 7,5 con una solución 0,1M de NaOH.

Cada concentración se ensayó con 5 replicados, de 5 organismos cada una. Las concentraciones testadas, tanto para AT como NE, fueron 0,2, 2, 20, 200, y 2000 mg/L.

La exposición se mantuvo durante 24h, a una temperatura de at 20 – 22 °C, pero en oscuridad total. Transcurrido ese tiempo, aquellos invertebrados que, tras aplicar una agitación, suave no mostraron movimiento durante 15 segundos, se consideraron inmóviles.

La CE_{50} y CE_{10} (es decir, aquellas concentraciones en las que se calculó una tasa de inmovilización del 50% y 10%, respectivamente) y sus intervalos de confianza (IC) se obtuvieron de las curvas dosis-respuesta para los ensayos de inmovilización de Daphnia magna, utilizando el software XLSTAT (versión 2014.5.03).

4.9.2 Ensayos de bioluminiscencia de *Aliivibrio fischeri*

Aliivibrio fischeri es una bacteria gramnegativa presente en la gran mayoría de ecosistemas marinos (Septer y Visick, 2024). Este organismo presenta bioluminiscencia de forma natural, proceso que parece estar ligado a la respiración celular (Abbas et al., 2018; Strotmann et al., 2020). La adecuación de esta bioluminiscencia al ámbito experimental lo convierten en uno de los bioindicadores más utilizados para estos ecosistemas (Mendonça et al., 2007). El ensayo de toxicidad aguda con esta bacteria consiste en la medición de la variación de esta bioluminiscencia después de la exposición a la sustancia a testar. El protocolo seguido fue el ISO 11348 (International Organization for Standardization, 2007)

Las bacterias (*Aliivibrio fischeri* 8NRRL B-11177) fueron adquiridas liofilizadas a la empresa Macharey-Nagel (ref. 945 006), y conservadas congeladas a −18 °C hasta el comienzo de los ensayos.

Todas las disoluciones stock se prepararon partiendo de una disolución de 20 mg/L NaCl. A partir de ellas, se realizaron diluciones para conseguir las 5 concentraciones a testar para cada sustancia, utilizando siempre el mismo solvente, y comprobando que el pH se mantuviera en los parámetros establecidos (6 – 8,5). No se empleó DMSO, ya que las pruebas de toxicidad indicaron que tiene repercusión en los resultados del experimento.

Para el TA, se testaron las concentraciones 0,2, 2, 20, y 200 mg/L. Para el NE, las concentraciones ensayadas fueron 0,4, 4, 40 y 400 mg/L. En el caso de las combinaciones sinérgicas, se expuso a los crustáceos a distintos porcentajes de la concentración de cada sinergia.

Las disoluciones se agitaron vigorosamente para mantener una correcta oxigenación del medio. Tras introducir el fondo de todos los viales en un baño a 15ºC (± 1 °C) para mantener la temperatura constante, se preparó la disolución de bacterias añadiendo 11 mL de medio de cultivo refrigerado al vial congelado provisto en el kit de *A. fischeri*, de acuerdo con las instrucciones del protocolo. Para cada concentración, se realizaron 4 replicados y se dejó uno que contendría sólo la disolución de bacterias como control negativo.

Se añadieron 500 µL de la disolución de bacterias a cada vial y se dejaron atemperar durante 10 minutos. Pasado este tiempo, se midió la bioluminiscencia basal producida por

la bacteria en cada uno de los viales. En este momento, se añadieron las alícuotas (500 µL) de sustancia a testar (diluyendo así las concentraciones finales a la mitad), y se dejó un tiempo de exposición de 30 minutos. Tras ello, se midió de nuevo la bioluminiscencia de cada vial, siendo el resultado del experimento su variación respecto a la primera medida.

Las concentraciones CE_{50} y CE_{10} corresponden este caso a las concentraciones responsables de una reducción de la bioluminiscencia del 50% y del 10%, respectivamente. Éstas y sus IC se obtuvieron a partir de las curvas dosis-respuesta para *A. fischeri* utilizando el software XLSTAT (versión 2014.5.03).

4.9.3 Ensayos de elongación radicular de *Allium cepa*

El ensayo con los bulbos de la planta de cebolla (*Allium cepa*) consiste en la evaluación de la toxicidad aguda mediante la medición del crecimiento de su sistema de raíces tras la exposición a la sustancia a testar. Este ensayo está ampliamente aceptado como indicador de la toxicidad citotóxica y genotóxica causada por contaminación acuática, aérea o terrestre (Barbério et al., 2011; Fiskesjö, 1993), estando validado por el Programa Internacional de Seguridad de las Sustancias Químicas (IPCS, WHO) y el Programa de las Naciones Unidas para el Medioambiente (UNEP)(Mónica et al., 2016).

El procedimiento se desarrolló siguiendo el protocolo elaborado por Fiskesjö, (1993) y consiste en la medida de la elongación de las raíces tras 72h de exposición continua a la sustancia a testar (Pino-Otín et al., 2021). Los bulbos adquiridos a la Compañía Fitoagrícola (Zaragoza), de la variedad Stuttgarter Riesen 14/21, fueron almacenados en un entorno seco para prevenir el crecimiento fúngico. El agua mineral en la que se situaron las plantas fue la proporcionada por la empresa Aguas de San Martín de Veri S. A. (Bisaurri, Huesca) (Productos Veri, 2022), ya que fue el agua comercial con un contenido en iones de calcio y magnesio más adecuado para un correcto crecimiento. Este agua fue utilizada también para el control negativo.

El primer paso para el desarrollo experimental fue el pelado de la parte externa de los bulbos, evitando dañar el anillo radicular, situado en la zona inferior, durante este procedimiento. A continuación, los bulbos, 12 replicados para cada concentración, se colocaron en la parte superior de tubos de ensayo Falcon de 15 mL. A su vez, estos tubos fueron llenados en su totalidad de la disolución a testar (o solamente de agua, en el caso de los controles negativos), asegurando que el anillo radicular de los bulbos entraba en contacto con el líquido. Finalizado este procedimiento, los bulbos se cultivaron 72 horas a 25ºC en total oscuridad, renovando las disoluciones a las 24 horas.

Las concentraciones ensayadas de AT fueron 0,2, 2, 20, 100 y 500 mg/L. En el caso de NE, las concentraciones fueron 0,03, 0,3, 3,30 y 300 mg/L.

Los valores de CE_{50} y la CE_{10} (las concentraciones efectivas de sustancia a testar resultantes en un 50% y 10% de crecimiento radicular) y sus IC se obtuvieron de las curvas dosis-respuesta para *A. cepa* usando el software XLSTAT (versión 2014.5.03).

4.9.4 Ensayos de supervivencia de *Eisenia fetida*

El ensayo con la lombriz de tierra común *Eisenia fetida* consiste en el recuento de organismos supervivientes después de 14 días de exposición a la sustancia a testar. El protocolo seguido para ello fue el OECD 207 (1984) (Organisation for Economic Co-operation and Development (OECD), 1984) del mismo modo que en estudios anteriores (Pino-Otín et al., 2015, 2021). La OECD define este animal como el principal bioindicador animal para ecosistemas terrestres. Como se detalló en la sección 1.1.4.b, son una parte fundamental de este tipo de ecosistemas, ya que degradan la materia orgánica, fertilizando el suelo, y muestran sensibilidad a los contaminantes expuestos.

Los anélidos adultos fueron adquiridos 15 días antes del comienzo de los ensayos a la empresa Toldo Verde (Zaragoza). Una vez recibidos, se acondicionaron en un sustrato de turba con sphagnum (de la empresa Verdecora Vivarium, Zaragoza). Se mantuvieron en un entorno estable a una temperatura de 18 – 25 °C, un pH de 7,8 – 8 y un nivel de humedad del 80–85%. Al comienzo del ensayo, se seleccionaron lombrices adultas de más de 60 días de edad. Todas ellas fueron de un tamaño similar, un peso de entre 300 y 600 mg, y mostraban un clitelo central.

De acuerdo con la norma OECD 207, la turba negra comercial (Verdecora Vivarium), la arcilla caolínica y la arena cuarcítica se combinaron en la proporción 7:2:1. El nivel de humedad de la mezcla de suelo se ajustó con aproximadamente un 40% en peso de agua desionizada. Una vez preparada la mezcla, se colocaron 600 mg de ella en envases de polipropileno de 1 litro de capacidad, al que se le practicaron agujeros para un correcto mantenimiento de la humedad y ventilación.

En cada recipiente se colocaron 10 lombrices adultas, a los que previamente se añadió la disolución de sustancia a testar, en varias concentraciones, repetidas todas ellas en 3 replicados. Tanto en el caso de AT como en el de NE, las concentraciones testadas fueron de 0,2, 2, 20, 200, y 2000 mg/L. Los controles negativos, también triplicados, fueron iguales, pero no se les añadió ninguna disolución. Durante el ensayo, las lombrices se mantuvieron en un entorno estable de 20 ± 2 °C, humedad relativa del 80 – 85%, y una luz de 400 – 800 lx. Tras 14 días de exposición, se recontó el número de anélidos supervivientes.

Del mismo modo que en los anteriores ensayos, las concentraciones CE_{50} y la CE_{10} (responsables de un 50% y 10% de supervivencia de lombrices) y sus IC se obtuvieron de las curvas dosis-respuesta para *E. fetida* usando el software XLSTAT (versión 2014.5.03).

4.9.5 Ensayos de rendimiento fotosintético con algas perifiton

El perifiton es una comunidad de algas diatomeas frecuentemente encontrada en ríos, utilizada como bioindicador para este tipo de ecosistemas. Estas comunidades se recolectan, como se ha descrito previamente (Navarro et al., 2002) dejando una serie de bastidores de metacrilato sumergidos en el agua. En la superficie de estos bastidores se va adhiriendo un biofilm de algas y bacterias, que forma el perifiton estudiado. Una vez

recolectados los bastidores, el ensayo consiste en la medición de la reducción del rendimiento fotosintético tras la exposición a la sustancia a testar.

Las muestras de perifiton se recolectaron, al igual que las muestras de agua, del río Gállego (Zaragoza). Los bastidores se colocaron a 15 cm por debajo de la superficie del agua y se retiraron 15 días después (el 24 de junio de 2019). En ese momento, la capa orgánica de perifiton fue de 0,75 mm de grosor. Esto significa que el biofilm algal mostraba comunidades de biomasa y dimensiones consideradas adecuadas para su análisis (Navarro et al., 2002; Pino-Otín et al., 2021), por lo que fueron recogidas y trasladadas al laboratorio.

Allí, y con ayuda de un microscopio, se identificaron y recontaron las algas de las muestras recolectadas, siguiendo protocolos anteriores (AENOR, 2007). En el caso de la diatomeas, se aplicó H_2O_2 sobre las muestras para obtener una suspensión oxidada y limpia de la frústula. Los resultados se dan como densidad (individuos/mL) (ver información suplementaria 8.1)

Las pruebas de toxicidad se realizaron en varios canales de metacrilato conectados a depósitos de agua (termostatizados a 23 °C) que aseguran un flujo continuo. Cada canal recibía 0,113 m^3/h del agua procedente de un circuito cerrado de agua, alimentado por una serie de motores conectados a los depósitos (cada uno, de 4 L). Las algas fueron colocadas en la base del canal de metacrilato, recibiendo luz de forma continua (lámparas específicas para algas de la marca Blau aquaristic, T5HO, 39 w/10 000 K, 80 µmol fotón/s-m^2 en la superficie del canal) para permitir a las algas realizar la fotosíntesis en condiciones lo más semejantes a su entorno natural. Los organismos de perifiton fueron expuestos a concentraciones del PN en dosis de 0,1, 1, 10, 100 y 1000 mg/L (tamponados con MOPS (3-ácido sulfónico propílico de morfolina) 0,01M y pH 7,5). Como control negativo, se utilizó un canal con MOPS, pero sin PN. La temperatura se comprobó con regularidad para asegurar la estabilidad del experimento.

De forma similar a lo descrito previamente por Pino-Otín et al., (2021), el efecto del PN sobre la eficiencia fotosintética del perifiton se evaluó midiendo el rendimiento fotosintético, que representa la efectividad del proceso fotoquímico de conversión de energía (Consalvey et al., 2004), por triplicado tras 1 y 2 h de exposición.

Los valores CE_{50} y CE_{10} (las concentraciones efectivas de PN que resultan en el 50% y el 10%, respectivamente, del rendimiento fotosintético) y sus Intervalos de Confianza (IC) se obtuvieron a partir de las curvas dosis-respuesta para el perifiton utilizando el software XLSTAT (versión 2014.5.03).

4.10. Estadística y representaciones gráficas

En los ensayos realizados con Biolog EcoPlate™, la significancia estadística de las diferencias observadas se evaluó mediante la prueba t de Student para dos muestras independientes, a fin de comparar las medias obtenidas entre los grupos analizados. Además, se verificó la homogeneidad de las varianzas entre los valores de AWCD (Average

Well Color Development) correspondientes a las 3 réplicas, con el propósito de asegurar que las condiciones de la prueba t se cumplieran adecuadamente. Por su parte, la significancia estadística de las curvas cinéticas de las combinaciones sinérgicas en comparación con el control se comprobó utilizando ANOVA para datos paramétricos, llevado a cabo con el software SPSS (versión 28.0.1.0)

Para los ensayos de inmovilización de *D. magna*, bioluminiscencia de *A. fischeri*, supervivencia de *E. fetida*, elongación radicular de *A. cepa*, y rendimiento fotosintético de las comunidades de perifiton se utilizó el software XLSTAT 2014.5.03 (Addinsoft 2023, Nueva York, NY, EE. UU.) (XLSTAT Standard – Sensory, consumer and market research), aplicando la regresión logística para crear las curvas dosis-respuesta de cada experimento. Esto permitió obtener los valores CE_{50} y CE_{10}. Los modelos dosis-respuesta se analizaron mediante la prueba *Chi-cuadrado* con el mismo software.

5. RESULTADOS

Atendiendo a los objetivos principales planteados en esta investigación (sección 3), se han agrupado los resultados en 5 capítulos:

Tabla 8. Relación de objetivos y resultados de la Tesis Doctoral:

	Objetivo	***Resultado***
Objetivo 1	*Determinar la ecotoxicidad de 8 ATBs de uso común, seleccionados como base para la búsqueda de sinergias con PN, utilizando comunidades microbianas no diana como bioindicadores*	***4.1. Evaluación de la ecotoxicidad de 8 antibióticos ampliamente utilizados sobre comunidades microbianas de ríos***
Objetivo 2	Identificar y caracterizar la eficacia y la cinética de combinaciones sinérgicas entre los 8 ATBs estudiados y los dos PN seleccionados (AT y NE).	***4.2. Mejora de la eficacia de los antibióticos con compuestos naturales: actividad sinérgica del ácido tánico y el nerol con antibióticos comerciales frente a bacterias patógenas***
Objetivo 3	*Evaluar la ecotoxicidad del AT en organismos y comunidades microbianas no diana*	***4.3. Estudio ecotoxicológico del ácido tánico en indicadores no diana de suelo y agua y su impacto en comunidades fluviales y edáficas.***
Objetivo 4	*Evaluar la ecotoxicidad del NE en organismos y comunidades microbianas no diana*	***4.4. Estudio ecotoxicológico del nerol en indicadores no diana de suelo y agua y su impacto en comunidades fluviales y edáficas.***
Objetivo 5	*Evaluar la ecotoxicidad de las combinaciones sinérgicas identificadas entre los ATB y los PN (AT y NE) en comunidades microbianas no diana*	***4.5. Estudio ecotoxicológico de combinaciones antimicrobianas sinérgicas de ácido tánico y nerol con antibióticos: ¿Una alternativa segura a los antibióticos comerciales?***

5.1. Capítulo 1. Evaluación de la ecotoxicidad de 8 antibióticos ampliamente utilizados sobre comunidades microbianas de ríos.

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- Resumen:

Para evaluar la ecotoxicidad que pueden generar 8 ATBs comerciales ampliamente usados en el medio ambiente (PEN, AMO, AMP, STM, GTM, ERY, TC y CHL), se emplearon comunidades microbianas naturales procedentes del río Gállego (Aragón) como bioindicadores. La caracterización genética de la comunidad bacteriana mediante secuenciación del gen ARNr 16S evidenció la prevalencia de géneros típicos de ecosistemas acuáticos, como cianobacterias, proteobacterias, actinobacterias y bacteroidetes. Mediante la técnica Biolog EcoPlate™, se estudiaron los efectos de distintas concentraciones de los ATBs sobre el metabolismo y crecimiento microbiano, observando una reducción significativa de la actividad metabólica a 100 mg/L para todos los compuestos, especialmente en la utilización de polímeros, carbohidratos y ácidos carboxílicos y cetónicos. CHL, ERY y GTM mostraron la toxicidad más acusada. A concentraciones bajas (0.1 mg/L), algunos ATBs estimularon levemente el crecimiento y la capacidad metabólica, salvo AMO, que redujo la actividad metabólica en todos los sustratos. Se analizaron además las correlaciones entre parámetros fisicoquímicos y mecanismos de acción para comprender la biodisponibilidad y toxicidad de los ATBs. Las propiedades fisicoquímicas, en particular la lipofia (log K/ow) y la acidez (pKa), parecen ser los parámetros que influyen decisivamente en la biodisponibilidad y toxicidad de estos ATBs en comunidades microbianas acuáticas. Esta comprensión es crucial para interpretar y predecir la ecotoxicidad ambiental de estos compuestos. Aunque las concentraciones detectadas en ríos son demasiado bajas para provocar toxicidad aguda, modificaciones en el funcionamiento microbiano pueden afectar el equilibrio ecológico, sugiriendo que el estudio de comunidades microbianas realistas resulta esencial para evaluar el impacto ambiental de los ATBs.

- Relación con la Tesis Doctoral:

El elevado consumo de ATBs y su liberación recurrente en ecosistemas fluviales generan efectos perjudiciales tanto a corto como a largo plazo en múltiples niveles tróficos. Este estudio, presentado como el primer capítulo de la presente Tesis, proporciona una evaluación profunda del impacto ambiental de 8 ATB comerciales sobre comunidades bacterianas naturales de ríos, que son clave para procesos esenciales como el mantenimiento de la calidad del agua, la recuperación de acuíferos y los ciclos biogeoquímicos en ecosistemas fluviales. Los resultados obtenidos constituyen una base fundamental, aportando evidencias cuantitativas de la ecotoxicidad ejercida por estos ATB en condiciones reales de comunidad microbiana y metabolismo. Esta base analítica es indispensable para el desarrollo de los siguientes capítulos de la Tesis, donde se estudiará

la ecotoxicidad de productos naturales individuales y sus combinaciones sinérgicas con dichos ATBs. De este modo, se establece un marco comparativo sólido que permite valorar y contrastar el grado de afectación microbiana, facilitando la identificación de alternativas o mitigaciones naturales frente a la contaminación por ATB.

- Importancia de los resultados:

Aunque abundan estudios sobre ecotoxicidad de ATBs que emplean indicadores individuales, esta investigación destaca la ventaja de usar comunidades microbianas naturales para obtener una visión ecosistémica más completa. Además, la secuenciación de las muestras permite conocer la estructura de la comunidad microbiana de partida e interpretar así los cambios metabólicos tras la exposición a los ATB. Los resultados permiten documentar alteraciones significativas tras exposiciones prolongadas incluso a dosis bajas (0.1 mg/L), así como efectos tóxicos evidentes a dosis mayores (100 y 1000 mg/L). Estas variaciones pueden desencadenar desequilibrios ecológicos con consecuencias complejas para la cadena trófica y la salud ambiental. Aunque los efectos de toxicidad aguda son menos probables, el reiterado aporte de ATB a los cauces pone en evidencia el riesgo de efectos acumulativos y crónicos. Por tanto, estos resultados contribuyen a justificar la necesidad de buscar alternativas a los ATB comerciales menos dañinas para el medio ambiente.

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Assessing the Ecotoxicity of Eight Widely Used Antibiotics on River Microbial Communities

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Abstract: Global prevalence of antibiotic residues (ABX) in rivers requires ecotoxicological impact assessment. River microbial communities serve as effective bioindicators for this purpose. We quantified the effects of eight commonly used ABXs on a freshwater river microbial community using Biolog EcoPlates™, enabling the assessment of growth and physiological profile changes. Microbial community characterization involved 16S rRNA gene sequencing. The river community structure was representative of aquatic ecosystems, with the prevalence of Cyanobacteria, Proteobacteria, Actinobacteria, and Bacteroidetes. Our findings reveal that all ABXs at 100 µg/mL reduced microbial community growth and metabolic capacity, particularly for polymers, carbohydrates, carboxylic, and ketonic acids. Chloramphenicol, erythromycin, and gentamicin exhibited the highest toxicity, with chloramphenicol notably impairing the metabolism of all studied metabolite groups. At lower concentrations (1 µg/mL), some ABXs slightly enhanced growth and the capacity to metabolize substrates, such as carbohydrates, carboxylic, and ketonic acids, and amines, except for amoxicillin, which decreased the metabolic capacity across all metabolites. We explored potential correlations between physicochemical parameters and drug mechanisms to understand drug bioavailability. Acute toxicity effects at the river-detected low concentrations (ng/L) are unlikely. However, they may disrupt microbial communities in aquatic ecosystems. The utilization of a wide array of genetically characterized microbial communities, as opposed to a single species, enables a better understanding of the impact of ABXs on complex river ecosystems.

Keywords: ecotoxicity; river microbial community; antibiotics; Biolog EcoPlates™; community-level physiological profiling

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1. Introduction

In recent decades, the scientific literature has reported the presence of micropollutants in different aquatic ecosystems. Among these compounds, known as "emerging contaminants", are antibiotics (ABXs), which have been widely used for the treatment of infections in humans and animals, to prevent damage by bacteria in plant cultures, and even as adjuvants in veterinary feed or food preservatives [1]. In 2013, it was estimated that 131,000 tons of ABXs were consumed worldwide, and this is expected to exceed 200,000 tons in 2030 [2].

ABXs, once consumed, become part of human and animal excretion, which constitute the main source of antibiotic release into the environment (approximately 80–90% of the total). These ABX residues can be directed to wastewater treatment plants (WWTPs) but, due their physicochemical properties, they cannot be partially or fully degraded via conventional treatments [3], allowing them to enter water bodies when wastewater is discharged into the environment [4–7]. Consequently, the amount and variety of ABXs, their metabolites, and degradation products that end up in various aquatic environmental compartments increase at a disturbing pace every year. This is the case, for example, with amoxicillin (AMO), erythromycin (ERY), and tetracycline (TC) [8,9]. ABX concentrations of around 50 µg/L [4] to 30 mg/L [10] have been reported in pharmaceutical and hospital

effluents; around 0.01 to tens of μg/L [11] in municipal wastewater; and a few ng/L in surface, ground and marine waters [12]. Such values may be considered quite low; however, it must be stressed that ABX exposure times could be exceptionally long, due to their persistence in ecosystems [13,14].

The chemical properties of antibiotics significantly impact their persistence in the environment and their vulnerability to degradation processes. Consequently, degradation by-products of ABXs have been identified not only in WWT systems but also in rivers, as evidenced by the detection of TC by-products [15,16]. Antibiotics, when dissolved in water, undergo alterations, including hydrolysis, as seen in the case of chloramphenicol (CHL), penicillin (PEN), or ampicillin (AMP), photolysis (ERY and TC), sorption, and biological degradation [17–19]. In fact, microbial populations have a central role in the biodegradation of organic materials [20,21]. These processes are influenced by both biotic and abiotic factors, which are contingent on environmental conditions such as sunlight exposure, water temperature, the presence of microorganisms, water chemical composition, sediment properties, and organic matter content. For instance, antibiotics like ERY and TC in river water appear to initiate degradation processes within approximately four days [22,23]. These metabolites may exhibit different properties compared to the parent compounds and can contribute to the overall environmental impact.

Furthermore, potential chemical interactions among different ABXs or with other chemical compounds are poorly understood and not easy to predict.

Given that there is still no European legislation on medium-term risk assessment of emerging contaminants (and ABXs in particular), systematic ecotoxicity studies of these pollutants are imperative to establish guidelines to regulate and restrict their use and subsequent uncontrolled release into the environment.

Reported evidence reveals that ABXs harm aquatic environments, affecting all trophic levels, from microalgae [24–27] and crustaceans [28,29] to fish [29,30] and even amphibians [31]. Some degradation products have also exhibited equal or greater toxicity than the original ABXs. For example, the primary degradation products of AMO [21] and clarithromycin [32] have shown significant toxicity to fish and cyanobacteria, respectively.

However, the literature regarding the toxicity to non-target organisms of many of the antibiotics detected in rivers has significant gaps and is still inconclusive. Additionally, most studies are based on individual indicator organisms, and very few examine whole river microbial communities as endpoints [33–35]. Freshwater microbial communities can serve as excellent bioindicators of the impact of antibiotics (ABXs) on the riverine ecosystem [36]. They form the basis of the food web, particularly among primary producers, and also play a significant role in organic matter decomposition, thus closing nutrient cycles and participating in energy exchange, as well as pollutant degradation [37,38]. Consequently, disturbances at this level can have repercussions on all riverine communities [36,39] with unpredictable consequences for the ecological balance of the aquatic environment [40]. Therefore, to achieve a better understanding of the impact of ABXs on the aquatic environment, it is necessary to consider the effects not only on an isolated indicator organism but also on whole communities [36,41].

Until now, the few studies regarding the impact of ABXs on freshwater microbial communities have mainly focused on changes in the relative abundance of prokaryotes, and only with one or very few ABXs [42,43].

In addition, beyond acute toxicity testing, long-term studies contemplating sublethal effects are imperative to assess the impact of ABXs over long periods of time, even if their concentrations are low [33–35].

In the present study, we selected eight of the most consumed ABXs, detected in rivers around the world and belonging to different families with different mechanisms of action, of both narrow and wide spectrums, with the objective of quantifying their impact on a real river freshwater microbial community. For this purpose, (a) the endpoint studied was the changes in the growth and physiological profile of the bacterial community using Biolog EcoPlates™; (b) the microbial community was characterized by 16S rRNA gene sequencing;

and c) the mechanism of action of each ABX was tested, as well as the values of the main physicochemical properties that might condition it (molecular weight, log Kow, and pKa), was correlated with its ability to reduce or slow down the growth of the entire community or alter its metabolic profile.

Biolog EcoPlates™ were selected for their ability to measure the capacity of a microbial community to metabolize a set of representative organic substrates [43–47], which indirectly provides relevant information about changes in their functional diversity [44]. Despite its great versatility, the main limitation of this approach is that it must be complemented with a taxonomic analysis of the variety of microorganisms present in the water sample. Therefore, in this study, microbial community taxonomy was also sequenced.

2. Results

2.1. 16S rRNA Gene Sequencing of River Microbial Communities

Figure 1 shows the abundance of taxa identified by DNA characterization in Krona chart plots, representing the relative abundance of microbial species detected in the samples. Input data were the average count for each taxon obtained from three studied replicates with an average of 24,818 reads. Only taxa with at least 1% total abundance are shown.

We found two predominant phyla: Cyanobacteria (56.73%) and Proteobacteria (19.77%). In addition, other phyla identified were: Actinobacteria (9.83%), Bacteroidetes (7.72%), Verrucomicrobia (4.68%), and a small proportion of Parcubacteria (1.27%).

All Cyanobacteria are oxygenated photosynthetic bacteria present in all aquatic environments, preferably in cold waters, and are an essential component of phytoplankton, which contribute to the primary production of aquatic food chains.

Proteobacteria are also very abundant in aquatic environments [48]. Based on 16S rRNA identification, this phylum is classified into three main families, all represented in our samples: Betaproteobacteria, (the most abundant, 15.49%), Alphaproteobacteria (2.92%), and a small proportion of Gammaproteobacteria (1.36%). Among the Betaproteobacteria, the only order identified was Burkholderiales, and all of these belonged to the Comamonadaceae family, among which the *Limnohabitans* genus, a relevant group of freshwater bacterioplankton [49,50], predominated (13.87%). *Hydrogenophaga*, a Gram-negative hydrogen-oxidizing bacteria, was the other genus identified within the Comamonadaceae and was much less abundant (1.62%). All Alphaproteobacteria identified belong to the genus *Paracoccus*, (Rhodobacteraceae family), a Gram-negative denitrifying bacterium with a high compound degradation capacity, useful in bioremediation [51].

All Gammaproteobacteria were Xanthomonadales and the genus identified was *Arenimonas*, a Gram-negative bacteria also found in fresh water [52].

The actinobacteria identified belonged to the order Actinomycetales, among which the genera *Rhodoluna* (4.32%) and *Cellulomonas* (3.5%) were identified. Both genera are frequent in freshwater habitats [53,54]. Actinobacteria are Gram-positive bacteria found mainly in soil and aquatic niches and play an important role as saprophytic organisms, participating in the decomposition of organic matter.

Flavobacterium (2.83%) and *Pseudarcicella* (1.65%) were the two genera identified among the phylum Bacteroidetes, belonging to the orders Flavobacteriales and Cytophagales, respectively. Bacteroidetes are a phylum of Gram-negative bacteria widely distributed in the environment, including in freshwater. Flavobacterium and Pseudarcicella are organotrophic aerobic bacilli that have been found to be widely distributed in freshwater [55,56]. Like most of the bacteria identified, the phylum Verrucomicrobia are Gram-negative bacteria frequently found in fresh waters; a potential role as polysaccharide degraders has been suggested for them [57]. In addition, some members of this phylum have been recently described as aerobic methanotrophs [58]. All bacteria identified belonged to the order Verrucomicrobiales and two genera were identified: *Haloferula* (2.35%) and *Luteolibacter* (1.3%).

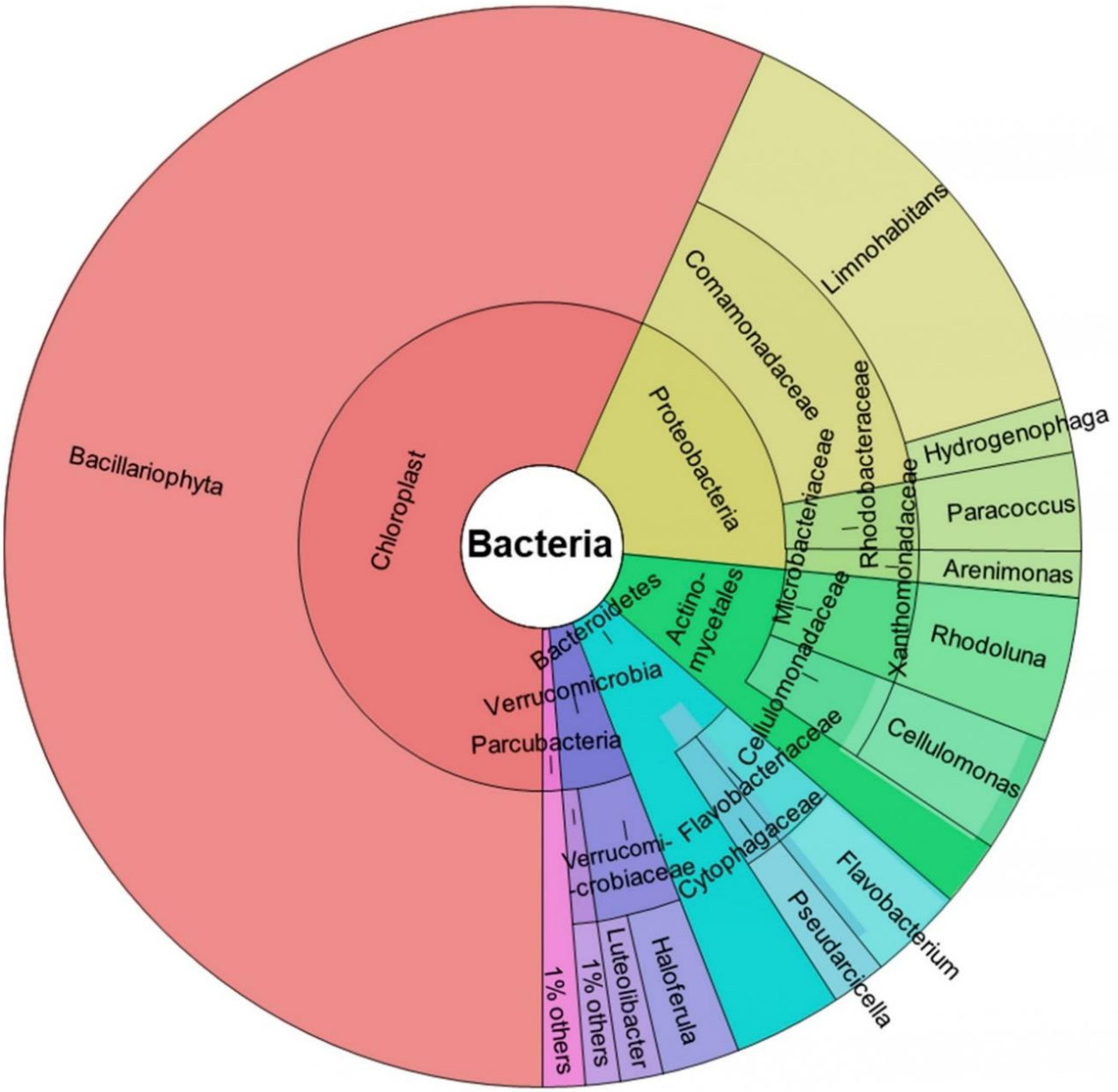


Figure 1. Taxonomic spectrum visualized with Krona chart of taxa showing percentage of the metagenome. Circles represent taxonomic classifications in ascending order up to the genus level (outermost circle). Only taxa with at least 1% of total abundance are shown.

2.2. Impact of Antibiotics on the Growth of River Microbial Communities

Figure 2 shows the impact of the eight selected ABXs on the growth kinetics of microbial communities obtained from river water after a seven-day incubation in Biolog EcoPlates™, measured as *AWCD*.

A clear dose-dependent response was observed in all cases(except for TC). Concentrations of 0.1 μg/mL were growth enhancers in all cases; however, with concentrations of 100 μg/mL, a clear decrease in microbial growth was observed, which was more pronounced at concentrations higher than 1000 μg/mL, except in the anomalous case of TC.

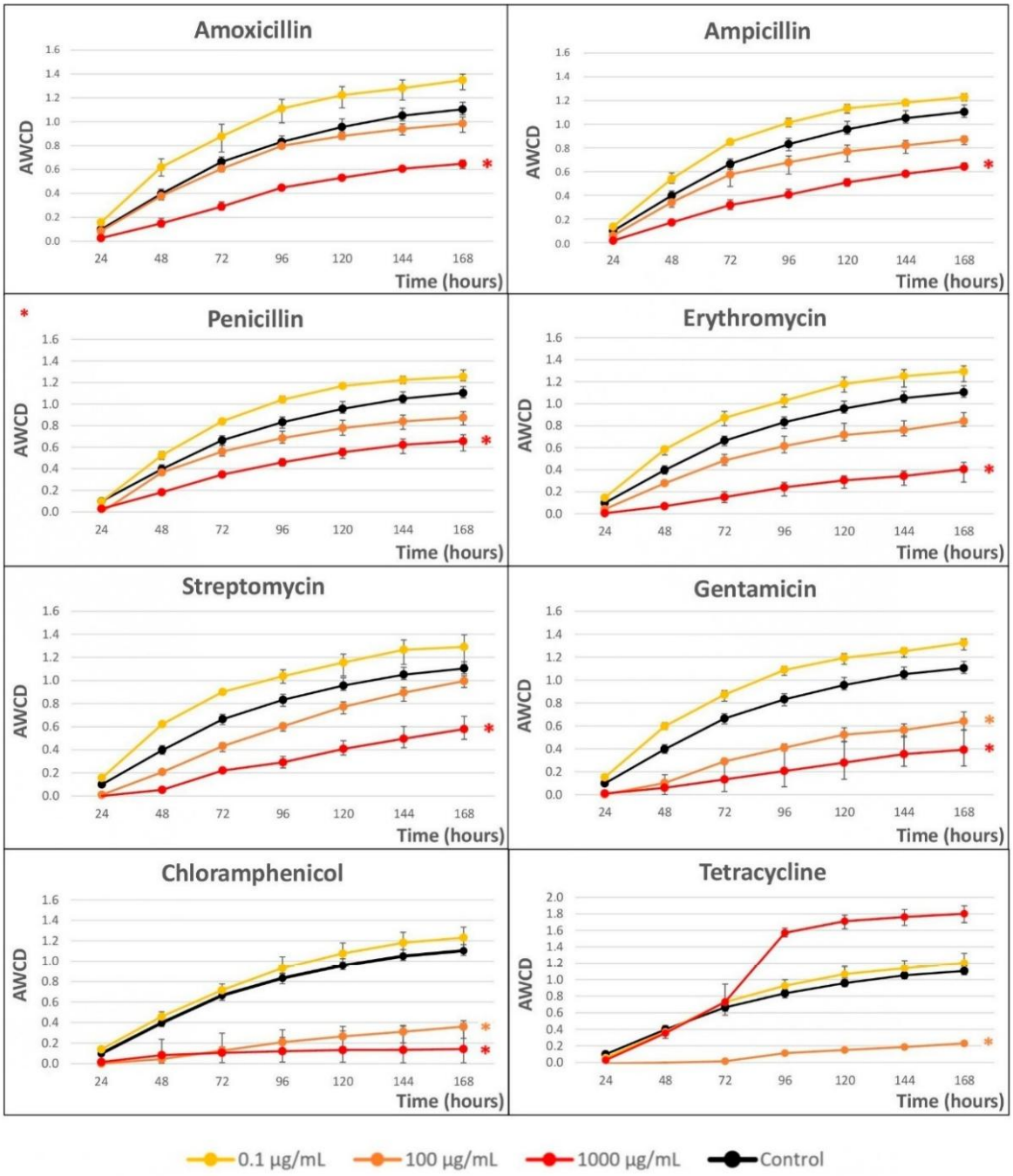


Figure 2. Average well color development (*AWCD*) vs. time (h) curves at three concentrations (0.1, 100, and 1000 µg/mL) for the eight antibiotics. Statistically significant differences between antibiotic exposure and control were analyzed using the Student's *t*-test. *p*-values < 0.05 have been marked with asterisks. Error bars represent the standard deviation of the mean of three replicates (n = 3).

To better compare the ABX effect, Cmax (maximum achievable population density) and r (intrinsic rate of population growth) were calculated from the curves as variable responses at 1000 µg/mL (see Table 1).

Table 1. Properties of the eight antibiotics assayed *.

Antibiotic Name	Abbr.	Family	CAS Number	MW (g/mol)	Water Solubility (mg/mL)	pKa		Log Ko/w	1000 µg/mL	
						pKa1	pKa2		C_{max} (1)	r (2)
Chloramphenicol	CHL	Amphenicols	56-75-7	323.1	2.5	7.5	−2.8	1.0	0.133	0.067
Tetracycline	TC	Tetracyclines	64-75-5	444.4	75.5	3.3	9.2	−2.0	1.801	0.070
Erythromycin	ERY	Macrolides	114-07-8	733.9	2.0	8.9	8.9	−3.0	0.645	0.043
Streptomycin sulphate	STM	Aminoglycosides	3810-74-0	581.6	75.0	10.9	12.0	−5.2	0.602	0.037
Gentamycin sulphate	GTM		1405-41-0	447.6	50.0	12.6	10.1	−2.4	0.420	0.035
Amoxicillin	AMO	Beta-lactams	26787-78-0	356.4	1.0	2.9	11.7	0.9	0.645	0.043
Ampicillin	AMP		69-53-4	349.4	10.0	2.6	7.2	1.4	0.640	0.037
Penicillin G sodium salt	PEN		69-57-8	356.4	75.0	3.5	−2.8	1.2	0.650	0.042
Control									1.081	0.043

* Physicochemical properties were obtained from PubChem and ChemicalBook databases. Cmax and r were obtained from the 1000 µg/mL *AWCD* curves. (1) Maximum achievable population density (carrying capacity). (2) Intrinsic rate of population growth.

Considering the Cmax values, from more effect (lower Cmax values) to less effect (higher Cmax values), the ABX that produced the greatest changes at 1000 µg/mL was CHL, and the least changes were produced by PEN, although all of them showed differences with respect to the control. At 100 µg/mL, the ABX TC presented the greatest growth inhibition but, surprisingly, promoted growth at 1000 µg/mL (see Figure 2).

On the other hand, GTM was the ABX that most affected the growth rate (lower r value) and AMO affected it the least (higher value of r), although the differences between them and the control were low.

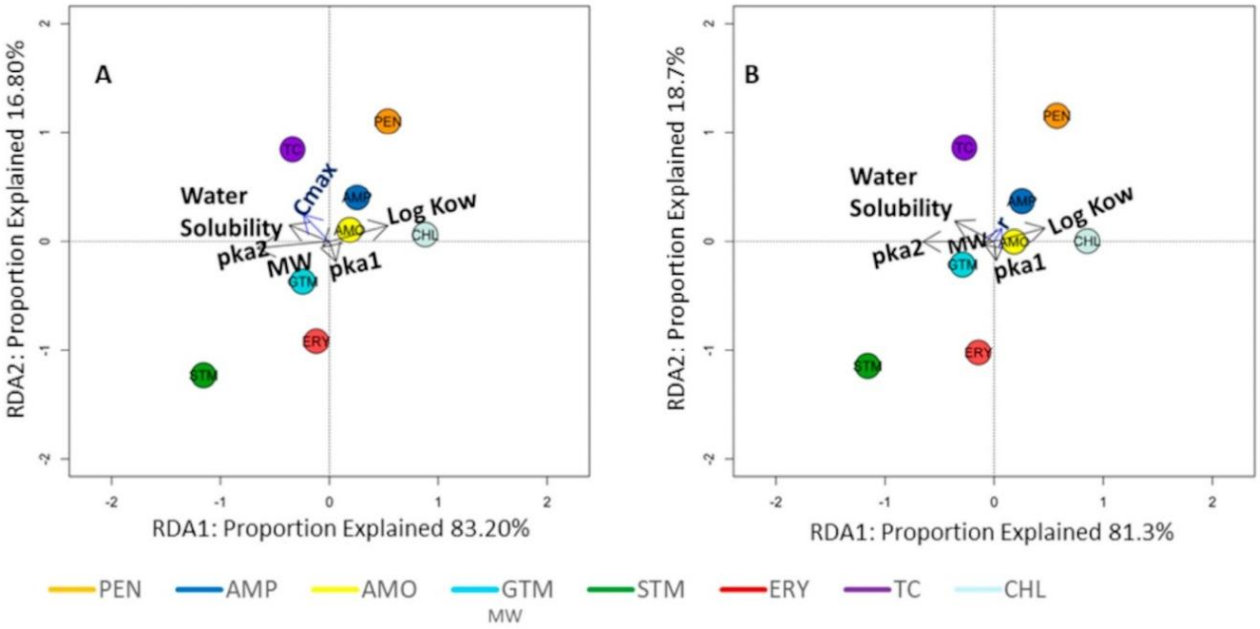
2.3. Relationship between the Physicochemical Properties of Antibiotics and the Impact on Bacterial Growth

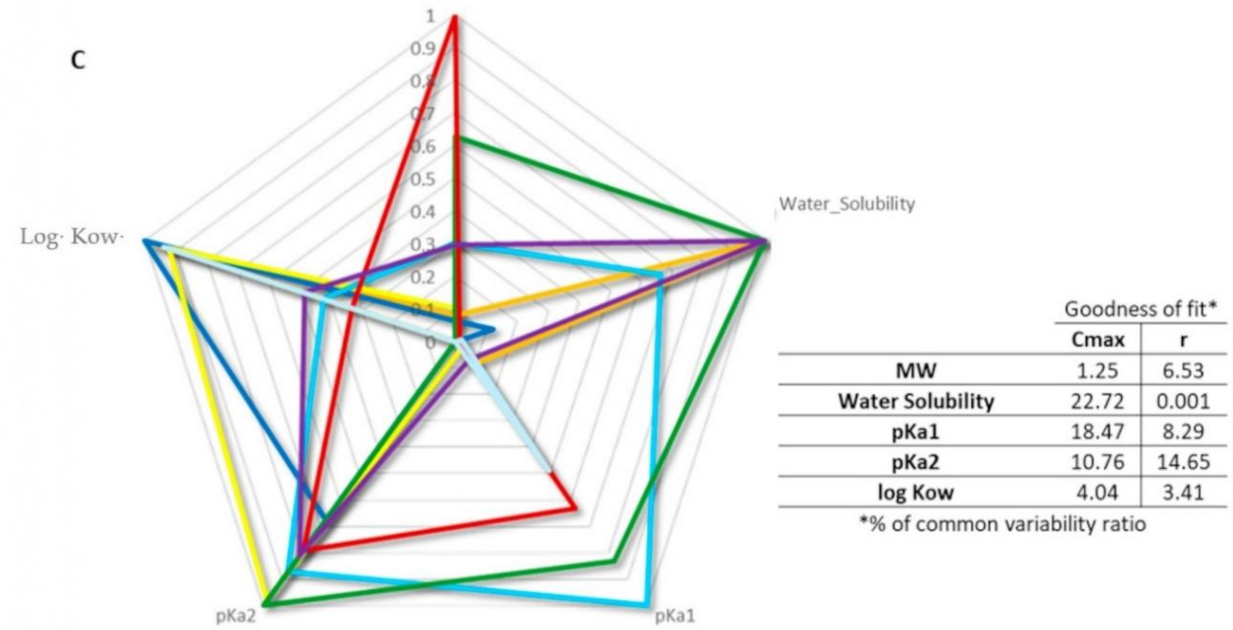
Our data have shown that the variables with the greatest weight in Cmax (Figure 3A), according to the length and perpendicularity of the arrows represented in the RDA graph, were pKa1 (pH at which the molecule under study loses its most acidic proton) and water solubility. Solubility explained 31% of the variability, while pKa1 accounted for almost 22.5%. In the case of r (slope of the AWCD line), shown in Figure 3B, the main factor was water solubility, which explained 15.16% of the variability. Following the directionality of the arrows (Figure 3A,B), we can see the comparative value and trend behavior of each ABX for each parameter; ABXs that are on the opposite side of the arrow directionality presented a lower parameter value and vice versa. As explained previously, for Cmax values, from more impact (lower Cmax values in the opposite direction to the arrow) to less impact (higher Cmax values in the same direction as the arrow), it can be seen that CHL was the ABX that produced the greatest effect and the least effect was produced by PEN.

In addition, the RDA graph allows us to see affinities in the behavior of the different ABXs according to their weight in Cmax, such that two groupings of ABXs can be identified (see ABXs grouped in red circles in Figure 3A).

In Figure 3C, we can easily observe the parameters representing the main physicochemical characteristics of the different ABXs, weighted to unify the scale. This makes it possible to observe which physicochemical characteristic is the most differential with respect to others. For example, for STM and PEN, their most remarkable parameter is water solubility; for CHL, it seems to be log Kow and, for ERY, molecular weight. The determination coefficient (goodness of fit) represents the percentage variability explained by the regression model. The percentage variability of Cmax/r was calculated for each

variable using SPSS (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, USA: IBM Corp.).





	Goodness of fit*	
	Cmax	**r**
MW	1.25	6.53
Water Solubility	22.72	0.001
pKa1	18.47	8.29
pKa2	10.76	14.65
log Kow	4.04	3.41

*% of common variability ratio

Figure 3. Radial graph. Redundancy analysis and physicochemical parameters representation. (**A**,**B**): redundancy analysis showing the relationship between the antibiotic and physicochemical parameters; antibiotic physicochemical properties were considered as exploratory variables and Cmax (**A**) and r (**B**) at 1000 µg/mL of antibiotic as response variables. Arrow length indicates the variance that can be explained by each parameter, with the perpendicular distance of the antibiotic to the arrow indicate the parameter's relative importance. The groups marked in graph A are significant ($p < 0.05$). (**C**) shows the radial chart that explores each one of the studied physiochemical parameters as a dimension, with % of common variability ratio for each of the parameters.

2.4. Impact of Antibiotics on Community-Level Physiological Profiling

The impact of the eight ABXs on the physiological diversity of the fluvial microbiota was evaluated by studying the changes in the ability of the microbial communities to metabolize different carbon sources in the Biolog EcoPlate™ (Figures 4–6).

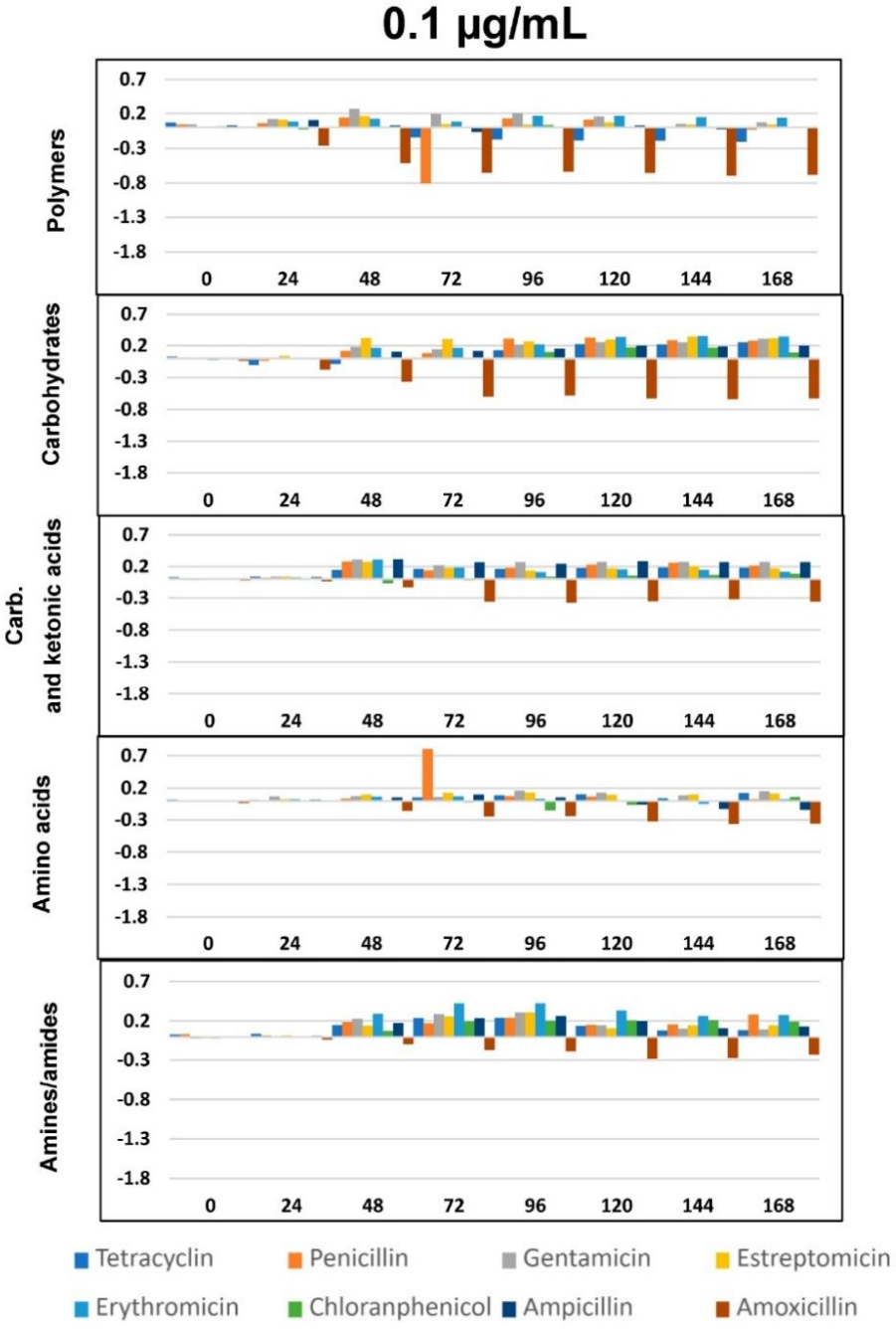


Figure 4. ΔOD vs. time (h) curves at 0.1 µg/mL for the eight antibiotics and five selected groups of metabolites. Plotted values were obtained by subtracting those from the negative control. Statistically significant differences between antibiotic exposure and control were analyzed using the T- Student's *t*-test. All differences with respect to the control show $p < 0.05$, except for: the chloramphenicol, ampicillin, and penicillin for polymers; tetracycline for carbohydrates; chloramphenicol for carboxylic and ketonic acids; and penicillin, chloramphenicol, and ampicillin for amino acids.

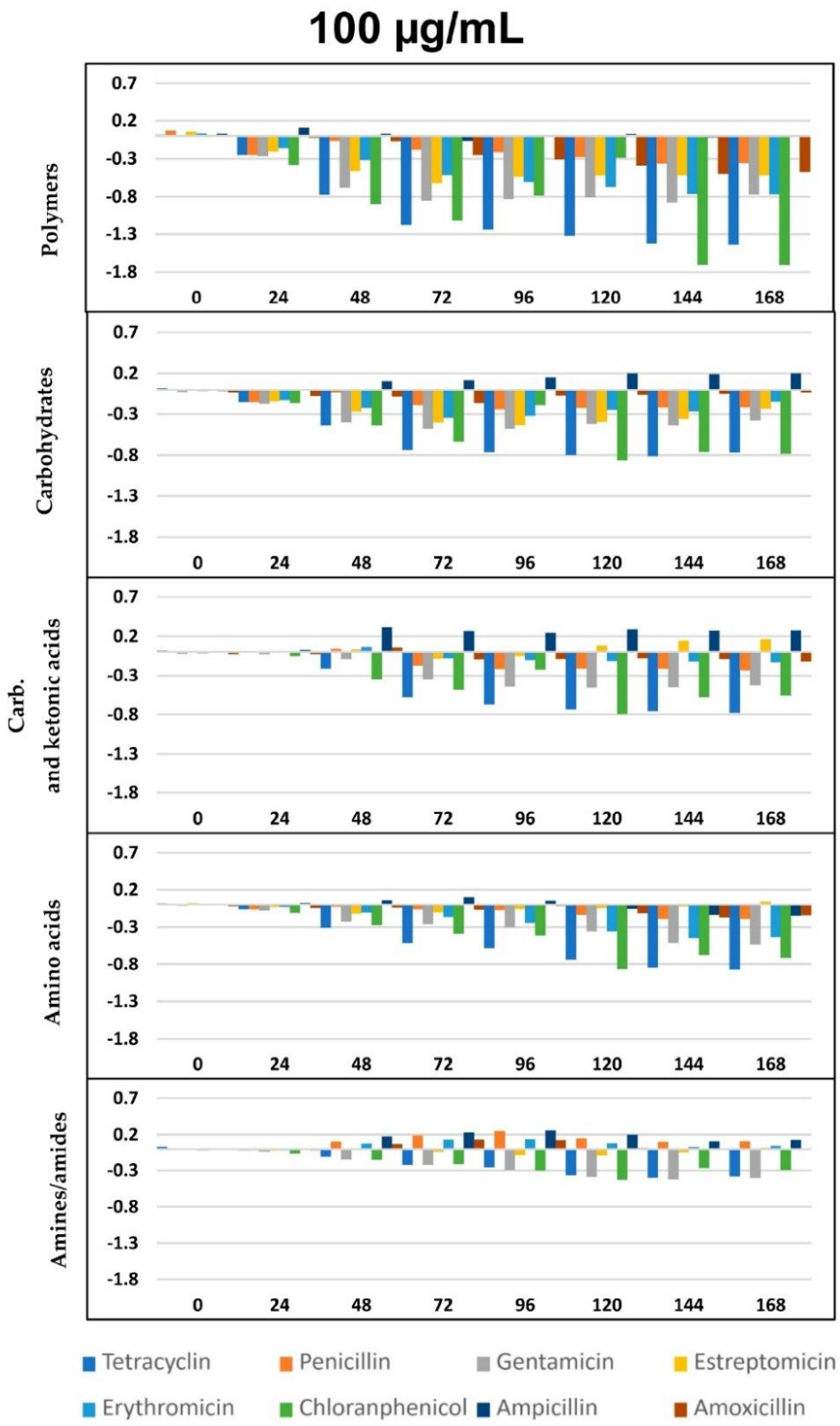


Figure 5. ΔOD vs. time (h) curves for the eight antibiotics and five selected groups of metabolites, as in Figure 4, but at the concentration of 100 µg/mL. All differences with respect to the control show $p < 0.05$, except ampicillin for polymers and amino acids.

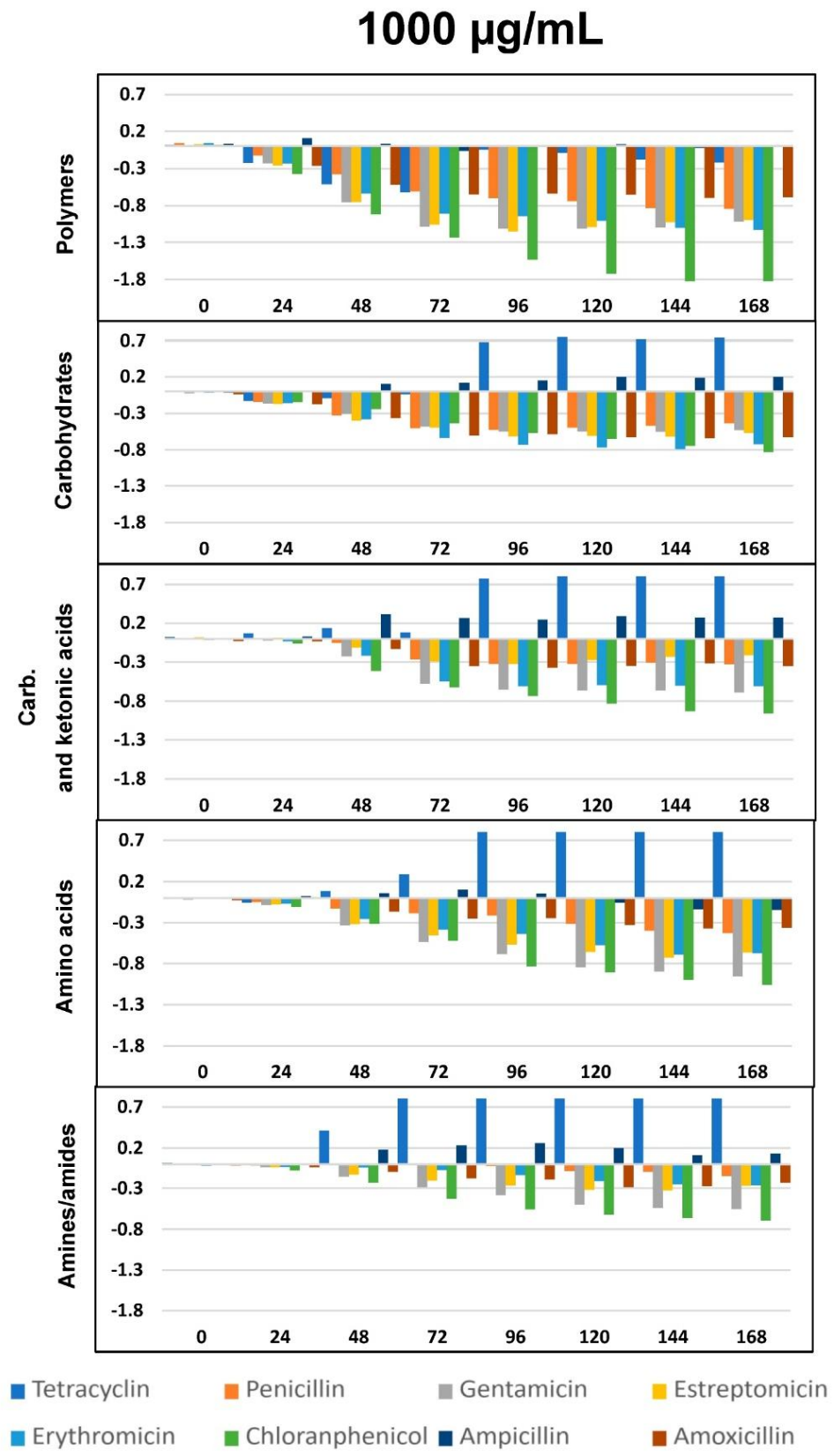


Figure 6. ΔOD vs. time (h) curves for the eight antibiotics and five selected groups of metabolites, as in Figure 4, but at the concentration of 1000 µg/mL. All differences with respect to the control show $p < 0.05$, except ampicillin for polymers and amino acids.

As per previous studies [44,59,60] Biolog's carbon sources can be grouped into five functional classes: polymers, carbohydrates, carboxylic and ketonic acids, amino acids, and amines/amides. Figures 4–6 represents optical density (OD) variation after seven days' exposure to three concentrations of the eight ABXs studied for each group of metabolites (subtracting values from the negative control). As can be seen, exposure to almost every ABX reduced the consumption of all metabolite groups, in particular with CHL (green bars). This effect was progressive over time; however, a moderate increase in the metabolism of amines, carboxylic and ketonic acids, and carbohydrates was observed for ampicillin after 48 h, as well as a major increase in all nutrient groups (except polymers) in the case of tetracycline.

3. Discussion

The results reported in this study revealed that the eight widely used ABXs had a significant impact on a natural river microbial community, in terms of growth kinetics and physiological profile, for concentrations ranging from 100 μg/mL. Interestingly, low concentrations (0.1 μg/mL) acted as a growth promoter.

All ABXs exhibited a dose-dependent response (except for TC), with the 1000 μg/mL dose showing the greatest reduction in microbial growth. However, both GTM and CHL displayed significant differences even at 100 μg/mL, indicating distinct mechanisms of action on microbial community growth.

To better understand these variances, growth kinetic parameters (Cmax and r) were considered, to evaluate ABX behavior.

Regarding Cmax values, from the highest to the lowest effect, ABXs were ordered as follows: CHL > ERY > GTM > STM > AMP > AMO > PEN. TC is not included in the list because it produced the greatest decrease in microbial growth at 100 μg/mL; however, it had an anomalous behavior that enhanced growth at 1000 μg/mL.

The sequence for the ability to diminish velocity growth (r) was GTM < STM < AMP < ERY < PEN < AMO; in all cases, r was lower than the control, except for CHL (but as soon as the ABX began to act, population growth practically stopped), and TC.

This impact on the metabolic profile of the microbial community was independently analyzed for each nutrient group, resulting in a general decrease in the ability to metabolize all metabolic groups after exposure to all ABXs, especially in the case of polymers at concentrations of 100 and 1000 μg/mL, with few exceptions, such as TC (Figures 4–6).

As already seen in the *AWCD* curves (Figure 2), concentrations of 0.1 μg/mL were growth-promoting, with a slight increase in metabolic capacity for all metabolites after exposure to all the ABXs tested, except AMO, which caused a decrease in metabolic capacity for all metabolites at all three concentrations.

The evaluation of physicochemical properties, together with genetic sequencing of the microbial populations for the eight ABXs, allowed for a better interpretation of these results, as described below. Besides Cyanobacteria, the predominant phyla were Proteobacteria, Actinobacteria, and Bacteroidetes, a distribution highly representative of river water [61–63].

3.1. Chloramphenicol Is the Antibiotic with the Greatest Impact on River Microbiota

CHL was the ABX with the greatest impact on microbial communities and was the fastest-acting. It also caused the highest decrease in the metabolic capacity of the five metabolic groups of river microbial communities, which was significant even at 100 μg/mL. It is an ABX with a broad spectrum of action that, together with its physicochemical properties (small molecular weight, weak acid strength [64], and predominance of non-ionized forms at an aqueous pH and solubility) surely makes it highly bioavailable and effective on a great variety of microorganisms. Moreover, its partition coefficient showed a solubility in organic solvents ten-fold higher than in water, allowing it to cross biological barriers [65].

These data are consistent with the RDA graphs, which showed that log Kow and pKa1 were the parameters that most influenced the effect of CHL at Cmax (graph 3a) and r (3b). Both graphs also showed that water solubility is not a factor for CHL, as it lay in the opposite direction to the arrow marked by this physicochemical property. In graph 3c, we again see that, among all the physicochemical properties for the different ABXs, which were weighted to unify the scale, both the partition coefficient and pKa1 were the main physicochemical properties, justifying the Cmax and r of CHL.

Therefore, it could cross the envelope of the predominant Gram-negative bacteria in our samples (Proteobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, and Parcubacteria) and move into the cytoplasmic environment until reaching its target, the 50s subunit of the bacterial ribosome. This would inhibit protein chain elongation by preventing the formation of the peptide bond [66].

Other complementary effects described for CHL that might contribute to the growth inhibition of exposed aquatic communities could be its inhibition of bacterial wall peptidoglycan and capsular polysaccharide synthesis [67,68].

The bilayer outer membrane of cyanobacteria (like that of Gram-negative bacteria) has a hydrophobic lipopolysaccharide that acts as a barrier to many drugs. However, it has been suggested that, for small non-lipophilic molecules, such as CHL, there may be a main route of entry into the cell cytoplasm of cyanobacteria, which could be the porin channels [69,70].

Nevertheless, a small proportion of microorganisms in the sample remained capable of growing in the presence of CHL, even at the highest concentration. It is common to find freshwater microorganisms that have developed resistance to CHL, including Bacteroidetes [71,72] or Actinobacteria [73].

3.2. Macrolides and Aminoglycosides Have a Great Impact on Microbial Growth

Despite belonging to different families (macrolides and aminoglycosides), ERY and GTM had very similar Cmax values at 1000 µg/mL, with ERY having a slightly lower value. GTM was also the ABX that most affected the microbial growth rate (it had the lowest r-value) and induced a significant reduction in growth even at 100 µg/mL, similar to CHL.

This can be seen in the RDA analysis, where both ABXs appear together in the lower left quadrant (Figure 3A in relation to Cmax) and the same quadrant, but slightly further apart (Figure 3B in relation to r). STM presented a somewhat lower effect, according to Cmax values, than ERY and GTM, but greatly affected the growth rate (it had the second lowest r value after CHL). We can also find it in the same RDA quadrant.

All three ABXs caused a decrease in the ability to metabolize all groups of metabolites at the two highest concentrations studied, especially polymers, in the case of ERY and STM, although GTM affected the metabolism of carboxylic and ketonic acids, amino acids and amines/amides slightly more, and ERY affected the metabolism of polymers more.

The mechanism of action of the two families was similar (inhibition of protein synthesis) but the ribosomal target is different. ERY acts by binding to the bacterial 50s ribosomal subunit and the aminoglycosides to the 30S s ribosomal subunit [74]. The spectrum of action is different too; STM has a broad spectrum of action, but ERY is effective mainly on Gram-positive bacteria, since it cannot pass through the cell wall of Gram-negative bacteria. For example, ERY was remarkably harmful to some groups of Actinobacteria (9.83% of our samples) [75].

GTM was usually indicated to be against Gram-negative bacteria, predominant in our samples. Previous studies [76,77] showed that several Cyanobacteria species (57% of our sample) were susceptible to GTM, which can cause cytotoxicity by the induction of reactive oxygen species, in addition to protein synthesis inhibition. Recently, Cyanobacteria were also found to be susceptible to STM, which acts as an antioxidant and disturbs photosynthesis [78].

It is worth noting that some bacteria in our samples were not affected by this ABX, as was reflected in the growth kinetic curves, and were not fully inhibited even at 1000 µg/mL,

as was the case, for example, with CHL. Cyanobacterial species resistant to all three ABXs have been detected [76,79,80], suggesting that they may present intrinsic resistance to GTM and STM [81]. Communities of actinobacteria resistant to GTM have also been detected [73], so it is difficult to establish which population dynamics might explain the greater effect of these ABXs.

In addition, the physicochemical properties of these ABXs will determine their ability to cross bacterial coatings in order to reach their ribosomal target.

Erythromycin was the ABX with the highest molecular weight, and, among these three ABXs, it had the lowest water solubility (2 mg/mL). Due to its pKa (8.9), it remains non-ionized in the physiological range; however, its log Kow of −3 was low, indicating that it is not very lipophilic. The aminoglycoside gentamicin had a higher water solubility (50 mg/mL) with a lipophilicity similar to erythromycin (log Kow = −2.4) and also remains non-ionized within the physiological range. Streptomycin was the most water-soluble of the three ABXs tested (75 mg/mL), was the least lipophilic of the three (log Kow = −5.2), and also remains non-ionized within the physiological pH range.

These physicochemical data agreed with those observed in RDA Figure 3A,B, where the parameters that most influenced these three ABXs were water solubility and pKa; log Kow was not such an influential factor, as it lay in the opposite direction to the quadrant where the three ABXs under study were located. This is corroborated by Figure 3C, where the parameters that contributed variability to the parameters studied were the acidity constants and molecular weight.

It has been suggested that aminoglycosides, being positively charged molecules, can pass through the wall of Gram-negative bacteria through electrostatic interactions with the negatively charged structures of the outer membrane of these bacteria, thereby deconstructing it and allowing the ABX to enter the peptidoglycan. From there, through the cell membrane via electrical gradients, they reach the target [82,83]. ERY cannot pass through the cell wall of Gram-negative bacteria because it is a weak base and its non-ionized form will only be predominant in basic environments [84], being more effective in Gram-positive bacteria. In the case of cyanobacteria, since all these ABXs are quite large molecules, it could be that access through porins is not as direct as in the case of CHLs. This mechanism has been suggested for aminoglycosides [85].

3.3. Beta-Lactams Have a Minor Effect on Microbial Growth Kinetics

AMO, AMP, and PEN presented analogous changes in the growth kinetics of microbial communities, with very similar growth inhibition curves (only significant at the higher concentration), as reflected in the Cmax and r values being very close. However, AMP seemed to affect the growth rate more, as indicated by a lower r. All three ABXs caused a moderate decrease in the ability to metabolize all metabolic groups. AMP had the least effect, even producing stimulation, except in the case of polymers.

The target of action of beta-lactams is the bacterial peptidoglycan wall. They produce competitive inhibition in the last steps of wall biosynthesis, producing an alteration of bacterial integrity, leading to cell lysis [86]. For this reason, they tend to be mainly effective against Gram-positive bacterial strains that have a thick peptidoglycan wall; they are less effective against Gram-negative bacteria that have, in addition to the peptidoglycan wall, a protective outer membrane that is not the target of the ABX [87]. AMP and AMO are broad-spectrum ABXs, but PEN would only be active against Gram-positives, which were less frequent in our samples. Nevertheless, cyanobacteria seem to show different degrees of sensitivity to AMP [88], PEN [89] and AMO [90].

Moreover, these ABXs are capable of generating resistance in freshwater microorganism communities [63] and some of the taxa identified in our samples have been shown to exhibit resistance, such as Limnohabitans [91,92]. Bacteroidetes are potential hosts of several ABX resistance genes (ARGs) in fresh water [93,94] and appear to play an important role in ABX clearance [95]. There are also indications that cyanobacteria may have intrinsic resistance to beta-lactamases [96].

Taken together, this may lead to a slightly slower action of these ABXs compared with aminoglycosides or CHLs, and also a slightly lower Cmax. However, the action may be sufficient to produce a reduction in functional diversity among resistant bacteria and lead to a loss of ecological fitness [97], reflected in the slowing down of population fraction and growth (Figure 2) and a decreased ability to metabolize substrates (Figure 4).

All three beta-lactams exhibited physicochemical properties that similarly affect microbial growth kinetics, as can be seen by their location in the upper right quartile of the RDA plots (Figure 3A,B), similarities that they also shared with TC and CHL. All had similar molecular weights, and, in all cases, their log Kow (Table 1) indicated a lipophilic character, which would facilitate crossing biological membranes. At physiological pH, these ABXs would be relatively ionized due to their pKa1, which facilitates crossing bacterial coatings and, thus, makes them more bioavailable [98]. In addition, the small size of beta-lactams would justify porin channels as one of the main access routes; however, the effect of these ABXs on cyanobacteria is highly variable and perhaps dependent on differences in the amount and type of porins in the outer membrane [85]. In contrast, none of these three ABXs were very soluble in water (Table 1), which would limit the diffusion of the ABX in the periplasmic space [99]. Figure 3C shows that the most important parameters for these ABXs were the partition coefficient and acidity constants, except in the case of PEN, where solubility presented with the same importance as the partition coefficient [64].

3.4. Tetracycline Has an Anomalous Behavior

Our results showed that TC concentrations of 100 µg/mL produced the greatest decrease in the growth of microbial communities, compared with the other ABXs studied (Figure 2). However, at concentrations of 1000 µg/mL, as of the third day, microbial growth increased, compared with the control. This was also seen in the effect of TC on changes in the metabolic profile of microorganisms exposed to this ABX (Figure 3), since at 100 µg/mL it reduced the ability to metabolize all metabolic groups, especially polymers, whereas at 1000 µg/mL, no decrease in polymers was observed and a significant increase in the metabolic capacity of all other metabolites was detected.

This lack of dose-dependent response for TC could be due to the physicochemical properties of this compound that may lead to a decrease in its bioavailability. On the one hand, TC binds strongly to proteins and silanol groups [100] and on the other hand, this ABX tends to form coordination compounds with divalent metal ions [101] present in organic substrates. Moreover, TC is photochemically unstable, being easily photodegraded, and the resulting metabolites, mainly lactones and carboxylic acids, [102,103] are not only bactericidal [104] but could be used as substrates by bacteria, which would explain this growth-enhancing activity at higher doses.

The target of action of TC is the 30S subunit of the ribosome, to which it binds, reversibly stopping protein synthesis [105]; it has a broad spectrum of action against Gram-positive and Gram-negative bacteria. It is a poorly water-soluble molecule that acts as a weak acid when ionized at a physiological pH and is not very lipophilic, but has a low molecular weight, which probably facilitates its ability to pass through microbial coatings to reach its ribosomal target [105]. The redundancy plots in Figure 3A,B placed TC in the upper left quadrant, where it showed a higher Cmax and r value and highlighted the influence of its pKa2 and log Kow properties.

Other studies reported that TC could affect the growth of cyanobacteria [77,78,106], disturb photosynthesis [35,107] and affect protein synthesis [108]. Also, it should not be forgotten that cyanobacteria can act as reservoirs that facilitate ARG dissemination in aquatic environments [109]. Other bacteria present in our samples, like Limnohabitans, could potentially exhibit ABX resistance after months of TC exposure [110].

3.5. Environmental Relevance

Our results reflected that ABX concentrations within the range of 0.1 to 100 μg/mL had a considerable impact on the growth and metabolism of representative taxa of the river nekton.

The ABXs selected are among the most widely consumed worldwide, both for human and livestock use. For example, penicillin consumption in Europe (25 countries) has been estimated to be slightly higher than 230 times the defined daily dose (DDD) in 2003 [111], whereas Spain, in 2017, was the European country with the highest consumption (14.23 DDDs per 1000 inhabitants per day) [112]. In 1991, the total outpatient consumption of ERY in Finland amounted to 2.06 DDDs/1000 inhabitants per day [113], and consumption has increased since then. According to data from the EU/EEA Annual Epidemiological Report for 2020 (ESAC-Net, 2020), total antimicrobial consumption in Spain is 18.2 and in Europe, it is 15 DDDs per 1000 inhabitants on average.

Most wastewater treatment plants are not able to remove these drugs [114], which end up in sludge, as occurs with TC [8]. The same is true for AMP in liquid effluent or ERY in both sludge and liquid effluent [9]. It is not uncommon, therefore, to find levels in the ng/L range in effluents containing high concentrations of most of the ABXs we have studied herein, such as CHL [115], AMP, and ERY [116] Levels of 759 ng/L for AMO and 1000 to 5000 ng/L for ERY [117,118] have been found in this type of effluent.

ABXs pass from effluents to rivers, where they have also been widely detected. Measured TC levels in European rivers are around 50 ng/L [119], although, in some Chinese rivers, they can reach 9500 ng/L [120], which is logical, given that China is the world's largest consumer of ABXs. In 2013, 53,800 tons were estimated to have been discharged into receiving waterways [121]. Up to 110,000 ng/L have been detected in Brazilian rivers [122]. ERY and AMP have been detected at 1149 and 184 ng/L, respectively, in rivers in Ghana [123] and AMO has been detected at up to 4630 ng/L in rivers in Brazil [124]. Some studies suggested that concentrations could reach between 0.1 and 1 μg/L at the most exposed locations of European rivers [125].

These data reflect that an acute ecotoxicity effect in rivers is not likely, since the levels of these ABXs detected in watercourses only exceed 0.1 μg/mL in extreme cases.

However, in our study, at these low concentrations, we observed an enhancement phenomenon in the ability to metabolize certain substrates for almost all ABXs, especially carbohydrates, carboxylic and ketonic acids, and amines and amides (Figure 4 at 0.1 μg/mL). On the other hand, at this same concentration, ABXs, like AMO, decreased the capacity to metabolize all substrates. This finding aligns with prior research, indicating that the metabolic functions of microbial communities are sensitive to environmental changes, and the response of specific functional flora to environmental changes is significant [126,127]. Specifically, it has been reported that environmental micropollutants can exert a significant influence on the bacterial community in surface water, even at low concentrations [128,129].

Therefore, at these low concentrations, changes occur in the growth and metabolic functions of river communities, but the extent of these changes is difficult to assess. Microbial communities play a key role in the ecological functions of the river, such as denitrification, methanogenesis, and sulfate reduction [130]. ABXs could lead to the loss or enhancement of microbial community functions, potentially affecting these ecological functions [131]. Impaired carbohydrate metabolism following exposure to AMO, for example, can impede the breakdown of organic matter in the ecosystem, thereby affecting nutrient recycling and the overall health of the ecosystem. Conversely, an increase in the capacity to metabolize these organic compounds can expedite the decomposition of organic matter within the river ecosystem, influencing the availability of resources for other organisms. This increased decomposition of organic matter may elevate the risk of eutrophication, potentially harming aquatic organisms such as fish and other vertebrates.

In these alterations, certain groups of microorganisms may derive greater benefits than others, potentially reshaping ecological interactions in the river and leading to phe-

nomena of aquatic microecological imbalance. Sensitive individuals may succumb to the selective pressure of ABXs and their derived effects, potentially being replaced by resistant opportunists [132,133].

This microbial community imbalance must also be considered in a river environment, where a great mixture of ABXs and other drugs may be present, in addition to other emerging contaminants. All may interact with each other, creating a low-dose ABX exposure that becomes prolonged over time. Taken together, all of this could increase the risk of ABXs in freshwater environments [125].

Some studies have highlighted the risk of ABX exposure in aquatic microorganisms [34,125,134,135]; however, studies generally focused on individual indicator organisms [136] that showed great variability in their sensitivity to different ABXs [23,85]. Días [78] reported that levels in the range of 0.1 μg/mL were necessary for GTM or TC to inhibit the growth of several Cyanobacteria; however, AMO appears to be more aggressive at lower concentrations. Similar results were found by Deng et al. [42] who showed that concentrations of 0.1 μg/mL were capable of inhibiting the growth of Microcystis aeruginosa. Other authors indicated that very low concentrations of TC (300 ng/L) seemed to have a growth-enhancing effect on Cyanobacteria [137], and that concentrations higher than 50 μg/mL were necessary for TC to have any effect on different Actinomycetes [138]. Zhou [137] found that levofloxacin and oxytetracycline concentrations higher than 0.1 μg/mL affected some aquatic microbial species but not others, even at 10 μg/mL. Therefore, it is difficult to extrapolate the dose results of the effect of ABXs on individual organisms to a microbial community where effects are much more complicated, as observed, for example, by Lu [129] with the ABX ciprofloxacin, which could generate reciprocal and antagonistic interactions between cyanobacteria, other bacteria, and eukaryotes.

However, ecotoxicity studies in aquatic bacterial communities have been conducted with very few ABXs and, to our knowledge, none of the ones analyzed in this study [32,129]. Other studies did not taxonomically characterize microbial populations [43,139] or focused on other microorganisms, such as diatomaceous algae [41]. Therefore, the results for these eight ABXs may contribute toward filling the gaps regarding the impact of ABXs on river microbial communities.

Finally, a serious effect of this permanent exposure to ABXs at the levels detected is that it may contribute to local environmental ABX resistance in microorganisms. The selection of potential ABX-resistant bacteria is a phenomenon that is in full expansion [140–143] and is considered a public health threat by the WHO [144]. In fact, in effluents where ABX concentrations are particularly high, such as in hospitals, the abundance of bacteria resistant to a given ABX is very high, as has been described, for example, in the case of AMP and AMO [145].

4. Materials and Methods

4.1. Antibiotics

The ABXs employed in this study included chloramphenicol (CHL), tetracycline hydrochloride (TC), erythromycin (ERY), streptomycin sulfate (STM), and gentamicin sulfate (GTM), all of which were supplied by Acofarma (Madrid, Spain). Amoxicillin (AMO), ampicillin (AMP), and penicillin G sodium salt (PEN) were obtained from Sigma Aldrich (Darmstadt, Germany). The purity of all the products was ≥95%. Their families, physicochemical properties, and other relevant characteristics are listed in Table 1.

Stock solutions of the ABXs were prepared in distilled water at the final concentrations of 0.1, 100, and 1000 μg/mL [136].

4.2. River Microorganism Sampling

Freshwater samples were collected in May 2021 from the Gallego river in Zaragoza (Spain, 41°41′58.0″ N, 0°49′51.7″ W). Two samples of 1.5 L were used for the physicochem-

ical analysis (see Table S1) and for the community-level physiological profiling (CLPP) assay, respectively. In addition, a third 5 L sample was taken for genetic analysis.

The bottles were unsealed at the time of sampling. Prior to sampling, the water bottles were homogenized with river water five times; the sample was taken from a representative area of the river [146] and immediately taken to the laboratory. In situ parameters were also measured. The water temperature was 13 °C (Nahita thermometer); the pH was 6 (PanReac AppliChem, Barcelona, Spain); and the conductivity was 2.73 mS/cm (conductivity meter Hanna HI8733).

4.3. River Sample Preparation for Genetic Analysis

On the same day as sampling, according to the methodology previously described in paper [47], the 5 L of fresh water were filtered (nylon 70 µm pore diameter filter, BD Falcon®®,Thermo Fisher Scientific, Waltham, MA, USA) to eliminate large solid debris. Next, the 5 L of water were filtered again with ultrafiltration equipment (0.22 µm pore diameter Sterifix®®, B. Braun Medical S.A.U, Rubí, Spain) to retain bacteria. Once the filter became saturated, they were washed with PBS (phosphate buffered saline, pH = 7.5) and the bacterial suspensions were collected in Petri dishes. Six filters were required to filter the 5 L. Bacterial suspensions were distributed into six 10 mL Falcon tubes and centrifuged at 5000 g for 10 min at room temperature (Heraeus Biofuge Primo R centrifuge, Thermo Fisher Scientific, Waltham, MA USA). After centrifugation, supernatants were removed with Pasteur pipettes, and 10 mL of sterile water added. This process was repeated five times and the final product (six Falcon tubes with the pellet of the bacteria) was stored at −80 °C (Froilabo Trust freezer, Collégien, France) for further genetic sequencing. Samples were always handled under sterile conditions.

4.4. River Sample Preparation for Community-Level Physiological Profiling of Freshwater Microbes with Biolog EcoPlates™

Biolog EcoPlates™ (Biolog Inc., Hayward, CA, USA), containing 31 triplicated carbon sources for microbial metabolism, allowed us to detect changes in the physiological profiling of river microorganisms exposed to different concentrations of the ABXs tested [47,147].

In order to prepare samples for the Biolog EcoPlate™ assays, 1 L of water was taken from one of the 1.5 L bottles sampled in the river and was filtered (using the previously described 70 µm pore diameter filters) to eliminate debris. Then, the water samples were transferred to the laminar flow biological safety hood (Model MSC Advantage 1.2).

Using a multichannel pipette, 75 µL of the water sample and 75 µL of ABX solution were placed in each well of the Biolog EcoPlate™. The final pH of the solution was measured with a pH meter (Hach, Sension+ pH3) to ensure it was in a neutral pH range. Each concentration was tested in triplicate. A plate with river water filtered in the same way and without antibiotic was used as a control.

The optical density (OD) of each well was measured at 590 nm (time 0 h) using the Bio-Tek Synergy H1 microplate reader (Agilent Technologies Inc., Santa Clara, CA, USA) and Gen5™ data analysis software. Biolog EcoPlates™ were incubated at 25 °C for seven days (J.P Selecta, Barcelona, Spain). The OD was subsequently measured every 24 h for seven days.

The average well color development (*AWCD*) was calculated, as given by [148,149]:

$$AWCD = \sum_{i=0}^{i=12} (OD_{t=xi} - OD_{t=x0}) \quad (1)$$

where OD_t = x0 is the optical density for a well at the beginning of the experiment and OD_t = xi is the value of the optical density for the same well at any given time.

4.5. Taxonomical Analysis: 16S rRNA Gene Sequencing

The solution obtained in Section 4.3 was sequenced at the ADM BIOPOLIS laboratories (Parc Cientific, Universitat de Valencia, Paterna, Spain). The DNA was extracted with the

High Pure PCR Template Preparation Kit (F. Hoffmann-La Roche AG, Basel, Switzterland) following the manufacturer's instructions. The DNA concentration and quality was checked by Nanodrop 2000c (Thermo Fisher Scientific, Madrid, Spain) and agarose gel electrophoresis (1% w/v in Tris-EDTA buffer). DNA was stored at −20 °C until it was used for sequencing. A total of 50 ng of DNA was amplified, following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (ILLUMINA, Inc., San Diego, CA, USA). Two consecutive amplification processes were carried out. In the first step, the primers designed were composed of two elements: a universal linker sequence, allowing amplicons for incorporation indexes and sequencing primers from the Nextera XT Index kit (ILLUMINA, San Diego, CA, USA), and 16S rRNA gene universal primers 341F/785R for the hypervariable region V3-V4 [141].

In the second step of the amplification process, assay amplification indexes were included. The quantification of the 16S-based libraries was carried out with a fluorimetry Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The sequencing process was conducted on the MiSeq platform (Illumina) with a 300-cycle paired-end read (300 bp). Libraries were pooled before sequencing. The Bioanalyzer 2100 (Agilent) and Library Quantification Kit for Illumina (Kapa Biosciences, Wilmington, MA, USA) were used to assess the size and quantity of the pool, respectively. The PhiX Control library (v3) (Illumina) was combined with the amplicon library (expected at 20%). Data quality assessment, base calling and image analysis were performed on the MiSeq instrument (MiSeq Control Software MCS v3.1). After approximately 56 h, the sequencing data became available.

4.6. Bioinformatic Analysis

The quality of the reads of the FASTQ files was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 21 September 2021). After the quality analysis, based on the results obtained, data preprocessing was carried out using Prinseq [150]. The preprocessing was performed by filtering the terminal indeterminations at both ends (N), and sequences with a mean quality (Phred quality score) lower than 26, with more than 5% of indeterminations, or sizes smaller than 150 bp were removed. Finally, bases at both ends with a quality lower than 28 were also trimmed.

The reads of the resulting files were merged, to obtain a read from overlapping paired-end Illumina sequencing reads' forward–reverse pairs; this procedure was carried out using STICH [151].

Taxonomy was assigned using the Ribosomal Database Project (RDP) release 11 (Bacteria+Archaea combination) as the reference database [152]. Taxonomical assignation was performed with a custom-made pipeline using VSEARCH [153]. Alignment was performed, establishing high stringency filters (≥90% sequence identity, ≥90% query coverage).

4.7. Data Representation

A hierarchical representation of the taxonomic assignment hierarchies of microbial communities was performed using Krona [154].

XLSTAT software Addinsoft 2021 was used to build the dose–response models and calculate the standard deviations of the replicated values. Growth as a function of time was represented by *AWCD* curves, which were fitted to a logistic model [155,156], described by Equation (2) with the Excel Solver add-in (Microsoft 365), https://www.microsoft.com/microsoft-365 (accessed on 30 January 2023):

$$AWCD = Cmax/(1 + e^{(}b - rt)) \quad (2)$$

where t is time, Cmax is the maximum achievable population density, also called carrying capacity, r is the intrinsic rate of population growth, and b is a necessary parameter for the sigmoid adjustment method, although it has no physical significance. Cmax and r were obtained for each ABX (Table 1).

The R package vegan (http://vegan.r-forge.r-project.org/, accessed on 30 January 2023) was used to perform the RDA. The radial graph was performed using normalized data for each ABX and physicochemical parameter. Normalization was carried out, considering the maximum value as one and recalibrating each value from this point.

The radial graph was constructed from the normalized physicochemical data with Excel software (office 365).

5. Conclusions

This study showed that eight widely consumed ABXs, present in watercourses around the world, could decrease the growth and metabolic capacity of river microbial communities, which are important as the basis of food webs and closure of carbon cycles in rivers. At ABX concentrations of 100 µg/mL, there was a reduction in microbial community growth and metabolic capacity, particularly for polymers, carbohydrates, carboxylic, and ketonic acids. CHL exhibited the greatest reduction in the capacity to metabolize all metabolites, followed by ERY and GTM. The differences detected in the toxicity of the eight ABXs likely stemmed from their distinct mechanisms of action and physicochemical properties, which may render them more or less bioavailable. A decrease in the ability to metabolize polymers or carbohydrates could potentially disrupt ecological dynamics within the river, impacting the breakdown of organic matter in the ecosystem and thereby influencing energy flow and nutrient recycling.

Yet, the impact of ABXs could already be detected at concentrations on the order of 0.1 µg/mL, where a slight increase in microbial growth and metabolism was observed (except in the case of AMO). This suggests that certain groups of microorganisms may experience benefits, leading to an imbalance in aquatic microecology that could impact the ecological functions of the river.

While acute ecotoxicity impacts are unlikely at the concentrations commonly found in rivers worldwide (except in extreme cases), the imbalance of communities at lower concentrations is not a negligible concern, especially considering the potential effects of long-term exposure to a complex mixture of ABXs and synergistic effects with other drugs and products present in the water.

This comprehensive study of a diverse range of ABXs, with different mechanisms of action on microorganisms, obtained from river waters with known taxonomy through 16S sequencing, fills the existing gap in understanding the effects of ABXs on river microbial communities and may help to better manage ABX residues to protect river ecosystems.

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5.2. Capítulo 2. Mejora de la eficacia de los antibióticos con compuestos naturales: actividad sinérgica del ácido tánico y el nerol con antibióticos comerciales frente a bacterias patógenas.

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- Resumen:

Este estudio evaluó la capacidad antimicrobiana de dos compuestos naturales, AT y NE, frente a 14 bacterias patógenas, incluyendo bacterias de la misma especie que en la lista de cepas prioritarias según la OMS. El AT mostró las CMIs más bajas, alcanzando 162,5 mg/L contra *Pasteurella aerogenes* y 187,5 mg/L contra *Acinetobacter baumannii*. Por su parte, el NE mostró CMIs de 500 mg/L frente a *Pasteurella aerogenes* y *Salmonella enterica*.

Se analizaron un total de 35 combinaciones de NE y 13 de AT con 8 ATB comerciales, sobre las bacterias seleccionadas. En combinaciones con NE, se detectaron 4 sinergias con STM y GEN frente a *Salmonella enterica*, *Bacillus subtilis* y *Streptococcus agalactiae*, con reducciones en las CMI de ATB entre 75% y 87,5%. AT, por su parte, mostró 6 sinergias con CHL, AMP, ERY y STM frente a las bacterias *Acinetobacter baumannii*, *Streptococcus agalactiae* y *Pasteurella aerogenes*, con reducciones de CMI de entre 75% y 93,7%. Además, se identificaron múltiples efectos aditivos.

Posteriormente, los estudios cinéticos de estas combinaciones sinérgicas mostraron una inhibición completa del crecimiento bacteriano, sugiriendo que los PN potencian el efecto de los ATB facilitando su acceso a las dianas celulares o previniendo la resistencia bacteriana. Tanto el AT como el NE cuentan con perfiles de seguridad reconocidos por la EPA y la FDA, lo que los convierten en candidatos prometedores para potenciar la eficacia de ATB comerciales, reduciendo las dosis necesarias y posiblemente mitigando la resistencia antimicrobiana.

- Relación con la Tesis Doctoral:

Este estudio constituye un punto central de la Tesis, ya que en él que se demuestra que tanto el AT como el NE pueden generar combinaciones sinérgicas con algunos de los ATB analizados en el primer bloque experimental. Estas sinergias permiten reducir la dosis necesaria del ATB entre un 75% y un 93,7%, manteniendo una eficaz actividad antimicrobiana a lo largo del tiempo. La presencia comercial de estos compuestos naturales en fragancias, cosméticos y productos farmacéuticos o alimentarios, junto con su reconocimiento por organismos internacionales como productos seguros para el consumo, los posiciona como candidatos realistas para disminuir el uso de ATB de manera segura.

No obstante, resulta imprescindible confirmar y cuantificar los posibles efectos ecotoxicológicos del TA, el NE y sus combinaciones sinérgicas con ATB para evaluar si su toxicidad ambiental es comparable, inferior o superior a la causada por los ATB comerciales empleados a dosis convencionales. Esta evaluación es fundamental para garantizar la viabilidad ambiental de estas alternativas terapéuticas y evitar impactos ecológicos no deseados.

Los siguientes capítulos de la tesis se dedican precisamente a esta cuestión, abordando el análisis ecotoxicológico detallado de estos compuestos y sus distintas combinaciones.

- Importancia de los resultados:

La importancia científica de los resultados de este trabajo radica en que aporta una evidencia clara y cuantificada sobre el potencial de dos compuestos naturales, el AT y el NE, para potenciar la eficacia de ATB comerciales a través de sinergias. Estas sinergias permiten reducir considerablemente las concentraciones necesarias de estos fármacos para inhibir bacterias patógenas relevantes, algunas de las cuales presentan cepas clasificadas por la OMS como de alta prioridad debido a su resistencia.

Además, este estudio incorpora análisis cinéticos detallados, que demuestran la inhibición completa del crecimiento bacteriano en las combinaciones sinérgicas, aportando información funcional crucial sobre el modo de acción en tiempo real. La posibilidad de disminuir dosis de ATBs también puede minimizar efectos secundarios y limitar la presión selectiva que promueve la resistencia.

Desde una perspectiva científica y médica, estos resultados amplían el conocimiento sobre la interacción entre fitoquímicos y ATB, ofreciendo bases sólidas para el desarrollo de terapias combinadas que puedan ser implementadas clínica y ambientalmente para combatir infecciones resistentes y reducir la contaminación por ATB. Este trabajo representa así un avance significativo en la búsqueda de alternativas terapéuticas y de estrategias integrales para el manejo de la resistencia antimicrobiana y la contaminación ambiental por ATB.

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Enhancing Antibiotic Efficacy with Natural Compounds: Synergistic Activity of Tannic Acid and Nerol with Commercial Antibiotics against Pathogenic Bacteria

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Abstract: The search for synergies between natural products and commercial antibiotics is a promising strategy against bacterial resistance. This study determined the antimicrobial capacity of Nerol (NE) and Tannic Acid (TA) against 14 pathogenic bacteria, including ESKAPE pathogens. TA exhibited the lowest Minimum Inhibitory Concentrations (MICs) at 162.5 µg/mL against *Pasteurella aerogenes* and 187.5 µg/mL against *Acinetobacter baumannii* (WHO priority 1). NE showed its lowest MIC of 500 µg/mL against both *Pasteurella aerogenes* and *Salmonella enterica*. A total of 35 combinations of NE and 13 of TA with eight commercial antibiotics were analyzed. For NE, combinations with Streptomycin and Gentamicin were effective against *Salmonella enterica*, *Bacillus subtilis*, and *Streptococcus agalactiae*, with antibiotic MIC reductions between 75.0 and 87.5%. TA showed six synergies with Chloramphenicol, Ampicillin, Erythromycin, and Streptomycin against *Acinetobacter baumannii*, *Streptococcus agalactiae*, and *Pasteurella aerogenes*, with MIC reductions between 75.0 and 93.7%. Additionally, 31 additive effects with antibiotics for NE and 8 for TA were found. Kinetic studies on these synergies showed complete inhibition of bacterial growth, suggesting that natural products enhance antibiotics by facilitating their access to targets or preventing resistance. Given their safety profiles recognized by the EPA and FDA, these natural products could be promising candidates as antibiotic enhancers.

Keywords: nerol; tannic acid; synergy; antibiotics; natural product

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1. Introduction

Antimicrobial resistance (AMR) has emerged as one of the greatest threats to global health, economy, and development, with antibiotics losing effectiveness over the past few decades [1]. In the past two decades, antibiotic consumption has significantly increased, with a particular rise in the use of aminoglycosides. The World Health Organization (WHO) has published a comprehensive list of antibiotic-resistant bacterial pathogens [2]. This list highlights 12 families of bacteria posing the greatest threat to human health including multidrug-resistant bacteria capable of resisting three or more classes of antimicrobial drugs. This group includes bacterial species such as *Acinetobacter*, *Pseudomonas*, and several *Enterobacteriaceae* (*Klebsiella*, *Escherichia*, *Serratia*, or *Proteus*).

The WHO has consistently urged the development of new antibiotic therapies, as the discovery rate of new antibiotics has significantly declined since 1960 [3], despite efforts from the pharmaceutical industry. One viable strategy to address this challenge is the exploration of natural products (NP) from plants, which are, historically, a rich source of active ingredients [4,5] and traditional phytotherapies [6]. Each plant species can produce between 500 and 800 different secondary metabolites [7,8], many of which have known antimicrobial properties [9,10]. Some commercial antibiotics exhibit synergistic effects when combined with NPs [11–13], resulting in enhanced efficacy compared to single agents [14–16]. Such combinations could supplement traditional antibiotic treatments [17,18] and reduce the

environmental impact of antibiotics reaching aquatic and terrestrial environments [19,20], as well as the spread of resistance genes. Furthermore, plant-based NPs align with the "One Health" concept, causing fewer side effects on human, animal, and environmental health [21,22].

Within the realm of plant secondary metabolites, many NPs derived from essential oils have demonstrated antifungal and antimicrobial properties [23,24]. These compounds, often extracted via hydrodistillation [25,26], include phenolic compounds, diterpenes, flavonoids, and volatile terpenes. While essential oils concentrate low-water-solubility NPs, some effective antimicrobial compounds are found in the aqueous by-products or hydrolates [27–30].

Nerol (NE), a volatile monoterpene (Z)-3,7-dimethylocta-2,6-dien-1-ol), is found in plants like lemongrass and hops and is widely used in food, cosmetics, and household products due to its Generally Recognized as Safe (GRAS) status by the FDA [31]. NE exhibits broad-spectrum antimicrobial activity against various Gram-positive and Gram-negative bacteria [32–34], as well as antifungal properties against *Candida albicans*, *Aspergillus* species, and others [34–38]. NE is a valuable ingredient in fragrances [31], cosmetics, soaps, and shampoos, and it is also present in cleaning products [39]. Notably, NE has shown synergistic potential with norfloxacin, significantly reducing its MIC against *S. Aureus* (*Staphylococcus aureus)* [40], and has been used in synergy with other natural products such as carvacrol against nosocomial pathogens [33,34].

Tannic Acid (TA), a naturally occurring polyphenol, is extracted from various plants used as food and feed [41,42]. Recognized for its antioxidant, antimutagenic, and antitumoral properties [43], TA also displays broad-spectrum antimicrobial activity against numerous bacteria and fungi [42,44]. Additionally, it has recently received attention due to its intrinsic properties such as polymerization, antioxidation, and metal chelation in applications of engineered advanced materials in biomedicine [41]. It is also marketed in pharmaceutical products for the treatment of diarrhea, such as Cesinex®, demonstrating a broad spectrum and a good safety profile [45]. Although the literature mainly focuses on synergistic effects with plant extracts containing this product as one of many constituents, there is some evidence in the literature suggesting a potential synergistic activity of TA with antibiotics against a few bacteria such as *P. aeruginosa (Pseudomona aeruginosa)* or *S. aureus* [44,46,47].

Therefore, these two natural products are good candidates in the strategy to reduce antibiotic consumption through synergistic combinations. However, studies on their antimicrobial capacity in combination with commercial antibiotics are scarce. They have only been conducted on a few bacteria and are rarely characterized beyond the calculation of Minimum Inhibitory Concentration (MIC) differences.

Hence, the aim of this study is to identify and characterize for the first time the potential synergies of these two natural products with a broad spectrum of commonly used antibiotics and pathogenic bacteria. To this end, (1) the antimicrobial effect (bactericidal and bacteriostatic) of NE and TA has been quantified on 14 reference bacterial strains responsible for the most common infections today [48], which have been described as having a high potential to develop antimicrobial resistance, according to the WHO's list of priority pathogens [2]; (2) the synergistic effects with eight commonly commercialized antibiotics (ABXs), which represent various modes of action, have been analyzed on these 14 bacteria; and (3) the kinetics of inhibition of the most promising combinations have been analyzed.

2. Results

2.1. Antimicrobial Activity of Tannic Acid, Nerol, and Antibiotics

The antibacterial activity measured as Minimum Inhibitory Concentration (MIC) of Nerol (NE) and Tannic Acid (TA) against 14 microorganisms is shown in Table 1. A previous toxicity test assessment of dimethyl sulfoxide (DMSO) showed that the growth of none of

the tested bacteria, except for *Proteus mirabilis* (and thus ruled out for the synergy tests) was affected by a concentration of 2.5% DMSO used to solubilize natural products (NPs).

Table 1. Antimicrobial activity of Nerol and Tannic Acid on selected pathogenic bacteria (μg/mL).

Microorganism	Nerol			Tannic Acid		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
Escherichia coli (ATCC 25922)	2000	2000	1	>2600	>2600	-
Salmonella enterica (ATCC 13311)	500	500	1	>2600	>2600	-
Klebsiella pneumoniae (C6)	1000	1000	1	>1387.50	>1387.50	-
Serratia marcescens (ATCC 13880)	>1000	>1000	-	>2600	>2600	-
Proteus mirabilis (ATCC 35659)	-	-	-	-	-	-
Pseudomona aeruginosa (ATCC 27853)	>1000	>1000	-	>2600	>2600	-
Klebsiella aerogenes (ATCC 13048)	2000	2000	1	>5200	>5200	-
Acinetobacter baumannii (ATCC 19606)	1000	1000	1	187.50	>187.50	>1
Bacillus subtilis (ATCC 6633)	500	>1000	>1	>3570	>3570	-
Staphylococcus aureus (ATCC 9144)	1000	1000	1	325	>325	>1
Enterococcus faecalis (ATCC 19433)	1000	1000	1	650	650	1
Streptococcus agalactiae (ATCC 12386)	1000	500	2	320	>320	>1
Pasteurella aerogenes (ATCC 27883)	500	>500	>1	162.50	>325	-
Candida albicans (ATCC 10231)	1000	1000	1	1800	>1800	>1

MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration; ATCC, (American Type Culture Collection).

Both NPs alone showed antimicrobial activity against most of the tested bacteria. TA had the greatest effect on the bacteria studied, presenting MICs with a value $\leq$ 500 μg/mL in four of the bacteria studied, where the lowest value was 162.50 μg/mL for *Pasteurella aerogenes* and 187.5 μg/mL for *Acinetobacter baumannii*, classified as priority 1 by WHO. NE had MICs of 500 μg/mL in two cases, *Salmonella enterica* and *Pasteurella aerogenes*.

The values of the ratio between the Minimum Bactericidal Concentration (MBC) and the MIC of NE and TA indicated that the activity was bactericidal in most cases (MBC/MIC $\leq$ 4) for both compounds.

Supplementary Table S1 shows MIC test results for the 8 commercial antibiotics (ABX) against 13 different pathogenic bacteria. These concentrations will be used to calculate the fractional inhibitory concentration index (FIC_I) for the combinations with synergistic effects.

2.2. Checkboard Tests for Synergy Assessments between Tannic Acid, Nerol, and Antibiotics

Potential reductions in MIC for commercial ABXs in the presence of NE or TA were evaluated by calculating the FIC for each combination, as shown in Tables 2 and 3. Although most interactions with NE were found to be additive, four combinations exhibited synergistic effects: *S. enterica (Salmonella enterica)* + Streptomycin (STM), *B. subtilis (Bacillus subtilis)* + Gentamicin (GTM), and both combinations for *S. agalactiae (Streptococcus agalactiae)* (with STM and GTM). FIC values were a maximum of 0.5, with ABX MIC reductions of 87.50% for *B. subtilis* and a reduction of 75.00% in all other cases. The NE reductions were 75.00% in all cases except for *B. subtilis*, which had a reduction of 87.50%.

Table 3 shows that six additional synergistic interactions were found for TA. Chloramphenicol (CHL), Ampicillin (AMP), and Erythromycin (ERY) exhibited synergism with *A.baumannii (Acinetobacter baumannii)*, while STM and CHL exhibited synergism with *S. agalactiae*, and CHL with *P. aeruginosa*. The nature of the remaining eight combinations tested was additive. The combination of TA and AMP with *A. baumannii* achieved the lowest FIC value (0.312). ABX MIC reductions ranged from 75.00% to 93.75%, while TA

reductions ranged from 75.00% to 87.50%. No antagonistic interactions were observed in the results.

Table 2. FIC values of Nerol and ABXs combinations.

	ABX	NE in Combination	ABX in Combination	FIC	Interpretation	ABX Reduction (%)	NP Reduction (%)
Escherichia coli (ATCC 25922)	STM	250	4.69	0.62	Additivity	50.00	75.00
	GTM	31.25	12.50	1.02	Additivity	0	98.44
	ERY	1000	150	1	Additivity	50.00	50.00
Salmonella enterica (ATCC 13311)	AMP	250	7.81	1	Additivity	50.00	50.00
	AMO	15.62	31.25	0.53	Additivity	50.00	96.87
	STM	**125**	**1.17**	**0.50**	**Synergy**	**75.00**	**75.00**
	ERY	500	1.17	1.03	Additivity	96.87	0
Klebsiella pneumoniae (C6)	AMP	15.62	62.50	1.02	Additivity	0	98.44
	AMO	15.62	250	1.02	Additivity	0	98.44
	STM	125	4.69	0.62	Additivity	50	87.50
	ERY	15.62	18.75	1.02	Additivity	0	98.44
Klebsiella aerogenes (ATCC 13048)	GTM	31.25	6.25	1.02	Additivity	0	98.44
	ERY	31.25	75	1.02	Additivity	0	98.44
	CHL	31.25	3.91	1.02	Additivity	0	98.44
	STM	1000	0.78	2.50	Additivity	50	50
Acinetobacter baumannii (ATCC 19606)	AMP	500	62.50	0.75	Additivity	50	75
	AMO	15.62	125	1.02	Additivity	0	98.44
	STM	62.50	25	0.62	Additivity	50	87.50
	ERY	125	3.12	0.75	Additivity	50	75
	CHL	250	31.25	0.75	Additivity	75	50
Bacillus subtilis (ATCC 6633)	STM	62.50	3.12	0.56	Additivity	50	93.75
	GTM	**125**	**0.78**	**0.37**	**Synergy**	**87.50**	**87.50**
	CHL	500	3.91	1.50	Additivity	0	50
Staphylococcus aureus (ATCC 9144)	STM	500	75	1.50	Additivity	0	50
	GTM	500	50	1.50	Additivity	0	50
	CHL	500	7.50	1.50	Additivity	0	50
Enterococcus faecalis (ATCC 19433)	STM	500	1.56	0.75	Additivity	75	50
	AMP	500	15.62	0.75	Additivity	75	50
	ERY	500	6.25	1.50	Additivity	0	50
Streptococcus agalactiae (ATCC 12386)	AMO	500	7.81	1.02	Additivity	50	50
	STM	**250**	**31.25**	**0.50**	**Synergy**	**75**	**75**
	GTM	**250**	**6.25**	**0.50**	**Synergy**	**75**	**75**
Pasteurella aerogenes (ATCC 27883)	STM	500	3.12	0.62	Additivity	87.50	50
	TC	250	1.56	0.75	Additivity	50	75
	GTM	250	3.91	0.75	Additivity	50	75

Concentrations of antibiotic (ABX) and Nerol (NE) in (µg/mL). STM (Streptomycin), GTM (Gentamicin), TC (Tetracycline), AMO (Amoxicillin), ERY (Erythromycin), AMP (Ampicillin), CHL (Chloramphenicol), FIC (fractional inhibitory concentration index), ATCC (American Type Culture Collection), NP (natural product).

From the synergy checkerboard results, isobolograms have been plotted for TA and NE (Figures 1 and 2) in cases where the $FIC_I \leq 0.5$. Four combinations show two synergy points, *A. baumanii* + TA + CHL, *A. baumanii* + TA + ERY, *S. agalactiae* + TA + CHL, and *B. subtilis* + NE + GTM. The lowest FIC_I was 0.250 for the combination *A. baumanii* + TA + CHL.

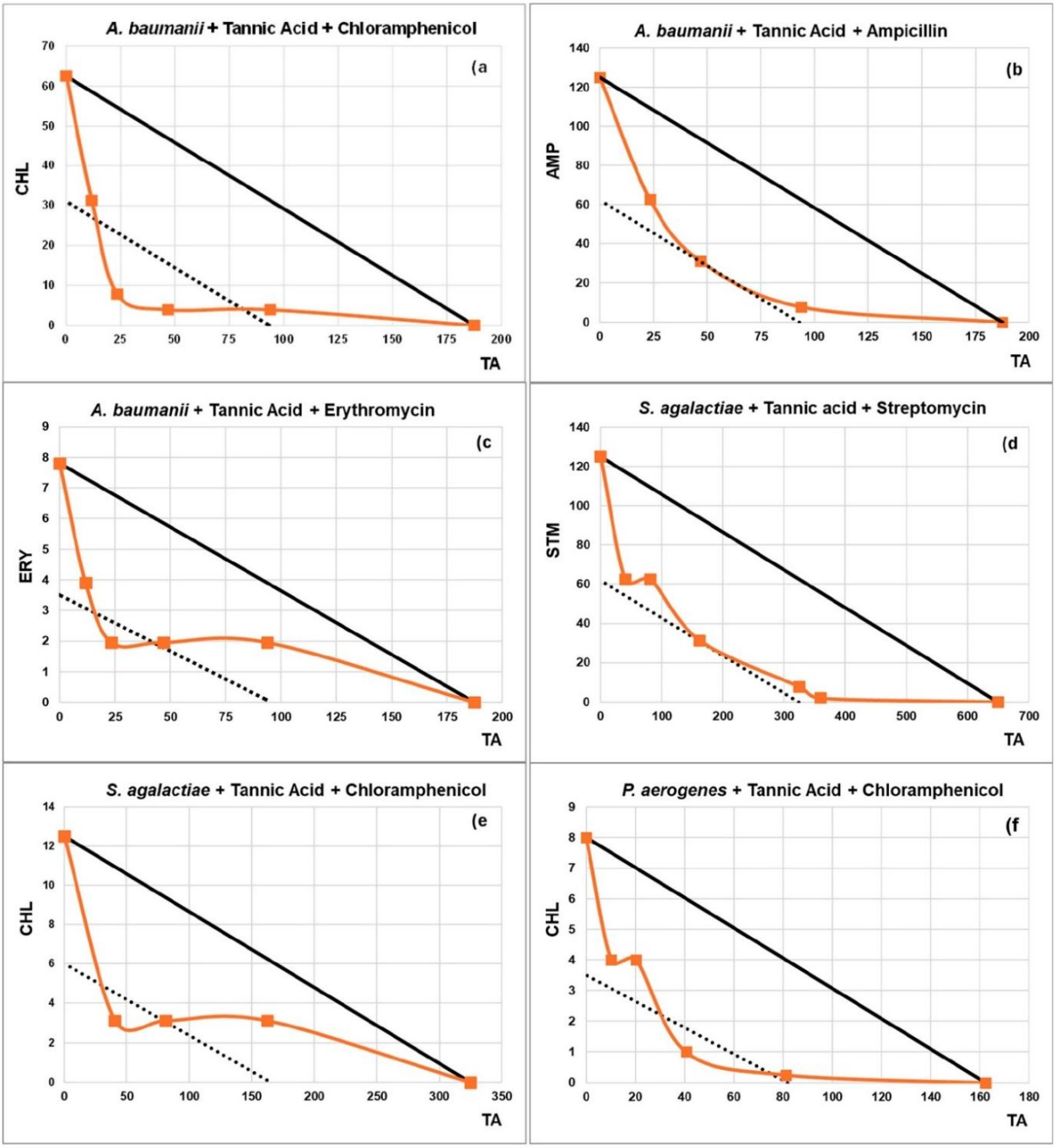


Figure 1. Isobolograms illustrate the interactions of Tannic Acid (TA) with the antibiotics (ABXs) where synergy was detected. The title of each subgraph indicates the bacterium studied along with the composition of the synergy showed in the isobologram.The x-axis represents TA concentrations, while the y-axis represents antibiotic concentrations. The solid line, known as the 'addition line', helps differentiate between additive effects—where points fall on or near this line—and synergistic effects, where concave isoboles are found below it. Additionally, there is a dashed line indicating the boundary of synergy. Points situated above or below this dashed line signify different degrees of synergistic interaction. Concentrations of ABXs and TA in (μg/mL).

Table 3. FIC values of Tannic Acid and ABXs combinations.

Microorganism	ABX	TA in Combination	ABX in Combination	FIC	Interpretation	ABX Reduction (%)	NP Reduction (%)
Staphylococcus aureus (ATCC 9144)	STM	40.62	25	0.75	Additivity	50	87.50
	CHL	40.62	3.75	0.62	Additivity	50	87.50
Acinetobacter baumannii (ATCC 19606)	STM	46.87	37.50	0.75	Additivity	50	75
	CHL	**46.87**	**3.91**	**0.31**	**Synergy**	**93.75**	**75**
	AMP	**46.87**	**7.81**	**0.31**	**Synergy**	**93.75**	**75**
	ERY	**23.44**	**1.95**	**0.50**	**Synergy**	**75**	**87.50**
	PEN	23.44	250	1	Additivity	50	75
Streptococcus agalactiae (ATCC 12386)	**STM**	**81.25**	**31.25**	**0.50**	**Synergy**	**75**	**75**
	CHL	**40.62**	**3.12**	**0.37**	**Synergy**	**75**	**87.50**
	GTM	31.25	25	1.03	Additivity	0	93.75
Pasteurella aerogenes (ATCC 27883)	STM	40.62	3.12	0.62	Additivity	87.50	50
	CHL	**40.62**	**1**	**0.37**	**Synergy**	**87.50**	**75**
	GTM	40.62	12	1.25	Additivity	0	75

Concentrations of antibiotics (ABX) and Tannic Acid (TA) in (µg/mL). STM (Streptomycin), GTM (Gentamicin), TC (Tetracycline), AMO (Amoxicillin), ERY (Erythromycin), AMP (Ampicillin), CHL (Chloramphenicol), FIC (fractional inhibitory concentration index), ATCC (American Type Culture Collection), NP (natural product).

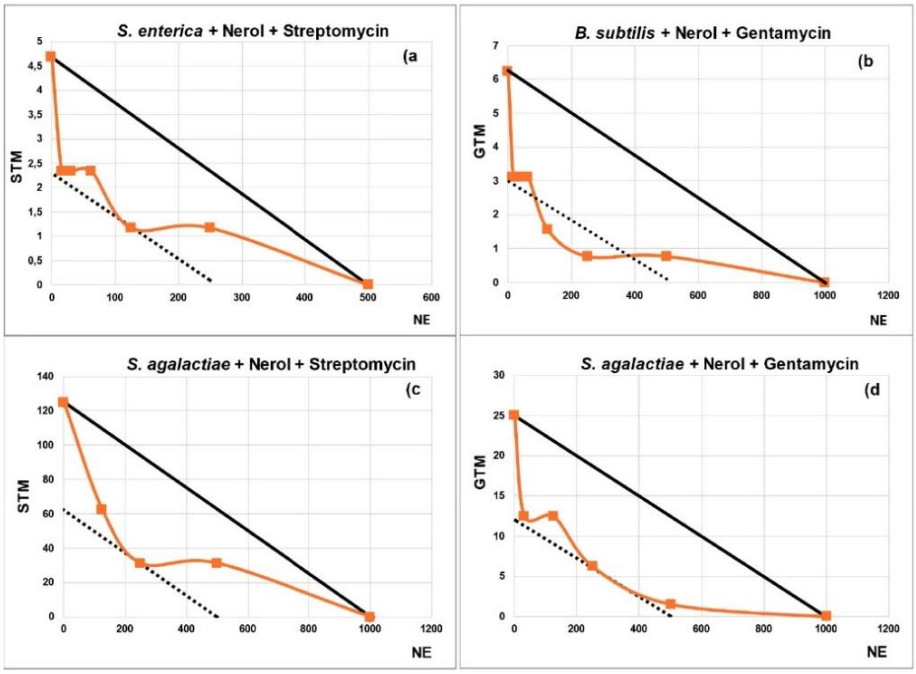
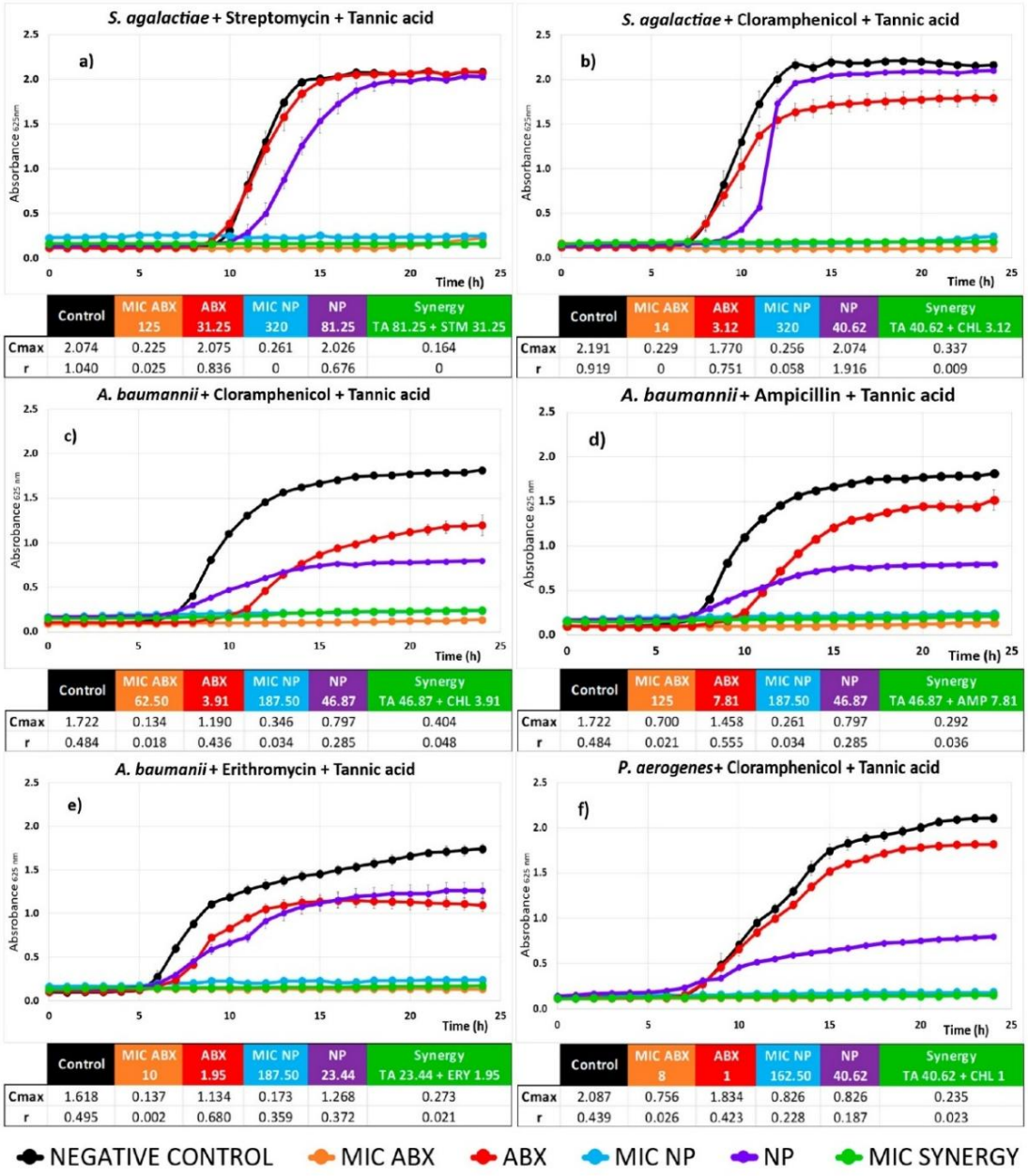


Figure 2. Isobolograms illustrate the interactions of Nerol (NE) with the antibiotics (ABXs) where synergy was detected. The title of each subgraph indicates the bacterium studied along with the composition of the synergy described in the isobologram.The x-axis represents NE concentrations, while the y-axis represents ABX concentrations. The solid line, known as the 'addition line,' helps differentiate between additive effects—where points fall on or near this line—and synergistic effects, where concave isoboles are found below it. Additionally, there is a dashed line indicating the boundary of synergy. Points situated above or below this dashed line signify different degrees of synergistic interaction. Concentrations of ABXs and NE in (µg/mL).

2.3. Synergy Kinetics Study

A kinetic study of the 10 different synergistic combinations was carried out, with the aim of gaining a deeper understanding of the behavior of each bacterium throughout its incubation period. Results are shown in Figures 3 and 4.



	Control	MIC ABX 125	ABX 31.25	MIC NP 320	NP 81.25	Synergy TA 81.25 + STM 31.25
Cmax	2.074	0.225	2.075	0.261	2.026	0.164
r	1.040	0.025	0.836	0	0.676	0

	Control	MIC ABX 14	ABX 3.12	MIC NP 320	NP 40.62	Synergy TA 40.62 + CHL 3.12
Cmax	2.191	0.229	1.770	0.256	2.074	0.337
r	0.919	0	0.751	0.058	1.916	0.009

	Control	MIC ABX 62.50	ABX 3.91	MIC NP 187.50	NP 46.87	Synergy TA 46.87 + CHL 3.91
Cmax	1.722	0.134	1.190	0.346	0.797	0.404
r	0.484	0.018	0.436	0.034	0.285	0.048

	Control	MIC ABX 125	ABX 7.81	MIC NP 187.50	NP 46.87	Synergy TA 46.87 + AMP 7.81
Cmax	1.722	0.700	1.458	0.261	0.797	0.292
r	0.484	0.021	0.555	0.034	0.285	0.036

	Control	MIC ABX 10	ABX 1.95	MIC NP 187.50	NP 23.44	Synergy TA 23.44 + ERY 1.95
Cmax	1.618	0.137	1.134	0.173	1.268	0.273
r	0.495	0.002	0.680	0.359	0.372	0.021

	Control	MIC ABX 8	ABX 1	MIC NP 162.50	NP 40.62	Synergy TA 40.62 + CHL 1
Cmax	2.087	0.756	1.834	0.826	0.826	0.235
r	0.439	0.026	0.423	0.228	0.187	0.023

Figure 3. Kinetic assay and Cmax and r values of Tannic Acid (TA) as natural products (NPs), antibiotics (ABXs), and their combinations against different bacteria. The title of each subgraph indicates the bacteria studied along with the composition of the synergy showed in the graph. Black represents the negative control, red represents the ABX concentration in the synergy, purple represents the NP concentration in the synergy, orange represents the Minimum Inhibitory Concentration (MIC) concentration of the ABX, and light blue represents the MIC concentration of the NP. The synergy is represented in green. Error bars indicate standard deviations ($n = 4$). Concentrations of ABXs and TA in (μg/mL). The color code used is described at the end of the chart.

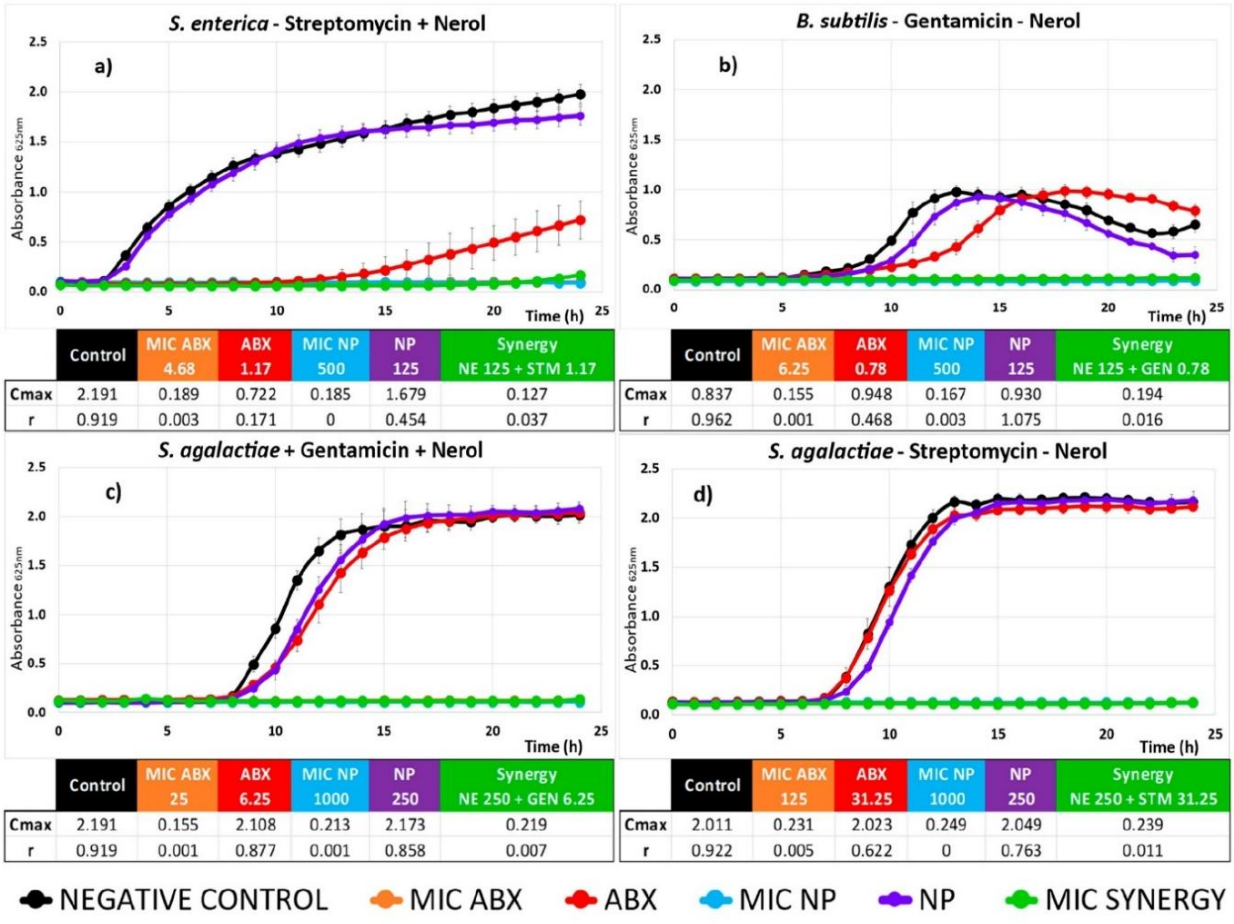


Figure 4. Kinetic assay and Cmax and r values of Nerol (NE) as natural products (NPs), antibiotics (ABXs), and their combinations against different bacteria. The title of each subgraph indicates the bacterium studied along with the composition of the synergy showed in the graph. Black represents the negative control, red represents the ABX concentration in the synergy, purple represents the NP concentration in the synergy, orange represents the Minimum Inhibitory Concentration MIC concentration of the ABX, and light blue represents the MIC concentration of the NP. The synergy is represented in green. Error bars indicate standard deviations (n = 4). Concentrations of ABXs and NE in (μg/mL). The color code used is described at the end of the chart.

3. Discussion

In this study, we investigated the ability of two plant-derived natural products to synergize with widely used commercial ABXs, aiming to reduce the required ABX dosage while maintaining efficacy. TA and NE were selected based on their demonstrated antimicrobial activity against various Gram-positive and Gram-negative pathogens [31,49,50]. The previous literature has also begun to suggest potential synergistic effects of these compounds with one or a few ABXs [51]. The use of these products offers a range of advantages: NE is abundant in essential oils from widely cultivated plants [52], while TA is prevalent in the bark of trees like oak and chestnut mahogany [53]. Therefore, their extraction from these inexpensive plant materials ensures high availability. Moreover, the essential oil industry enables cost-effective NE production, often as a byproduct of other compounds. Similarly, byproducts from the wine and wood industries can be used to extract TA, promoting sustainable production [54].

The antimicrobial capacity of both products was tested against a comprehensive and representative panel of 14 pathogenic bacteria to determine the MICs. This allowed us

to conduct a synergy study with 8 ABXs, resulting in a total of 48 combinations. The synergies (4 for NE and 6 for TA) were further analyzed by monitoring their growth kinetics. A reference bacterial strain was selected for each bacterium studied, enabling comparison with results from the literature, such as those involving other natural products or combinations with different ABXs.

3.1. Antimicrobial Activity of Tannic Acid and Nerol

NE and TA exhibit antimicrobial activity against most of the tested bacteria and the fungus *Candida albicans*: NE showed activity against 11 out of 14 organisms, with MIC ranges from 500 to 2000 µg/mL, and TA showed activity against 6 out of 14 organisms, with MIC ranges from 325 to 1800 µg/mL (Table 1).

The results obtained from MICs have been compared with the literature; however, the existing literature is scarce, as it mainly focuses on essential oils rather than pure compounds. The value of 500 µg/mL has been considered a reference value to consider a strong effect of NP according to bibliographic criteria [55].

The MIC values obtained for NE against *S. enterica* and *P. aerogenes* (500 µg/mL) are very similar to those found in the literature (441 µg/mL and 600 µg/mL) [32,56]. Similarly, the effect of NE on *E. faecalis(Enterococcus faecalis)*, *P. aeruginosa*, *S. enterica*, *K. pneumoniae (Klebsiella pneumoniae)*, and *C. albicans* has been documented in the literature, showing very similar MICs (600 µg/mL vs. 1000 µg/mL) [56].

Regarding TA, the literature shows a MIC for *S. aureus* between 40 and 160 µg/mL [44], which differs significantly from our results (325 µg/mL). There are also differences compared to *A. baumannii*, where our results show 187.5 µg/mL, while the literature reports a MIC of 600 µg/mL [57]. The existing variability may be due to the technique used, the solvent, the strain, or the culture medium [58].

To our knowledge, there are no bibliographic MIC values for the other bacteria studied in our work. There are studies that use essential oils containing these products, but these results are not comparable, as essential oils contain other compounds that can affect the calculation of MICs. In the case of *P. mirabilis (Proteus mirabilis)*, we were unable to calculate MICs because the concentration of DMSO needed to dissolve our NPs affected the bacteria.

Considering these results, two inclusion criteria were established to test synergies.

- ABXs with a MIC below 4 µg/mL for certain bacteria were discarded, mainly due to the difficulty in assessing reductions in such low concentrations;
- Bacteria susceptible to DMSO at concentrations $\geq$2.5% were discarded, as it was not possible to have stable dilutions of either NE or TA.

Once these criteria were applied, 35 interactions with NE and 13 with TA were selected for testing in checkerboard assays (Tables 2 and 3). The synergies identified between natural products and antibiotics have been represented in isobolograms, which allow for a more intuitive visualization of the relationship between individual data points and a reference line (the addition line), making it easier to understand the degree of synergy for dose combinations that fall below this line.

3.2. Kinetic Study of the Obtained Synergies

In all cases, as expected, no bacterial growth was observed for the MICs of ABXs and NP alone (orange and light blue lines in Figures 3 and 4). Additionally, while the ABX and NP separately produce a significant antimicrobial effect at the synergistic concentration compared to the control (red and purple lines in Figures 3 and 4), their combination at the same concentrations (green line) achieves complete inhibition of the growth of all tested bacteria. Consequently, these last three growth curves, which represent nearly complete inhibition of microbial growth, often overlap. Although natural products have shown clear antimicrobial activity on their own, the required concentrations are high. Combining them with ABXs not only reduces the MIC of the ABX, which is our primary goal, but also lowers the necessary concentrations of the natural product while maintaining the same antimicrobial effectiveness. This allows for a synergistic combination with low doses of

both agents. Additionally, the individual curves of NP and ABX indicate the potential mechanisms of action of each product separately, as explained below.

3.3. Tannic Acid and Nerol Synergies with Antibiotics against Gram-Positive Bacteria

The antimicrobial capacity of TA has been explained on the basis of its ability to damage the cell membrane, causing lysis of the bacteria, inhibiting enzymes of bacterial metabolism, affecting protein synthesis and bacterial growth [59], disrupting oxidative phosphorylation in mitochondria [60], chelating metal ions necessary for bacterial growth [17], and inactivating bacterial adhesins, which are responsible for the adherence of bacteria to the host [61,62]. Other suggested impacts of TA on microorganisms include the inhibition of efflux pumps, as observed in the case of *S. aureus* [46]. In addition to all these mechanisms, the literature shows a dependence on the concentration present, the pH, and the matrix in which it is found [63]. Recent studies on the genomics and transcriptomics of the mechanism of action of Tannic Acid and other Gram-positive cocci, such as *S. aureus* [64], have revealed that the integrity of the cell envelope is affected by a decrease in the expression and protein abundance of enzymes involved in the synthesis of peptidoglycans, teichoic acids, and fatty acids. Additionally, there is a reduction in ribosomal components that impacts protein synthesis.

The mechanism of action of NE is also based on interaction with the cell membrane. Similar to other acyclic monoterpenes, it is capable of interfering with the integrity of the membrane, leading to the development of pores and even destruction, resulting in leakage of cell contents [65], which has been primarily studied in fungi [34,38]. NE has been shown to form aggregates of antimicrobial–lipid complexes, reducing lipid packing efficiency, increasing membrane fluidity, and altering the total dipole moment of the membrane [66]. Its lipophilicity also enables it to partition into the lipophilic lipids of the mitochondria, disturbing these structures.

Gram-positive bacteria are affected by TA, as demonstrated in our results for *S. agalactiae* (Figure 3a,b). TA has been reported to influence the growth of other Gram-positive bacteria such as *Listeria monocytogenes* [42] and *S. aureus* [67,68], where it can also inhibit biofilm formation [69]. NE has also shown its antimicrobial capacity against Gram-positive bacteria, including *S. aureus* [32] and *S. epidermidis (Staphylococcus epidermidis)* [39].

In Figure 3a,b, TA at a synergistic concentration, while having minimal impact on total growth (Cmax similar to control), can delay growth by a few hours (r_{TA} = 0.676 compared to $r_{control}$ = 1.040, in Figure 3a, for example), likely due to cell envelope damage that the bacteria eventually recover from. For NE with *S. agalactiae* (Figure 4c,d), a similar but less pronounced effect is observed.

The ABXs (STM, CHL, and GTM) at this sub-MIC synergistic concentration show little impact on microbial growth. Only CHL (Figure 3b) significantly reduces the total population growth ($Cmax_{CHL}$ = 1.770 compared to $Cmax_{control}$ = 2.191). These ABXs target bacterial ribosomes. CHL is a broad-spectrum ABX that inhibits protein synthesis by binding to the 50S subunit of bacterial ribosomes, preventing peptide bond formation. Streptomycin (STM) and Gentamicin (GTM) both target the 30S subunit, disrupting protein synthesis by causing mRNA misreading and incorporating incorrect amino acids, leading to defective proteins and bacterial cell death [70]. Both ABXs are more effective against Gram-negative bacteria, and at this sub-MIC concentration, they possibly do not reach their target, which together explains their minimal effect on bacterial growth that we observed.

However, in synergy with TA and NE, the effect of these ABXs is enhanced to the point of completely inhibiting bacterial growth (green line in Figures 3 and 4). The most plausible mechanism for this synergy could be that the membrane-disrupting activities of both TA and NE facilitate the ABXs' access to their intracellular ribosomal targets, making the ABX effective even at sub-MIC concentrations. Additionally, TA can inhibit genes that regulate the synthesis of proteins composing the *S. aureus* 50S and 30S ribosome, as well as genes for proteins such as the translation initiation factor IF-2, which is involved in regulating the efficiency and fidelity of translation–initiation complex formation [64]. On

its own, TA would only cause the growth delay we observed, but in combination with the ABX, the effects of both products on the ribosomal target would accelerate the impact on protein synthesis, reducing bacterial survival. Furthermore, it has been reported that TA could enhance the inhibitory effect of ABXs targeting ribosomal sites, such as Erythromycin, on drug-resistant *S. aureus* by inhibiting bacterial virulence factors like efflux pumps [46], which would promote ABX accumulation in the cytoplasm, making it effective even at sub-MIC concentrations.

In the case of *B. subtilis* exposed to sub-MIC concentrations of NE (Figure 4b), we observe minimal changes compared to the control. To our knowledge, there are no previous studies specifically examining the activity of NE against *B. subtilis*, except for essential oils containing fractions of this product, which have demonstrated slight antimicrobial activity against *B. subtilis* [71]. When exposed to sub-MIC concentrations of GTM, however, we observe that the ABX can delay growth for several hours, but the bacteria eventually recover, reaching a Cmax that is like or even higher than the control (Figure 4b). This recovery might be due to enzymatic modification of the ABX (aminoglycoside-modifying enzymes), alteration of the target site, or active efflux of the ABX, as described in other cases for this ABX in aminoglycosides [72]. It is possible that after prolonged exposure to the ABX, resistant strains emerge through competitive selection. However, this strain typically does not have intrinsic resistance to Gentamicin (https://genomes.atcc.org; 8 July 2024), and horizontal gene transfer is unlikely in a reference strain, necessitating further studies to clarify the mechanisms of recovery at sub-MIC concentrations. In synergy, however, NE can eliminate this effect by enhancing the ABX's activity, possibly by facilitating its access to the ribosomal target, or by inhibiting bacterial resistance mechanisms.

3.4. Tannic Acid and Nerol Synergies with Antibiotics against Gram-Negative Bacteria

Gram-negative bacteria have a more complex envelope, with an external lipid bilayer and a peptidoglycan layer [73–75]. It would be expected that they are somewhat more resistant to the membrane disruption effects of TA. However, TA appears to be much more effective against *A. baumannii* and *P. aerogenes (Pasteurella aerogenes)* (Figure 3c,d,f) than we observed with Gram-positive bacteria. This is consistent with the literature describing TA's effects on various Gram-negative bacteria, such as *E. coli (Escherichia coli)* [51] and *S. enterica* [76].

Despite its high molecular weight (1701.2 g/mol) [77] and high hydrophilicity (log Kow = −0.19) [77], TA contains multiple hydroxyl groups that can electrostatically interact with the phosphate and carboxylate groups present in the LPS of the Gram-negative outer membrane. This interaction could facilitate the binding of TA to the bacterial surface, affecting the permeability and integrity of the outer membrane, allowing it to penetrate through the peptidoglycan layer to reach the cell membrane and its cytoplasmic targets. Interestingly, quorum-sensing disruption effects might also occur, as observed with TA in a P. aeruginosa strain [78].

In the case of *A. baumannii* (Figure 3c,d), the effect of sub-MIC concentrations of TA appears more detrimental to the bacterial population from the onset of bacterial growth following the lag phase, resulting in a lower $Cmax_{TA}$ compared to the control in all three cases. Additionally, it is evident that both AMP and CHL inhibit bacterial growth for 3 to 4 h longer than TA, reflected in a higher r. However, the microbial population exposed to ABX is able to recover better, with Cmax values closer to the control.

CHL targets the 50S subunit of the bacterial ribosome, while AMP is a broad-spectrum penicillin ABX that disrupts bacterial cell wall synthesis by binding to bacterial penicillin-binding proteins (PBPs), preventing cross-linking of peptidoglycan chains, and leading to bacterial lysis [79]. It seems that the bacteria can recover from ABX exposure after 5 to 10 h, possibly due to resistance mechanisms. Indeed, *A. baumannii* possesses various intrinsic resistance genes against all three ABXs. For instance, it has inactivating enzymes against CHL (chloramphenicol acetyltransferase) and efflux pumps that expel CHL and ERY from the cell, as well as beta-lactamases for AMP, among others [80].

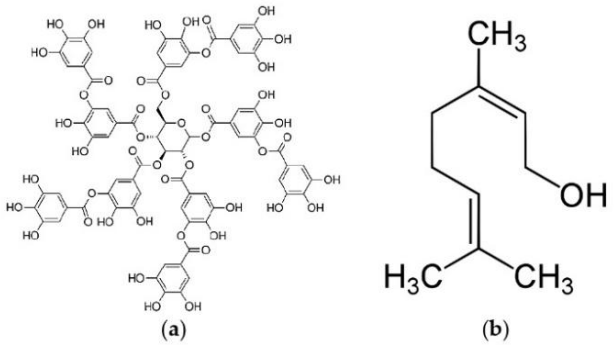
We observe that the growth of *A. baumannii* is halted after exposure to TA from 10–15 h onward, indicating that the effect of this natural product remains consistent over time, possibly due to its lower potential to induce resistance, as seen with other similar natural products [24].

The effect of ERY on the same bacteria (Figure 3e) shows less growth delay compared to the previous cases, despite the ABX mechanism of action being similar to that of CHL. ERY is a broad-spectrum ABX of the macrolide family, which acts by binding to the 50S ribosomal subunit of sensitive microorganisms, similar to CHL, but at a different site, inhibiting ribosomal translocation and thus the incorporation of new amino acids, ultimately resulting in the arrest of peptide chain elongation.

When *P. aerogenes* is exposed to TA and CHL (Figure 3f), we observe that TA not only delays microbial growth more than the ABX (r_{TA} = 0.187 vs. r_{CHL} = 0.423), but also results in a more pronounced inhibition of the total microbial population (C_{maxTA} = 0.826 vs. C_{maxCHL} = 1.834) compared to the previous cases. It is important to note, however, that in both cases, the dose of TA in synergy is somewhat higher than that of the ABX. The action of the ABX on this bacterium is also somewhat less effective.

As seen in the case of Gram-positive bacteria, the combination of the ABX and the natural product leads to complete inhibition of bacterial growth (green line in Figures 3 and 4). Combinations of TA likely enhance or facilitate the interaction of an antimicrobial agent with its target inside the pathogen, thereby preventing the emergence of resistance. For CHL and ERY, TA seemingly facilitates their access to their intracellular ribosomal targets by damaging the cell membrane. In the case of AMP, its mechanism of inhibiting peptidoglycan wall synthesis prevents the bacteria from maintaining cell wall integrity, leading to unbalanced osmotic pressure within the cell, ultimately causing cell lysis, which is probably further accentuated by TA's membrane-altering effects.

In the case of NE, its antimicrobial activity against Gram-negative bacteria such as *E. coli* and other intestinal bacteria [49], as well as *S. enterica* [32], has been reported. However, at the synergistic concentrations used in our study, we detected very little effect of NE on *S. enterica*, with growth kinetics like the control (Figure 5a). The properties of NE, such as its high lipophilicity (log Kow = 3.47) [77] and its low molecular weight (154.25 g/mol), facilitate its interaction with cell membranes. Gram-negative bacteria have porins in their outer membrane that act as selective channels, allowing the passage of certain small hydrophilic solutes. NE, being lipophilic, may have difficulty passing through these porins, although its small size might help. The outer membrane of Gram-negative bacteria and the presence of liposaccharids can present an additional barrier that may reduce the efficiency with which NE traverses these structures.



(**a**) (**b**)

Figure 5. Chemical structure of Tannic Acid (**a**) and Nerol (**b**).

However, the antimicrobial effect of STM is very pronounced, both in reducing the time to the onset of growth by 12–13 h and in decreasing C_{max} by more than 50% compared to the control, probably because NE's membrane-altering activity facilitates the access of STM to its 30S ribosomal target in the bacteria. In addition, genes such as aadA1, aadA2, and strA are associated with STM resistance in various *Salmonella* strains [81] as well as in

ours (https://genomes.atcc.org). These genes typically encode enzymes that modify and inactivate streptomycin, preventing it from binding effectively to the bacterial ribosome.

Finally, it is worth noting that although our study has focused on synergies, given that additive interactions may not be as effective as synergistic interactions, the latter are far more numerous in our results and therefore deserve attention.

As seen in Tables 2 and 3, they also allow for a reduction in the concentration of ABX, and in many cases, the concentration of the adjuvant needed to achieve additivity in combinatory treatment might even be lower than what is observed in synergistic interactions.

Although the mechanisms of additive activities have been little studied, we hypothesize that one possible cause could be that the action of the natural product only slightly damages the membrane or causes some intracellular damage, which by itself does not facilitate the ABX's action but simply adds to the damage inflicted on the bacteria. Additionally, due to the broad, nonspecific mechanisms associated with natural products, there may not be an opportunity for the combined activities of these compounds to exceed the sum of their parts, as has been suggested in the case of disinfectant combinations [82]. However, another study [83] argues that the effects on the membrane from the additive interaction of cinnamon bark oil and meropenem are very similar to those observed in previously reported synergistic combinations, indicating that further studies are indeed necessary to clarify this point. It is important to assess the therapeutic potential of additive interactions alongside synergistic ones, as many studies on natural ABX adjuvants, including this one, report an equal or greater number of additive interactions [83–88].

3.5. Future Perspectives

These results propose both TA and NE as enhancers of the activity of commercial ABXs as well as Antimicrobial Resistance Modifying Agents.

In addition to the previously mentioned advantages in their production, TA and NE likely have a lower potential for inducing resistance compared to commercial ABXs as a result of several factors [89]. First, natural compounds often have more complex and diverse chemical structures than synthetic ABXs, making it harder for bacteria to develop effective resistance mechanisms, as they would need to adapt to multiple sites or modes of action, which is more challenging. Additionally, while synthetic ABXs typically target a single cellular process, natural products like TA and NE act on multiple fronts, as we have seen. This multifaceted approach further reduces the likelihood of resistance, as bacteria would need to simultaneously mutate in several areas. Moreover, NE and TA can disrupt bacterial membranes, affecting resistance mechanisms associated with these membranes, such as efflux pumps. Another key point is that natural compounds have been in contact with microorganisms for millennia, possibly leading to an evolutionary balance where bacteria are less prone to develop resistance. In contrast, synthetic ABXs are more recent and often used in large quantities, which can exert intense selective pressure, quickly fostering the development of resistant strains. When natural products are used in synergy with ABXs, the required ABX doses can be significantly reduced, as seen in this study, decreasing the chances of resistance development. Finally, the simultaneous action of natural products and ABXs on different cellular targets makes it more difficult for bacteria to develop resistance strategies against both, a benefit that is less common in synthetic ABXs typically used as monotherapies.

Although further studies are necessary to clarify the mechanisms of action of these products in synergy with each type of ABX, the fact that both products can be considered safe for human use and are already marketed as health products makes them very promising candidates for clinical application. TA is already marketed as a medical product [90] and is recognized as safe in the US by the FDA (Food and Drug Administration) [91] and in the European Union by the EFSA (European Food Safety Authority) [92]. On the other hand, NE was approved by the Food and Drug Administration as a flavor (21 CFR 172.515) and is recognized as a safe flavor ingredient—GRAS 3 (2770)—by the Flavor and Extract Manufacturers Association (FEMA, https://www.femaflavor.org/flavor-library; 6 June

2024). Although it is not specifically approved as a food additive by regulatory agencies such as the FDA or the EFSA, the recommended doses for its use are much higher than the concentrations we have obtained in our synergies [31].

In general, natural products require higher doses compared to ABXs to achieve antimicrobial activity. However, the advantage of synergistic combinations is that they can reduce not only the concentration of ABXs but also the amount of the natural product needed, as demonstrated by our results.

Typically, these synergies are considered for use in topical formulations such as lotions, ointments, gels, or creams for skin infections, wounds, and ulcers [89]. They could also be applied in mouthwashes and oral rinses [93]. Oral solutions like Cesinex® [45] are already marketed for gastrointestinal conditions, with Tannic Acid as one of their components.

Additionally, there is potential for these combinations in veterinary feed to treat animal diseases, which is relevant given EU Regulation 2019/6 [94], which emphasizes reducing ABX use in livestock to mitigate environmental impact [20] and microbial resistance [95]. Furthermore, these synergies could be valuable as disinfectants to inhibit microbial biofilm formation on stainless steel surfaces [96].

The effective doses found in the synergies between natural products and ABXs are very low, ranging from 23.44 to 81.25 µg/mL for TA and from 125 to 250 µg/mL for NE. The ABXs in synergy further reduce their concentrations significantly, with ranges between 0.78 and 31.25 µg/mL. Topical ABXs available on the market, for example, are used at concentrations several orders of magnitude higher than the synergistic concentrations obtained in this study. Most of the available ABX ointments (such as those with mupirocin or sodium fusidate) and antifungal ointments (like ketoconazole) contain a concentration of 20 mg/g. Other formulations contain even more, such as oxytetracycline or clindamycin ointments, which typically have 30 mg/g of the ABX. Commercially available ABX eye drops have similar concentrations, often 3 mg/mL (or 3000 µg/mL). Some formulations of Gentamicin even reach up to 5000 µg/mL of the ABX. Therefore, it is pharmacologically realistic to prepare topical, veterinary, or even oral formulations containing these ABXs and natural products at the effective concentrations found in the synergies of our study.

New perspectives on the antimicrobial use of these natural products are emerging. TA, a type of polyphenol, can form metal complexes through coordination, as has been observed with other polyphenols such as gallic acid [97,98]. TA can form coordination compounds with silver and iron, leading to nanoparticles with antimicrobial activity [99,100]. This opens the door to potential new antimicrobial applications for these natural products, such as in wound management [101] or in shoe insoles and fruit preservation [100]. Additionally, the efficacy of these nanoparticles could potentially be enhanced by combining the natural product with an ABX to achieve synergistic effects.

However, before developing clinical formulations, it is crucial to assess the potential toxicity of the combination, as well as its bioavailability, through clinical trials [102], since the physicochemical and pharmacological properties of these combinations may differ from those of the individual products [103]. Formulations must also take into account organoleptic properties and stability. Finally, within a One Health framework, it is important to evaluate whether these synergies are more environmentally friendly compared to using higher doses of ABXs alone.

4. Materials and Methods

4.1. Antimicrobial Compounds

Two NPs, NE and TA, and eight ABXs were selected as antimicrobials for this study. Figure 5 shows the chemical structures of both NPs. CAS number, provider, and some chemical properties of each antimicrobial are compiled in Table 4. DMSO (Fisher Bioreagents) has been used to solubilize the NP solutions. The natural product solutions have been prepared with a concentration of 5% DMSO.

Table 4. Chemical details for the antimicrobials used.

Antibiotic/ Natural Product	Abbreviation	Chemical Family	CAS-Number	Supplier	Purity	Molecular Weight (g/mol)	Range of Concentrations Tested (µg/mL)
Gentamycin	GTM	Aminoglycosides	1403-66-3	ACO-FARMA	≥97.0%	447.60	250—0.19
Streptomycin	STM		57-92-1		≥97.0%	581.6	400—0.79
Chloramphenicol	CHL	Amphenicols	56-75-7		97.50%	323.1	250—0.23
Amoxicillin	AMO	B-Lactams	26787-78-0	SIGMA-ALDRICH	96–102%	365.4	500—1.95
Ampicillin	AMP		69-53-4		≥90.00%	394.4	500—7.81
Penicillin G	PEN		69-57-8		96-102%	356.4	1000—7.81
Erythromycin	ERY	Macrolides	114-07-8	ACO-FARMA	95.90%	733.9	600—1.17
Tetracycline	TC	Tetracyclines	64-75-5		99.20%	444.4	100—1.56
Tannic Acid	TA	Polyphenols	1401-55-4		99.00%	1701.2	2600—10.16
Nerol	NE	Monoterpenes	106-25-2		99.00%	154.2	2000—15.62

4.2. Microorganisms

Fourteen reference bacterial strains, all well-known human and veterinary pathogens, were chosen for this study. Selected strains included both Gram-positive (*Staphylococcus aureus* ATCC 9144, *Streptococcus agalactiae* ATCC 12386, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC 19433, and *Bacillus subtilis* ATCC 6633) and Gram-negative (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC13311, *Pseudomona aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606, *Klebsiella aerogenes* ATCC 13048, *Pasteurella aerogenes* ATCC 27883, *Klebsiella pneumoniae C6*, *Proteus mirabilis*, ATCC 35659, and *Serratia marcescens* ATCC 13880).

All microorganisms were purchased as freeze-dried Culti-LoopsTM bacteria from Thermo Scientific (Dartford, UK), rehydrated, and kept at −80 °C in cryovials (Deltalab S.L. Barcelona, Spain) until used. Thermo Scientific and ATCC product sheet instructions for each strain were followed for rehydration process and culture conditions, as well as antibacterial activity testing (www.atcc.org, accessed on 20 May 2024).

4.3. Determination of the Antimicrobial Activity: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

To study antimicrobial properties, Minimum Inhibitory Concentrations (MICs) were determined using the broth microdilution method in 96-well round-bottom microplates (Deltalab S.L. Barcelona, Spain), according to the ISO 207776-1 [104] and the Clinical and Laboratory Standards Institute's [105] (CLSI, M100-S15 2018) guidelines. Each step of the procedure was performed in a flow chamber (Model MSC Advantage 1.2) under sterile conditions. The MBC (Minimum Bactericidal Concentration) index, which indicates the minimum concentration capable not only of stopping bacterial growth, but also of eliminating bacteria, was calculated by culturing the contents of several wells of MIC plates in Petri dishes for 24 h.

ABX stock solutions were prepared in distilled water (SIEMENS Ultra Clear™), adding 5% of DMSO (CAS: 67-68-5) from Fisher Bioreagents (Madrid, Spain), with a purity of 99.7% for the stock solutions of NE and TA. A toxicity assessment of DMSO was previously tested on every bacterial strain, assessing that its highest concentration in the wells (2.5%) did not affect microbial growth [12].

Using a BioTekTM Synergy H1 hybrid multimode microplate reader (625 nm), bacterial cultures were previously adjusted to the McFarland standard (CLSI, 2018) [105] to achieve a standard initial bacterial concentration per well of roughly 2.5×10^5 CFU/mL.

Microplates were cultured for 24 h at the appropriate temperature for each bacterium in a bacteriological culture incubator (Incuterm, Trade Raypa®). According to CLSI guideline M07-A9 (2018), the MIC was defined as the lowest concentration that visibly inhibited

microbial growth. The absorbance of each well was also measured at 625 nm using a microplate reader to provide a more precise measurement of microbial growth.

MBC/MIC ratio indicates the bacteriostatic or bactericidal activity of a compound on each bacterium, i.e., whether it causes the death of microorganisms or only inhibits their growth. If this ratio is ≤4, a substance is considered to have bactericidal activity [106,107].

4.4. Determination of the Natural Product-ABX Combination Behavior

4.4.1. Checkboard Assays and Fractional Inhibitory Concentration Index

Synergies to be tested were selected based on MIC experiment results for ABXs and NPs, choosing those combinations with a suitable solubility for the NP. To measure these potential synergies between ABXs (drug A) and the NP (drug B), the checkerboard method was chosen. NP (NE or TA) were diluted vertically, from columns 1 to 7 of the 96-well plate. The matching ABXs for that synergy were then diluted vertically from rows A to G. Stock concentrations prepared for every substance were four times higher than its MIC for each bacterium tested, to ensure a reliable synergy value.

The FIC_I index, which defines the type of interaction produced between two drugs, was calculated as follows:

$$FIC_I = FIC_A + FIC_B = \frac{MIC_{A+B}}{MIC_A} + \frac{MIC_{B+A}}{MIC_B} \quad (1)$$

In this equation, drug A is a NP (NE or TA), and B is a commercial ABX. Then, FIC_A is the quotient between the MIC of drug A in the presence of drug B (MIC_{A+B}) and the MIC of drug A alone (MIC_A). FIC_B, on the other hand, is the quotient between the MIC of drug B in the presence of drug A (MIC_{B+A}) and the MIC of drug B alone (MIC_B).

Following the guidelines from the European Committee on Antimicrobial Susceptibility Testing [108], a synergy is defined as an interaction with a FIC_I value of ≤0.5. Values between 0.5 and 1 correspond to additivity, values ranging from >1 to 2 indicate no significant interactions, and values ≥2 indicate antagonistic effects [109,110].

4.4.2. Growth Kinetic Tests

To provide a more accurate evaluation of the effects of synergistic combinations (those with a FICI ≤ 0.5), growth kinetics tests were conducted. As described in Section 4.3, bacterial cultures were adjusted to the McFarland standard. Then, based on the data provided by the checkerboard test, new 96-well microplates were prepared, exposing bacteria to various inhibitory and sublethal concentrations of ABXs, NPs, and their combinations [93]. Afterward, the plates were then introduced to the absorbance reader Synergy H1 (Biotek), which incubated them for 24 h at specified temperatures and simultaneously took one absorbance reading every hour. Data were plotted as absorbance vs. time, obtaining the growth/time curves. Each absorbance reading consisted of four replicates. Kinetic curves were fitted to a logistic model (Equation (2)) for sigmoid microbial growth:

$$\text{Absorbance} = \frac{C_{max}}{1 + e^{b-rt}} \quad (2)$$

where C_{max} is the maximum population density achievable, r is the rate of population increase, and b is the fitting parameter. C_{max} and r were calculated to determine the kinetics growth of synergistic combinations.

The significance of Kinetic curve differences compared to the control was assessed using ANOVA for parametric data, conducted with SPSS software (version 28.0.1.0, 142).

4.4.3. Isobolograms

An isobologram is a graphical representation that allows the observation of ABX-natural product interactions from the results obtained in the checkerboard tests used to obtain the synergies [111,112]. In this work, only isobolograms whose FICI is ≤0.5 are plotted.

To draw an isobologram, the MIC of the NP is placed on the X-axis and the MIC of the ABX on the y-axis. The graph is plotted with the combinations obtained in the checkerboard tests that inhibit bacterial growth. It includes an 'addition line' (solid line) that helps differentiate between additive effects (where points fall above or near this line), synergistic effects (indicated by concave isoboles below the line), and antagonistic effects (shown by convex isoboles above the line). Additionally, there is a lower dotted line that marks the boundary of synergy. Points situated above or below this dotted line represent different degrees of synergistic interaction.

5. Conclusions

Although natural products have repeatedly demonstrated antimicrobial capabilities in the literature, the fact is that they require high doses that hinder clinical application, making it difficult for them to replace much more efficient commercial ABXs. However, the strategy of combining natural products with commercial ABXs may be a good solution to combat antimicrobial resistance. Our results show that both TA and NE, in combination with widely used commercial ABXs, are capable of completely inhibiting microbial growth, reducing the ABX dose by margins from 75.00% to 93.75%. Additionally, the dose of the natural product is also reduced by about 75% in the synergies. The growth kinetics of the microbes when treated with the two products separately and in synergy suggest different mechanisms of action, some of which indicate the natural product's ability to enhance the ABX's activity by making the target more accessible or even acting as Antimicrobial Resistance Modifying Agents.

The fact that both natural products are considered safe by international official agencies and that their doses are significantly reduced in synergy, thereby reducing their toxicity, makes their application as enhancers of commercial ABXs very promising for clinical, food, or veterinary use.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants13192717/s1, Table S1. Antimicrobial activity of antibiotics (MIC, µg/mL) on pathogenic bacteria tested; Table S2. Microorganisms reference and culture conditions according to American Type Culture Collection (ATCC) datasheets for each microorganism.

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5.3. Capítulo 3. Estudio ecotoxicológico del ácido tánico en indicadores no diana de suelo y agua y su impacto en comunidades fluviales y edáficas

Artículo publicado en Plants (Q1, FI: 4,1)

- Resumen:

AT es un PN de origen vegetal, conocido principalmente por su interés en la industria del curtido de cuero. Pese al interés creciente que sus distintas propiedades han generado recientemente (entre ellas, antimicrobianas o anticarcinogénicas), se ha destinado poca atención al impacto medioambiental que este PN puede tener en el medioambiente.

En este estudio, se llevaron a cabo ensayos de ecotoxicidad con varios indicadores, como bacterias marinas (*Aliivibrio fischeri*), crustáceos (*Daphnia magna*), lombrices (*Eisenia fetida*) y plantas (A*llium cepa*), además de pruebas en comunidades microbianas naturales de agua y suelo, incluyendo el perifiton.

Los resultados revelan que el AT presenta toxicidad especialmente sobre *A. fischeri* y *D magna* con CE_{50} de 30.038 y 22.000 mg/L respectivamente. Aunque los valores obtenidos suponen cierta toxicidad (European Chemicals Agency (ECHA), 2008), apenas tiene efecto sobre el perifiton (CE_{50} > 100 mg/L) y requiere dosis elevadas (CE_{50} > 200 mg/L) para detectarse efectos en comunidades microbianas del necton fluvial. Respecto a los bioindicadores de suelo, no tiene efectos sobre *E. fetida* (> 2000 mg/kg) y es poco tóxico para el crecimiento de los bulbos de *A. cepa* (CL_{50} = 0,2 mg/L). Sin embargo, presenta una toxicidad elevada (CL_{50} = 0,2 mg/L) sobre microorganismos del suelo, afectando el crecimiento de estas comunidades, así como al metabolismo de aminas y amidas y polímeros. Este trabajo subraya la relevancia de examinar compuestos naturales como el AT desde una perspectiva ecotoxicológica completa, abarcando distintos niveles tróficos y ambientales, para valorar posibles riesgos ambientales derivados de su uso y vertido.

- Relación con la Tesis Doctoral:

Una vez comprobado que existen combinaciones sinérgicas de AT con varios ATBs, y conocido el impacto ecológico de los ATB comerciales seleccionados, es necesario estudiar el impacto que este PN puede producir en el medioambiente. Estos resultados permiten cumplir el tercer objetivo de la Tesis, evaluando de forma sistemática la toxicidad ambiental del AT y aportan información clave para los siguientes objetivos, ya que establecer los perfiles de ecotoxicidad del AT resulta esencial antes de considerar su aplicación en combinaciones sinérgicas con ATBs. Además, este capítulo establece el marco para comparar la toxicidad de este producto natural respecto a la de los ATBs utilizados en las sinergias que fueron evaluados en el capítulo primero.

- Importancia de los resultados:

Este es el primer trabajo que evalúa de forma sistemática la toxicidad de AT frente a indicadores de diferentes niveles tróficos (productores y consumidores), diferentes ecosistemas (fluvial y edáfico) e indicadores no diana estándar individuales, así como comunidades microbianas complejas, proporcionando información valiosa acerca de sus efectos en el medioambiente. Esta visión, que contempla diferentes elementos de la cadena trófica, tanto en ecosistemas acuáticos como terrestres, muestra un enfoque innovador e integrador que permite tener una visión precisa del posible impacto ambiental de este producto. Identificar que ciertas comunidades microbianas de suelo son especialmente sensibles al AT implica un posible riesgo para las funciones ecológicas del suelo, como la mineralización y el ciclado de nutrientes, así como para la fertilidad y calidad del propio suelo. Por otra parte, la demostrada resistencia de las comunidades microbianas acuáticas, atribuible a mecanismos de biodegradación y resiliencia funcional, aporta información crucial para el manejo de riesgos ambientales asociados al aumento previsto en el uso industrial y agrícola de taninos. Los resultados destacan no sólo la susceptibilidad diferencial entre ambientes (mayor sensibilidad en el suelo frente al agua), sino también la necesidad de incluir comunidades y organismos no diana en las evaluaciones de riesgo. Además, subraya la importancia de una regulación adecuada para productos naturales, tradicionalmente percibidos como inocuos, pero que pueden afectar los ecosistemas, sobre todo en escenarios de aumento de usos industriales y comerciales de estos compuestos.

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Ecotoxicological Study of Tannic Acid on Soil and Water Non-Target Indicators and Its Impact on Fluvial and Edaphic Communities

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Abstract: Tannic acid (TA) is a key tannin extensively used in the leather industry, contributing to around 90% of global leather production. This practice leads to the generation of highly polluting effluents, causing environmental harm to aquatic ecosystems. Additionally, tannins like TA degrade slowly under natural conditions. Despite efforts to reduce pollutant effluents, limited attention has been devoted to the direct environmental impact of tannins. Moreover, TA has garnered increased attention mainly due to its applications as an antibacterial agent and anti-carcinogenic compound. However, our understanding of its ecotoxicological effects remains incomplete. This study addresses this knowledge gap by assessing the ecotoxicity of TA on non-target indicator organisms in both water (*Vibrio fischeri*, *Daphnia magna*) and soil environments (*Eisenia foetida*, *Allium cepa*), as well as natural fluvial and edaphic communities, including periphyton. Our findings offer valuable insights into TA's ecotoxicological impact across various trophic levels, underscoring the need for more comprehensive investigations in complex ecosystems. Our results demonstrate that TA exhibits ecotoxicity towards specific non-target aquatic organisms, particularly *V. fischeri* and *D. magna*, and phytotoxicity on *A. cepa*. The severity of these effects varies, with *V. fischeri* being the most sensitive, followed by *D. magna* and *A. cepa*. However, the soil-dwelling invertebrate *E. foetida* shows resistance to the tested TA concentrations. Furthermore, our research reveals that substantial TA concentrations are required to reduce the growth of river microbial communities. Metabolic changes, particularly in amino acid and amine metabolism, are observed at lower concentrations. Notably, the photosynthetic yield of river periphyton remains unaffected, even at higher concentrations. In contrast, soil microbial communities exhibit greater sensitivity, with significant alterations in population growth and metabolic profiles at a very low concentration of 0.2 mg/L for all metabolites. In summary, this study offers valuable insights into the ecotoxicological effects of TA on both aquatic and terrestrial environments. It underscores the importance of considering a variety of non-target organisms and complex communities when assessing the environmental implications of this compound.

Keywords: tannic acid; soil ecotoxicity; water ecotoxicity; fluvial communities; edaphic communities

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1. Introduction

Tannins, among which tannic acid (TA) is found, are mostly used in the leather industry. Around 90% of global leather undergoes a process where tannins are used in combination with chromium salts [1–3], and according to Kanth et al. [4], between 350,000 and 400,000 tons of vegetable tannins are spent on leather processing every year [4]. This industry is especially relevant in developing countries, like Brazil [5] or Morocco [6].

Effluents from this process are highly pollutant because of these compounds [7]. There are many studies reporting the environmental damage provoked by these effluents on fish, invertebrates, bacteria, or algae [6,8–14].

Apart from effluents, this industry discards 40–50% of tanned leather, producing about 600,000 tons of solid waste per year worldwide [15], which constitutes one more source of surface and groundwater [16–19] and soil pollution [15]. In fact, in a simulation study performed by Qiao et al. [15], where the migration of leather tannins and chromium salts in soils was studied, they found two interesting facts: (i) tannins slowly degraded in natural conditions and (ii) the total chromium leaching efficiency was promoted by tannins in the leachates. This means that this kind of lixiviate and the previously cited effluents deserve more attention from an ecotoxicological point of view [15,20].

To generate less pollutant effluents, many researchers focus their investigations on the replacement of chrome–tannin complexes with other tannin–chrome-free structures [21,22]. However, none of these studies have inquired into the effect of tannins, themselves, in the environment. In addition to the presence of tannins in leather-processing by-products, they are also found in waste from the cork [23] and food industries [24].

When studying tannins from different points of view, TA is their representative compound [24–29]. TA is a water-soluble gallotannin that belongs to the hydrolysable class of tannins [30]. It is a secondary metabolite of many plants, which can be found in natural surface waters [31,32].

TA has received increasing interest in recent years. Searching in the Web of Science for the term 'tannic acid' in the 'topic' field, the number of results rose from 4628 in the first decade of the 2000s (2000–2009) to 14,844 in the second decade (2010–2020), with most of them concentrated in the following areas: agriculture, plant sciences, chemistry and biochemistry, pharmacology, food science and technology, environmental sciences, and nutrition.

This growing interest is focused, for instance, on its antibacterial activity alone or incorporated in nanoparticles, biofilms, or other biostructures [33–39], its anti-carcinogenic properties [40–42], its heavy metal removal capacity in wastewater [43], and its role in antibiotic soil sorption [29] or degradation in aquatic environments [27].

Whatever the application, TA will end up in the environment in one way or another. The fact that it is a natural compound does not mean there is an absence of ecotoxicity, as previously demonstrated with other natural products [44]. There are some studies that shed some light on the toxicity of TA in some aquatic organisms. Zhao et al. [45], for example, found significant toxicity in *Cytophaga columnaris* fish species. Goel et al. [46] studied the hepatotoxic effects of TA in two teleost fish, Varanka et al. [47] assessed the toxic effects in *Cyprinus carpio* fish, and Xie et al. [48] examined the same effects in zebrafish and Saha et al. [49] in *Oreochromis mossambicus* fish, as well as chronic effects after 90 days' exposure. Although limited, some literature is available about the toxicity of TA in soil biomarkers, for example, on legumes [50].

However, there is still no complete ecotoxicological study of it, a need that Pautou already pointed out in 2000 [28]. So, these findings suggest that TA may also affect ecosystems at higher levels, highlighting the need for further study in complex communities.

Therefore, the objective of this study was to evaluate the ecotoxicity of TA in the environment using non-target indicator organisms in water and soil, as well as in fluvial and edaphic natural communities, in order to have a realistic and complete view through the different levels of the trophic chains of these environments.

2. Results

2.1. Genetic Analysis of Microbial Populations

2.1.1. River Communities

Phylogenetic analysis of the DNA sequences of the 16 S rRNA genes led to the identification of the taxonomic classification of the microorganisms in our river samples, which can be seen in the graphs in Figure 1.

The percentage of total reads classified in the different taxonomic levels, including kingdom, phylum, class, and order, were in the range of 99.59% to 93.06%. However, the sequencing was less successful for family, genus, and species, with percentages of 62.87, 56.35, and 20.38%, respectively. In Figure 1, the top taxa abundance of different taxonomic levels can be seen.

The dominant phyla were Cyanobacteria, Proteobacteria, and, to a lesser extent, Bacteroidetes, which is a highly representative pattern of freshwater environments [51].

Among Cyanobacteria, the predominant class was Oscillatoriophycideae (over one-third of cyanobacteria), which belong to the order Chroococcales, a dominant cyanobacterial group in freshwater ecosystems [52], characterized by coccoid cells often surrounded by a mucilaginous envelope [53].

The Beta- and Alphaproteobacteria classes are usually the most abundant Proteobacterias in bacterial communities in freshwater followed by Gammaproteobacteria [54], as we found in our samples. Within the Betaproteobacteria, almost all of them belonged to the order Burkholderiales, which is prevalent in various rivers [55,56], and to the family Commamonadaceae, the most abundant family found in our samples. The order Rhodobacterales (practically all of them being Rhodobacteriaceae) dominated among Alphaproteobacteria. This order was found to be predominant in other rivers in Spain [57].

The classes Flavobacteria and Sphingobacteria were the most abundant within the Bacteroidetes phylum, similarly to other studies in river waters [58–60].

2.1.2. Soil Communities

Kingdom, phylum, class, order, and family were optimally sequenced (above 81% of taxa), and only genus and species were identified below this ratio (76.85 and 36.10%, respectively).

As can be seen in Figure 2, the predominant phylum identified was Actinobacteria (approximately half of bacterial reads), followed by Proteobacteria and Firmicutes, which is a type and proportion of microorganisms characteristic of many edaphic ecosystems [51,61,62].

Actinobacteria are an important phylum of common Gram-positive bacteria in soil and plant matter [63]. More than half of the Actinobacterias identified in our samples belong to the order Actinomycetales, with two families identified: Nocardioidaceae and Pseudonocardiaceae. Actinomycetales are a ubiquitous order in soil environments [64–66]. The class Thermoleophilia (all of them belonging to the order Solirubrobacteales) had the second highest abundance in the phylum Actinobacteria and also have been identified in different types of soils [67].

The main class of Proteobacteria identified was Alphaproteobacteria, followed by Deltaproteobacteria and Gammaproteobacteria. The predominant orders of these families are Rhizobiales, Myxococcales, and Xanthomonadales, respectively. However, the three classes show a great diversity at the level of family, genus, and species, resulting in fewer clearly dominant groups.

The phylum Firmicutes, also frequent in different types of soils [68–73] and in soils in Spain similar to that studied here [74], has the class Clostridia (with two main orders, Clostridiales) as the most abundant in our samples.

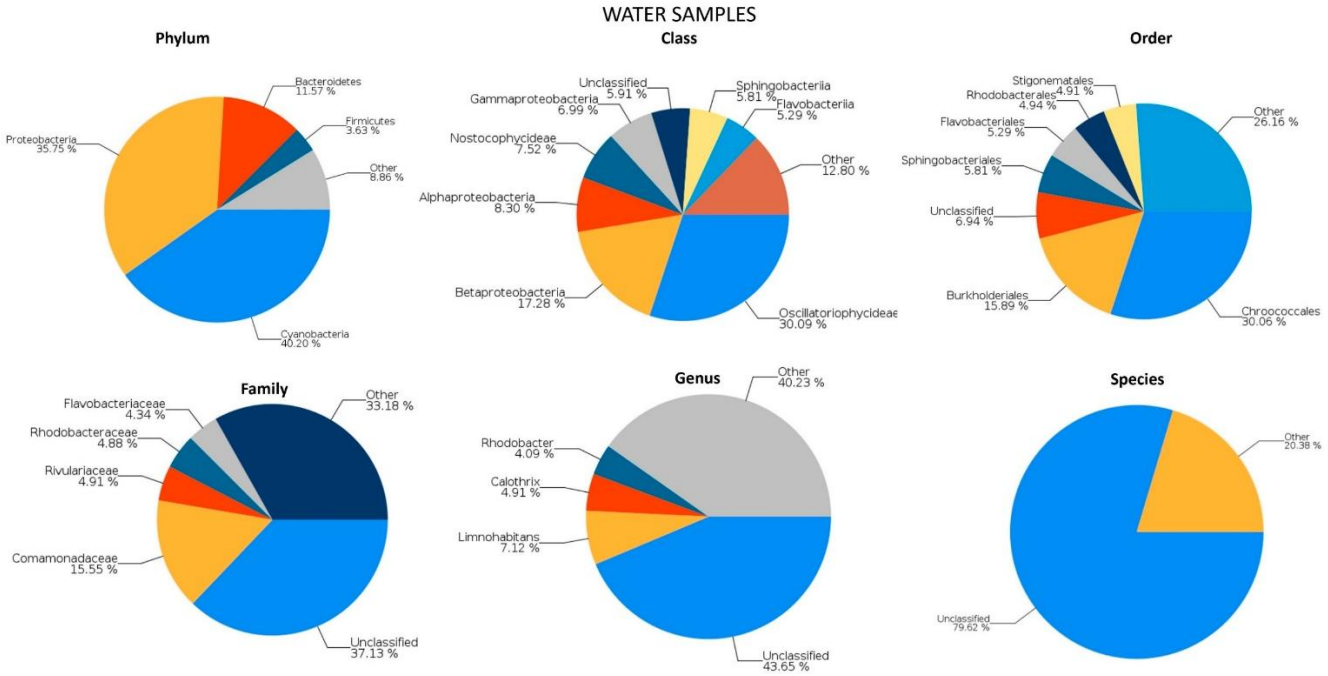

Figure 1. Phylum, class, order, family, genus, and species classification results for water samples.

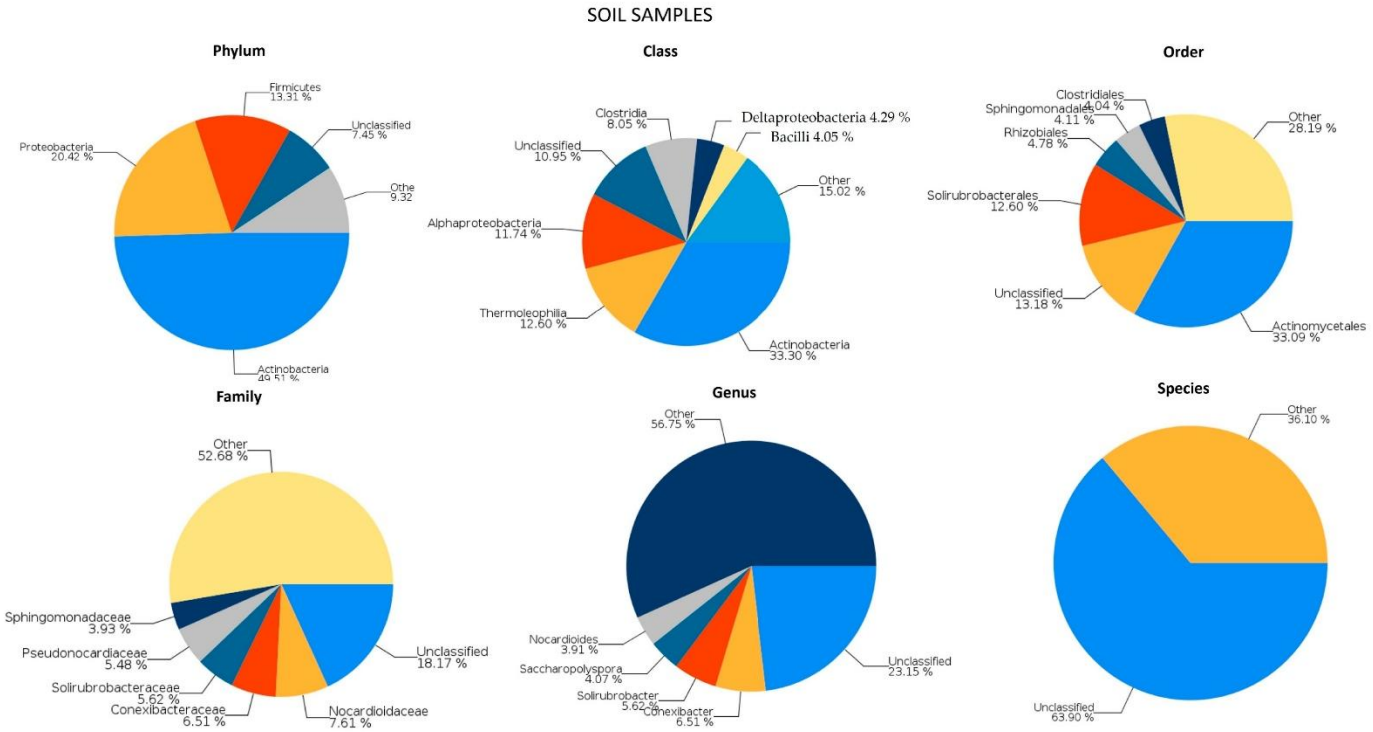

Figure 2. Phylum, class, order, family, genus, and species classification results for soil samples.

2.2. *Average Well Color Development (AWCD) of Microbial Populations*

2.2.1. Water Samples

Figure 3 shows the impact of increasing concentrations of TA on the growth of bacterial communities from a river water sample for a 144 h incubation period in Biolog Ecoplates® (Hayward, CA, USA). Only the highest dose (200 mg/L) provoked a significative decrease in AWCD ($p < 0.05$) with respect to the control (black line).

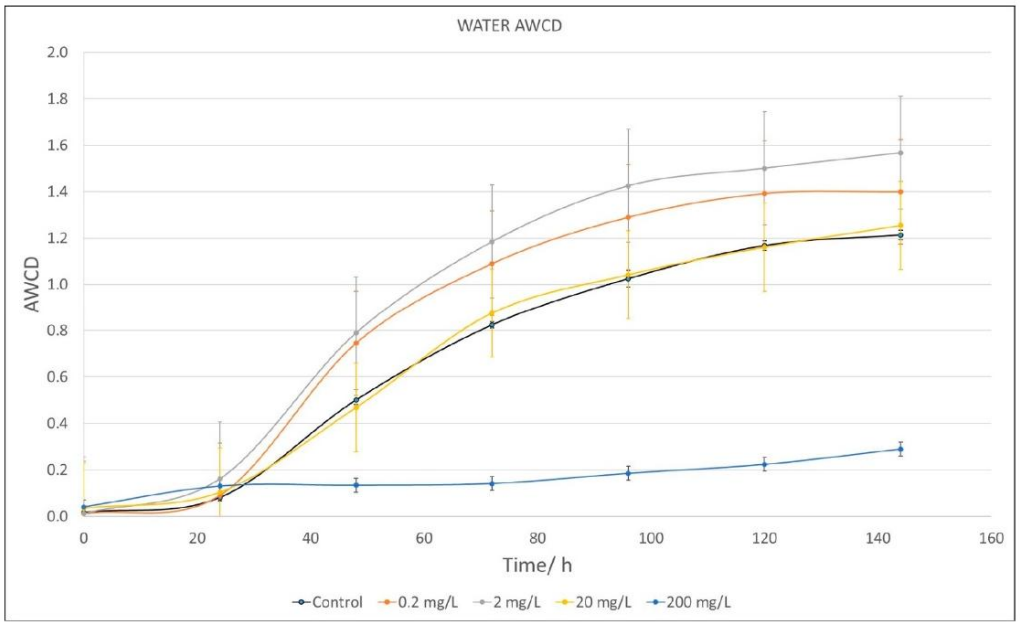

Figure 3. Average well color development (AWCD) of metabolized substrate in Biolog EcoPlates® by river water microorganisms after 144 h exposure to different concentrations of tannic acid (TA) (shown at the bottom of the figure). Values are compared to a control value as reference (river communities not treated with TA). Each point is an average of three replicates and includes the error bars representing their standard deviation.

2.2.2. Soil Samples

The behavior of the AWCD with time (144 h) for the microbial communities obtained from soil samples exposed to different concentrations of TA is illustrated in Figure 4. As can be seen, the effect of TA on the soil communities was stronger than on the river water ones. In this case, every concentration showed lower microbial growth than the control curve (black). This decrease was easily detectable as early as 0.2 mg/L, even though only 200 mg/L of TA showed significant differences with respect to the control ($p < 0.05$).

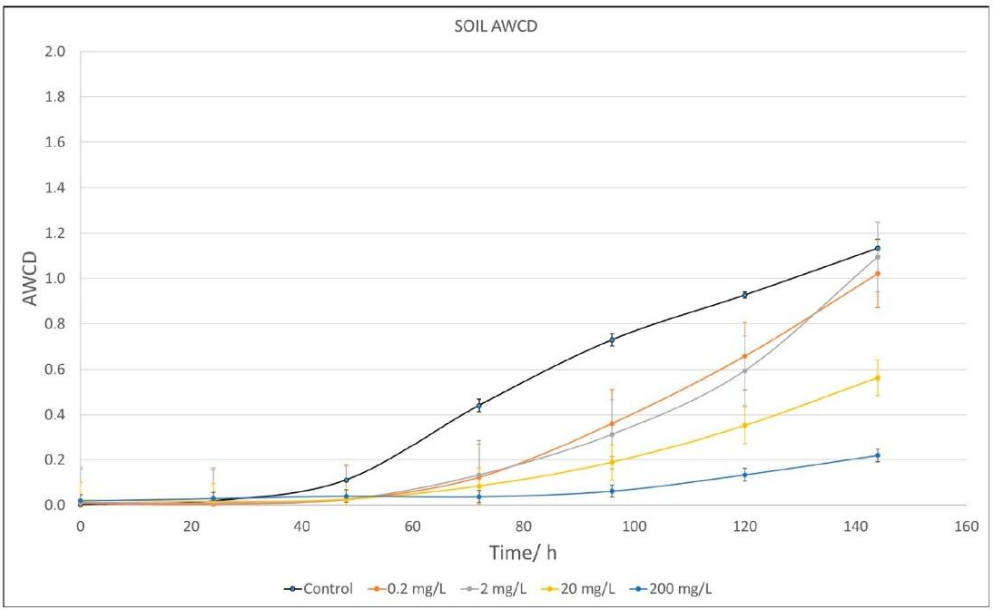

Figure 4. Average well color development (AWCD) of metabolized substrate in Biolog EcoPlates® by soil microorganisms after 144 h exposure to different concentrations of TA (shown at the bottom of the figure). Values are compared to a control value as reference (soil communities not treated with TA). Each point is an average of three replicates and includes the error bars representing their standard deviation.

2.3. Community-Level Physiological Profiling (CLPP)

2.3.1. Water Samples

Figure 5 gives information about changes in the metabolic profile of river water microorganisms at different TA concentrations when they are grouped according to the type of the carbon source with which they were supplemented in BIOLOGs ecoplates®, that is, polymers, carbohydrates, carboxylic and acetic acids, amino acids, and amines, for 144 h. The first bar graph represents the control (river microorganisms not exposed to TA).

For the lowest concentrations (0.2 and 2 mg/L), no significant changes were observed for any group, although at 20 mg/L, the microbial capacity to metabolize amines and amino acids showed a small decrease, which, however, was only significant ($p < 0.05$) for polymers and carboxylic and acetic acids at the highest concentration (200 mg/L).

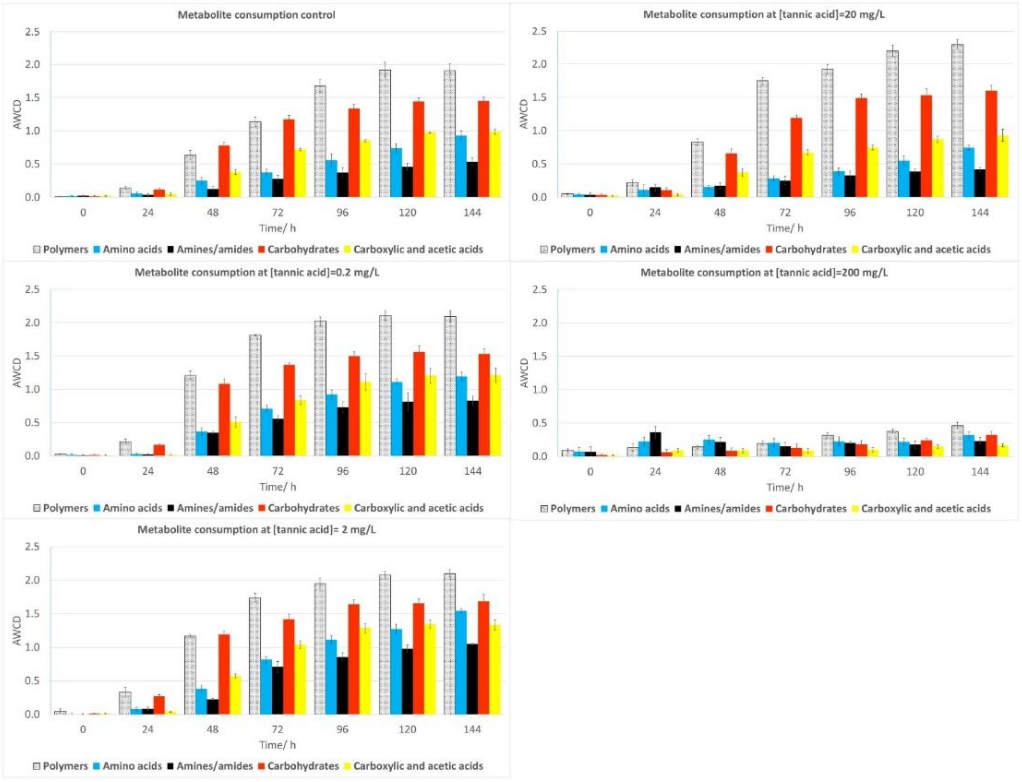

Figure 5. Metabolic profile of water samples after 144 h exposure to TA. Bars represent the AWCD growth for each group of metabolites of water bacteria. Each value is an average of three replicates and includes the error bars representing their standard deviation.

2.3.2. Soil Samples

Information about changes in the physiological profiling of the soil microorganisms with time at the TA doses tested is provided in Figure 6 for the five classes of metabolites used in Biolog Ecoplates®. As can be seen, soil microorganisms were more sensitive to TA exposure than river microorganisms.

In general terms, the ability of the soil microorganisms to metabolize substrates was reduced with higher TA concentrations for the cited metabolite classes, reaching almost no metabolic consumption for the highest concentration (200 mg/L). The most significant changes ($p < 0.05$) were detected for polymers, carboxylic and acetic acids, and amines at 200 mg/L.

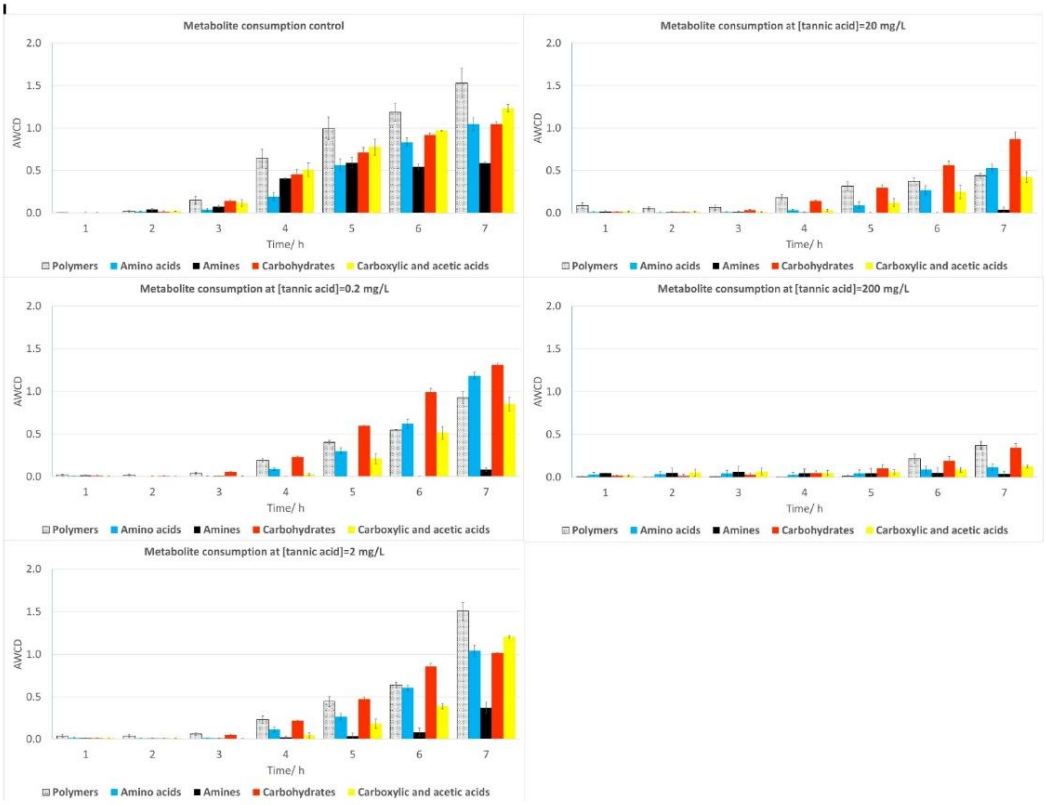

Figure 6. Metabolic profile of soil samples after 144 h exposure to TA. Bars represent the AWCD growth for each group of metabolites of soil bacteria. Each value is an average of three replicates and includes the error bars representing their standard deviation.

2.4. Periphyton

No effect of TA was observed on periphyton species at the concentrations tested (Supplementary Information Figure S1), with $LC_{50} > 1000$ mg/L and $LC_{10} = 7.02$ (CI:0.36–46.44) mg/L.

2.5. Daphnia magna

Daphnia magna serves as a robust indicator of water quality due to its exposure to toxins via two pathways: surface contact and ingestion as a filter feeder, making it highly sensitive to environmental changes. Additionally, its swift reproductive cycle, widespread presence in diverse aquatic environments, and significant role in the food chain—being a staple for numerous aquatic organisms—underscore its importance. Its reaction to contaminants not only informs us about the quality of the water but also about the potential impact of the contaminants on the broader ecosystem [75].

Figure 7 shows the dose–response curve for the D. magna immobilization test after 24 h exposure to TA. The results were analyzed with a chi-squared test, indicating high significance ($p < 0.0001$). TA influenced D. magna's mobility, with EC_{50} and EC_{10} values of 32.038 mg/L (CI: 18.101–47.441 mg/L) and 5.606 mg/L (CI: 0.699–11.784 mg/L), respectively.

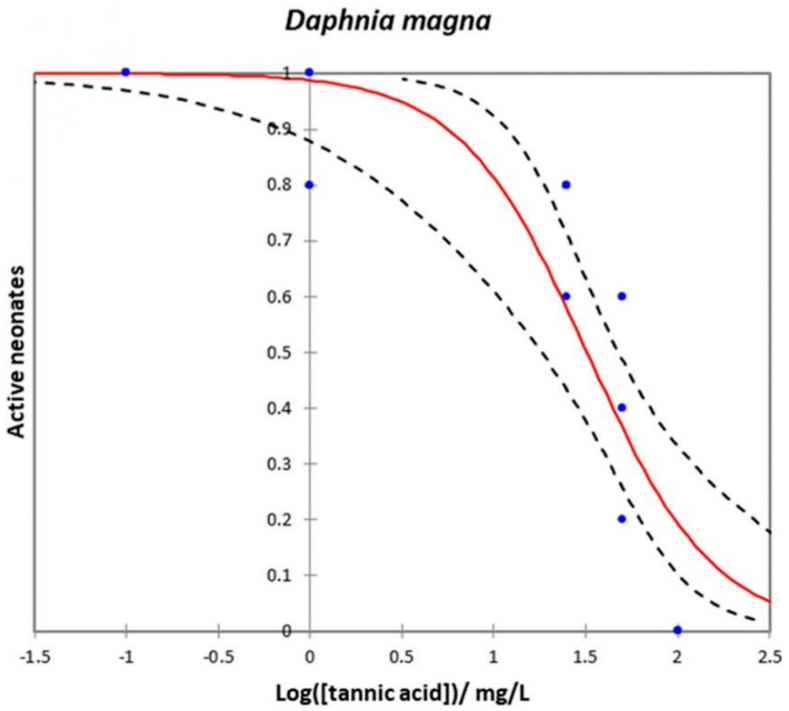

Figure 7. Dose–response curve for Daphnia magna test after 24 h–exposure to TA. Curves are the average of 5 replicates. Red line is the model, blue dots are experimental data, and dashed lines are the inferior and superior confidence limits (95%).

2.6. Vibrio fischeri

The Vibrio fischeri assay is a straightforward and economical method commonly used for screening and evaluating a diverse array of potentially harmful substances in ecotoxicology. It is known for its high sensitivity, ease of use, reliability, and consistency, making it a convenient addition to a test battery employed in assessing the ecotoxicity of various compounds. However, this assay, as is the case for D. magna, does not accurately reflect the chronic or long-term effects of contaminants [76].

The dose–response curve for the bioluminescence assay carried out on *V. fischeri* is displayed in Figure 8. A significance of $p < 0.0001$ was obtained when the dose–response values were analyzed. The EC_{50} and EC_{10} values were 22.000 mg/L (CI: 17.075–28.444 mg/L) and 0.121 mg/L (CI 0.028–0.198 mg/L), respectively.

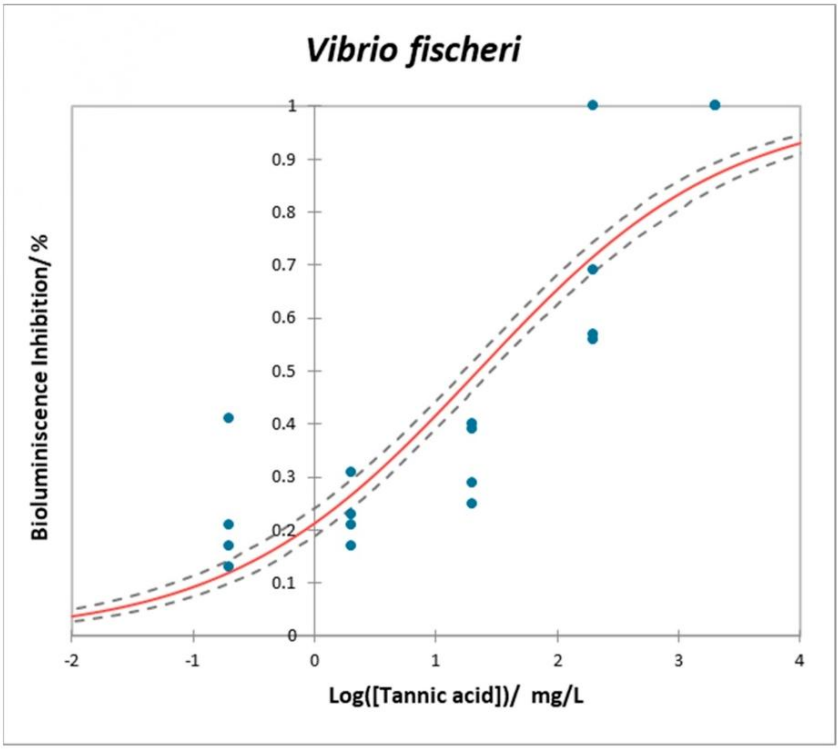

Figure 8. Dose–response curve for the bioluminescence assay for *Vibrio fischeri* after 24 h–exposure to TA. Curves are the average of 4 replicates. Red line is the model, blue dots are experimental data, and dashed lines are the inferior and superior confidence limits (95%).

2.7. Eisenia foetida

Eisenia foetida activity significantly impacts soil health, nutrient cycling, and overall ecosystem productivity in terrestrial environments. Their roles as decomposers, soil engineers, and contributors to nutrient cycling make them crucial in maintaining healthy and productive ecosystems [77]. *E. foetida* is commonly employed in terrestrial ecotoxicology [78,79] due to its high sensitivity as an indicator of soil quality and the possibility of conducting long-term assays. This species is exposed to contaminants in the soil through two pathways: firstly, through the skin via a thin epidermal cuticle and a glandular orifice that connects the worms with the surrounding environment, and secondly, through ingestion while burrowing and stirring the soil.

E. foetida survival was not influenced by any concentration of TA within the range studied (Supplementary Information Figure S2) with $LC_{50} > 2000$ mg/L.

2.8. Allium cepa

Finally, the *Allium cepa* root growth assay has also been frequently used [80–82] as a phytotoxicity test due to its sensitivity, swift response, ease of handling, and representation of effects on higher plants.

Figure 9 offers the dose–response curve for A. cepa after 72 h of incubation with TA. A. cepa root elongation was modified when exposed to the TA concentrations. The EC_{50} value was 133.389 mg/L (CI: 90.364–210.467 mg/L) and EC_{10} value was 3.415 mg/L (CI: 1.236–6.693 mg/L). The results' significance was evaluated using a chi-squared test, which showed high significance ($p < 0.0001$).

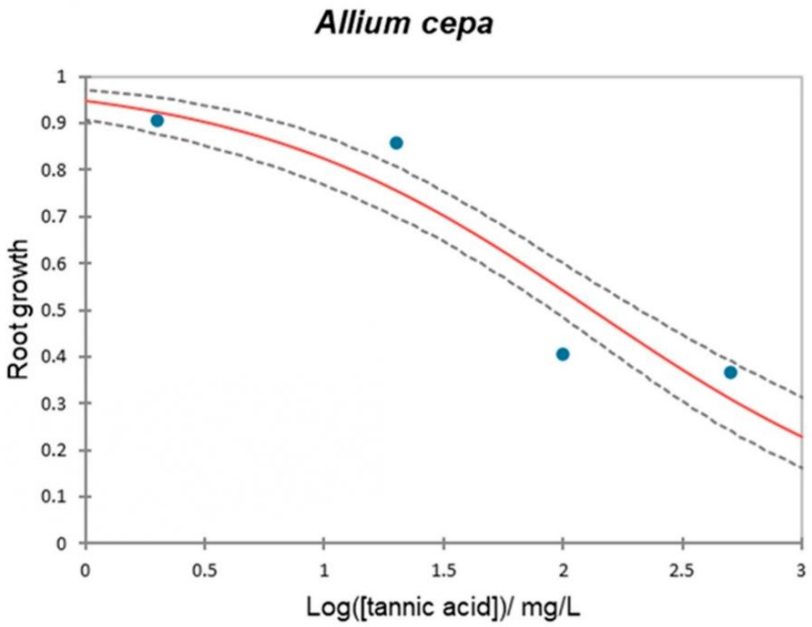

Figure 9. Dose–response curve for *Allium cepa* test after 72 h exposure to TA. Curves are the average of 12 replicates. Red line is the model, blue dots are experimental data, and dashed lines are the inferior and superior confidence limits (95%).

3. Discussion

Our results show that TA exhibits ecotoxicity towards individual non-target water organisms such as *V. fischeri* and *D. magna*, as well as phytotoxicity towards *A. cepa*. From most to least ecotoxic (according to their LC_{50} values), the order is as follows: *V. fischeri* > *D. magna* > *A. cepa*. However, it did not display toxicity on the soil invertebrate *E. foetida* at the concentrations tested.

Our study also reveals that very high concentrations of TA are needed to produce a reduction in the growth of the river microbial population (of the order of 200 mg/L), although at 20 mg/L, some changes can be seen in the metabolic profile compared to the control, especially in the ability to metabolize amino acids and amines. There were also no changes in the photosynthetic yield of the river periphyton at the highest concentrations tested (100 mg/L). However, soil microbial communities appeared to be more sensitive to TA, modifying population growth with an intense change in the metabolic profile at 0.2 mg/L for all metabolites.

3.1. The Effects of TA on Water Bioindicators and Water Ecosystems

D. magna is a planktonic crustacean that is used as a highly reliable indicator for testing water quality contamination. It is a filter-feeding organism that feeds by straining suspended particles from the water and, thus, accumulating potential contaminants. Although it is unlikely that TA can cross membranes via simple diffusion due to its physicochemical properties, such as its high molecular weight and high hydrophilicity, and the fact that it contains multiple hydroxyl groups, which can ionize and carry a negative charge at physiological pH, *D. magna* may be exposed to this compound through the digestive tract, which may explain its toxicity in this aquatic invertebrate.

To our knowledge, there is only one study that has reported the EC_{50} values of *D. magna* exposed to AT [24], which are very similar to ours (EC_{50} = 50 mg/L). Studies of plant extracts, such as *Pelargonium graveolens*, in which TA is the main component, have shown an LC_{50} (48 h) of 203 mg/L [83]. Other studies have also been conducted with wastewater from the tanning and cork industries, like the one by DeNicola et al. [6], who reported

100% immobilization of *D. magna* individuals at 12.5% of traditional tannery effluents containing up to 0.4 g/L of vegetable tannins. On the other hand, Libralato et al. [84] reported an LC_{50} value, for tannery wastewater, of 26 mg/L for *D. magna* too [23] and provided EC_{50} values ranging from 2.3 to 24.2% dilutions when studying cork-boiling wastewaters containing tannins.

V. fischeri is a Gram-negative bacillus widely distributed in marine ecosystems. Its natural bioluminescence is metabolically linked to the cellular respiration AS [76,85] and has been adopted internationally [86] as an indicator of toxicity to marine ecosystems [23].

To the best of our knowledge, this is the first research that provides ecotoxicity data of TA on *V. fischeri.* However, Jochimsen et al. [87] tested tannery wastewaters containing tannins on *V. fischeri*, but they did not provide any EC_{50}.

Although is unlikely that TA is able to cross membranes via simple diffusion (as stated before), it seems to be able to disrupt the bacteria envelope and produce cell-oxidative damage [88], which could explain the toxicity effect observed for *V. fischeri*. Samoilova [89] also suggests that the strong oxidative properties of TA override stress responsiveness and alter membrane potential. In this regard, some other studies can be found. For example, TA has significant toxicity in *Staphylococcus aureus* [30], *Escherichia coli* [90,91], *Salmonella enterica* [92], or *Lysteria monocytogenes* [91] strains and *Saccharomyces cerevisiae* yeast [93]. Also, detrimental effects for the normal functioning of *P. aeruginosa* [33] can also be found. However, to the best of our knowledge, there is no available literature regarding the effects of TA on any complex aquatic microbial community.

Our results show that the impact of TA on the river microbial community is significantly lower than in the case of the isolated bacteria *V. fischeri*. This phenomenon has also been observed in other bacteria, such as cyanobacteria [94,95] and bacteria of the *Flavobacteria* genus [96], both of which were present in our samples. All of them exhibited sensitivity to TA when exposed individually, but when they were associated within a community, they appeared to modulate the impact of this compound.

The river community, as revealed in the genetic study (Supplementary Information Table S2), exhibits a wide diversity of taxa that may display varying sensitivities to this product [97]. So, some bacteria can be directly affected by the disruption of their envelope or intracellular processes. However, those that are more resistant can survive, proliferate, and occupy the niches left by the former. This may explain the limited effect of TA on bacterial growth or even the slight increase observed in comparison to the control (Figure 3), as microbial biomass can be maintained, even though there are changes in biodiversity [98].

On the other hand, microorganisms capable of biodegrading TA have been detected, an effect that can be enhanced when the product is exposed to a diverse community of microorganisms rather than a single species [99–101].

Similarly, periphyton, a complex community of aquatic organisms, comprising species of algae, plants, bacteria, fungi, protozoa, and invertebrates, appears to buffer the impact of TA. Periphyton is a basic link in aquatic ecosystems and serves as a valuable indicator of toxicity [102] and water quality [103]. It has been seen that TA can reduce the growth rate and photosynthetic yield of the cyanobacteria *Microcystis aeruginosa* and the unicellular green alga *Desmodesmus armatus* at concentrations ranging from 1 to 20 mg/L, with cyanobacteria being the most sensitive. In fact, the use of these compounds to prevent cyanobacterial and algal growth in freshwater has been proposed [104,105]. However, until now, the effect of TA on river periphyton systems, which integrate the responses of numerous species and their interaction, had not been directly studied. The literature reported that macrophytes heavily colonized by periphyton may have developed a xenobiotic strategy [106] for the release of TA and other polyphenols as a tool in this competition scenario. This strategy may not be enough in highly eutrophicated waters since colonized macrophytes end up reducing their growth until they eventually completely decline [107] and are not able to eliminate the periphyton that covers them, even though it can easily be reached by plants' freshly released xenobiotics.

According to our results, the architecture of the periphyton itself and the association of several organisms to create these mats with a wide diversity of taxa exhibiting different sensitivities (Supplementary Information Table S2) might provide stronger protection against potentially toxic products, depending on the multispecies competition that is established [108].

The density and extracellular polymeric substance (EPS) content can also condition the diffusion properties of periphyton. The EPS improves cell attachment [109] and is a crucial structural property influencing architecture and stability [110], but it also acts as a mechanism of protection for the biofilm microorganisms against toxicants [108–111]. The protective effect of mucilage has been shown in several studies with chlorine [112] or zinc [113], which causes less damage in biofilms with higher EPS. Recently, it was found that the amine, carbonyl, carboxyl, and hydroxyl groups in EPS were the main functional groups contributing to the interaction of TA with EPS. The existence of EPS reduced the toxicity of TA to algal cells [114].

On the other hand, interactions between periphyton species need to be taken into account when investigating the response to toxic compounds. These interactions can be very complex depending on the taxon composition and the physicochemical conditions of the environment, and they are not well understood in periphyton. It has been reported that interactions with green algae can reverse the inhibitory effects of polyphenols on cyanobacteria, such as *Microcystis aeruginosa* [115]. A similar scenario could be considered in our case, following exposure to TA, among the predominant cyanobacteria in our samples, which are more sensitive to TA [104], and the unicellular algae also present.

Finally, the various taxonomic groups that make up the periphyton exhibit wide metabolic diversity and could potentially play a role in biodegradation. Recent studies have shown that microalgae and cyanobacteria, when associated with bacteria, seem to contribute to the biodegradation of TA [116].

3.2. The Effects of TA on Soil Bioindicators and Soil Ecosystems

To the best of our knowledge, this is the first research about TA ecotoxicity in *E. foetida*. This earthworm is defined by the OECD as the main soil model animal and test organism [117]. These earthworms are a vital part of soil ecosystems, mainly due to their ability to degrade organic matter, ensuring their fertility and quality [118]. The fact that they have a very permeable cuticle and a glandular orifice, in addition to a digestive route when they feed in the soil, turns them into a good indicator of soil quality because they are constantly exposed to possible contaminants. However, they seem to be resistant to the effects of TA at the concentrations tested, so it is possible that the effects are not serious enough to cause death.

Nevertheless, these earthworms could experience some non-observable sublethal effects, such as alterations in their gut microbiota, an effect described in exposure to certain antibiotics, such as oxytetracycline, to which they also appear to be resistant [119]. In fact, TA seems to induce some form of irritation in earthworms, as evidenced by the registration of a product containing this compound to inhibit these terrestrial organisms in 1996. This product was applied by spraying or injecting into the soil to achieve the mentioned effect.

The *Allium cepa* root elongation test has been widely accepted for cytotoxic assessment and genotoxic influence caused by soil, air, and water contamination [120,121]. It has been validated for chemical toxic screening by the International Programme on Chemical Safety (IPCS, WHO) [122] and the United Nations Environment Programme (UNEP) [123]. Our results show, for the first time, that TA presents phytotoxicity against this terrestrial plant, affecting its seed germination. This effect was already registered but in few terrestrial plants, such as legumes of the species *Vigna unguiculata*. Jadhav et al. [50] found inhibitory effects on the seed germination and root and stem growth of this plant at concentrations of TA of 100 mg/L. Seed inhibition by TA was also observed in

Alternanthera tenella [124]. Further studies are required to clarify the specific mechanisms of action of TA in plant cells.

Finally, the effect of TA on soil microorganisms is stronger than that in river communities, according to some authors who have pointed out that microorganisms present in sediments are much more vulnerable to the stress of potentially toxic elements than aquatic organisms [125,126].

Some evidence has been reported in the literature indicating that pine needle tannin extracts, as well as condensed and hydrolysable tannins, exhibit inhibitory activity or induce alterations in nitrogen fixation in soil microorganisms such as Rhizobiales, which are also present in our samples [127,128]. However, this is the first comprehensive study of genetically identified soil communities in this context.

In addition to damaging bacterial cover, other mechanisms of action of TA may explain its antimicrobial effects, especially in communities with high biodiversity, as studied in this research (Figure 2). An important characteristic of tannins that provides a possible explanation for their mode of action on microorganisms is their astringent capacity, which allows them to precipitate proteins by binding to them. This binding could lead to the inhibition of extracellular microbial enzymes [129,130]. These authors suggest that this astringent capacity tends to increase with molecular weight, as is the case with TA. For instance, Mandal et al. [131] demonstrated the inhibition of important resistance-related enzymes, such as beta-lactamases and carbapenemases. It has also been suggested that TA may affect microorganisms by limiting the availability of substrates required for microbial growth, such as via metal deprivation, as tannins can form organo-metallic coordination compounds with some of them. Furthermore, according to the literature, this chelating action increases with the number of oxygen-diphenol groups, which are abundant in TA [132,133]. The observed changes in the metabolic profile may also result from a direct impact on microbial metabolism through the inhibition of oxidative phosphorylation. Other suggested impacts of TA on microorganisms include the inhibition of quorum sensing in bacteria [33] and efflux pumps, as observed in the case of *S. aureus* [134].

All this could help explain why this edaphic community seems to be more sensitive than the river community. The literature also reports studies suggesting that soil microorganisms may exhibit somewhat greater sensitivity to potentially toxic compounds compared to aquatic microorganisms [125,126]. This observed effect aligns with the findings of our previous studies involving microbial communities exposed to various compounds, such as plant-based products or extracts [44,135]. However, it is important to consider that several microorganisms have evolved mechanisms to tolerate these high concentrations of tannins [97]. There is also evidence that certain soil bacteria are capable of degrading TA [136,137]. These mechanisms may mitigate its impact on these communities, including the dynamics of replacing sensitive species with more resistant ones.

This is the first study that assesses the ecotoxicity of TA in both aquatic and soil environments using a range of aquatic and terrestrial bioindicators, as well as genetically identified communities. This comprehensive approach provides a more holistic understanding of the potential effects of TA on different ecosystems, which is of utmost interest given the increasing commercialization of this product. Furthermore, the study reveals different sensitivities of various organisms towards TA, presenting valuable information on variable levels of susceptibility among different species.

In this initial study, a wide range of TA concentrations was sought to better evaluate its ecotoxicity. However, it may be valuable to explore, in further research, the effects at lower and more environmentally relevant concentrations to comprehensively evaluate the potential risks associated with even minimal TA exposure. Furthermore, it is essential to consider the impact of environmental factors such as temperature variations, the presence of other contaminants, and ecological interactions, which could significantly influence the actual impact of TA, a consideration that may not be fully captured in a laboratory setting.

Finally, while the study primarily focuses on the acute effects of TA exposure on various organisms, it could be beneficial to examine potential long-term chronic effects and the possibility of bioaccumulation in the ecosystem. Considering these aspects could offer an alternative perspective on the overall environmental risk associated with technical assistance.

4. Materials and Methods

4.1. Chemicals

All chemicals used in this research, as well as some of their properties, are listed in Table 1.

Table 1. Name of the chemical, CAS number, purity, and company where all the chemicals used in this research were purchased from.

Name of the Chemical	CAS Number	Purity	Company
Tannic acid	1401-55-4	ACS Reagent (>95%)	Sigma-aldrich (Burlington, VT, USA)
NaOH	1310-73-2	98%	PanReac (Barcelona, Spain)
HCl	7647-01-0	37%	Fisher Chemical (Pittsburgh, PA, USA)
PBS (phosphate-buffered solution)	-	-	Sigmaaldrich (Burlington, VT, USA)
MOPS (4-Morpholinepropanesulfonic acid, 3-(N-Morpholino) propanesulfonic acid)	1132-61-2	≥99.5%	Sigma-aldrich (Burlington, VT, USA)
Ethanol	64-17-5	≥99.9%	Supelco by Sigma-aldrich (Burlington, VT, USA)

4.2. Daphnia Magna Tests

To perform the *D. magna* tests (water flea, from Vidrafoc, Spain, ref. DM121219), the OECD 202 (2004) guidelines [138,139] and the standard operational procedures of the Daphtoxkit FTM magna (1996) were followed. Briefly, the planktonic crustaceans were stored at 5 °C until use. *D. magna* eggs were incubated for 72 h at 20–22 °C using 6000 lx light in a TOXKIT model CH-0120D-AC/DC incubator (supplied by ECOTEST, Valencia, Spain). Then, they were fed with spirulina, provided in the kit, 2 h prior to TA exposure.

TA was dissolved into synthetic sterile freshwater (ISO 6341 2012 [131]) to final concentrations of 0.1, 1, 10, and 100 mg/L. This water was also used as a negative control. The pH was adjusted up to 7–7.5 with a 0.1 M NaOH solution. Each concentration was tested with five replicates of five organisms each.

In absolute darkness and for 24 h of exposure at 20–22 °C, daphnids were incubated at the cited concentrations. The organisms that could not swim for 15 s, when gentle agitation was applied, were considered immobile.

The corresponding EC_{50} and EC_{10} (the effective concentration of TA resulting in 50% and 10% immobilization, respectively) values and their Confidence Intervals (CIs) were obtained from the dose–response curves for the *D. magna* mobility tests using the Xlstat software, Addinsoft (2023), New York, NY, USA (https://www.xlstat.com/essoftware, accessed 27 September 2022).

4.3. Vibrio Fischeri Bioluminescence Assay

This experiment was based on the determination of inhibition of the marine bacteria *Vibrio fischeri* (NRRL B-11177) and, therefore, assessed the toxicity of any given contaminant to this marine bioindicator.

Lyophilizate bacteria were purchased from Macharey-Nagel (ref. 945 006) and stored frozen at −18 °C. The experiments were carried out according to the protocol ISO 11348 [86].

A stock solution of 4000 mg/L of TA was prepared by diluting pure TA in an aqueous solution of 20 g/L NaCl. From this, serial dilutions (0.4, 4, 40, and 400 mg/L) were prepared using the same solvent. The pH of the sample was checked to ensure that it remained within the established parameters (6–8.5) and shaken vigorously for adequate oxygenation. At the same time, the bacterial solution was prepared by adding the culture medium (about 10 mL) provided by the *V. fischeri* kit (purchased from Macharey-Nagel (ref. 945 006) to the freeze-dried vial. All samples and solutions were kept at a moderate temperature (15 ± 1 °C), and four replicates were measured for every sample dilution.

The experiment consisted of determining the bioluminescence of the bacterial solution after a short resting period (10 min). At this point, aliquots of serial dilutions of TA were then added to an equal volume (1 mL) of the bacterial solution, and the change in bioluminescence was measured after 30 min of exposure. Therefore, the concentrations tested were half of the above-mentioned serial dilutions (0.2, 2, 20, 200, and 2000 mg/L).

The EC_{50} and EC_{10} (the effective concentrations of TA resulting in an inhibition of the bioluminescence of 50% and 10%, respectively) values and their Confidence Intervals (CIs) were obtained from the dose–response curves for *V. fischeri* using the Xlstat software cited before.

4.4. Periphyton Communities Assay

4.4.1. Colonization

Periphyton communities were collected from the Gállego River, as previously described [44]. Racks were placed 15 cm below the water surface and were removed 15 days later (24 June 2019). At that time, the thickness became 0.75 mm. This meant that the algal biofilm showed communities of similar biomass and physical dimensions [44,140]. Then, the periphyton colonies were taken to the laboratory and prepared for the taxonomical analysis.

4.4.2. Water Samples

A water sample was collected at the same time and place as the periphyton slides, and the physicochemical parameters of the river were measured (see Supplementary Information Table S1).

4.4.3. Taxonomic Identification

The identification and counting of algae from the periphyton samples were performed in the laboratory following the Utermöhl technique adapted to inverted microscopy (UNE-EN 15204, 2007 [141]. H_2O_2 was applied on the samples to obtain an oxidized and clean frustule suspension. To identify, count, and interpret diatoms in the sample, hydrogen peroxide was mounted on slides with Naphrax© resin (UNE-EN 15204 [133], UNE-EN 13946 [142], and UNE-EN 14407 [143]).

Diatom cell count and identification were carried out with a Leica light microscope at 1000 magnification, while other algae needed 100, 400, and 1000 magnifications.

The results are given as density (individuals/mL) (see Supplementary Information Table S2).

4.4.4. Dose and Time Response Curves in Flow-Through Artificial Channels

The toxicity tests were conducted in methacrylate flow-through pipes that were connected to various water reservoirs. Every channel received 0.113 m^3/h of water from a closed water circuit that was fed by a series of motors connected to various reservoirs. A thermostatic bath kept the water at a constant 23 °C. The volume of each reservoir was the same (4 L).

The slides colonized with periphyton recently brought from the river were laid horizontally in an acclimatization channel at 23 °C before the ecotoxicology studies began. The slides were then positioned horizontally at the base of six fake flow-through tubes (mesocosms).

The light was delivered via lamps (Blau aquaristic, T5HO, 39 w/10,000 K, 80 µmol photon/s·m^2 on the channel surface) designed specifically to produce algae, which had a light spectrum similar to that of sunshine. This allowed periphyton communities to carry out photosynthesis in a manner that was representative of actual environmental conditions. The periphyton organisms were treated with TA at doses of 0.1, 1, 10, 100, and 1000 mg/L in a buffer solution (MOPS, 0.01 M) that had been pH-adjusted to 7.5, using either NaOH or HCl. As a negative control, one channel with MOPS but no TA was utilized. To ensure steadiness during the experiment, the temperature was checked on a regular basis.

Similarly to that previously described by Pino-Otín et al. [44], the effect of TA on the photosynthetic efficiency of the periphyton was assessed by measuring the photosynthetic yield, which represents the effectiveness of the photochemical energy conversion process [144], in triplicate after 1 and 2 h of exposure.

Since the structure of each community can differ and have an impact on the toxicity of the chemicals, a control sample was taken at time 0 for each measurement.

The EC_{50} and EC_{10} values (the effective concentrations of TA resulting in 50% and 10%, respectively, of photosynthetic yield) and their Confidence Intervals (CIs) were obtained from the dose–response curves for periphyton using the Xlstat software cited before.

4.5. Water and Soil Microorganism Tests

4.5.1. Water Samples

In accordance with established protocols (ISO 19458:2006 [145]), water samples were taken from the Gállego River in Zaragoza, Spain, at the same time as the periphyton samples (Table S3).

Microorganisms were isolated for genetic analysis from 5 L of river water that had been filtered using a 0.22 mm cellulose nitrate filter (Sartorius), resuspended in a sterile Falcon tube with 50 mL of phosphate-buffered saline (PBS), sterilized, and centrifuged for 10 min at 5000× *g*. Before sequencing, the pellet was kept at −80 °C, while the supernatant was discarded.

1 L of river water was filtered to remove debris using a 70 mm nylon sieve (BD Falcon) and then stored at 4 °C in the dark until use in the ecotoxicity experiments.

4.5.2. Soil Samples

The soil was taken from a crop field devoid of pesticides or other contaminants (CITA, Zaragoza, NE Spain). The composition of the soil was examined (CITA Soil and Irrigation Unit, Table S4).

For the genetic study, we followed previous procedures [44]. Briefly, 20 g of soil was mixed with 100 mL of sterile water for 30 min before being allowed to stand for an additional hour. The sample was then separated into 10 mL Falcon tubes and centrifuged at 1000× *g* for 10 min after being subjected to a 1 min sonication. The supernatant was sterilely obtained. The soil microorganisms were recovered by filtering the supernatant using a vacuum Büchner flask and a 0.22 mm Sartorius cellulose nitrate filter. After

carefully washing the filter material in sterile PBS, the sample was centrifuged at 5000× *g* for 10 min. A dropper was used to extract the supernatant, and the pellets were then kept at −80 °C until sequencing.

Using a 2 mm sieve, 10 g of soil was filtered (Becton Dickinson, Zaragoza, Spain) before the ecotoxicity tests. The 95 mL of sterile water was added to the 10 g of pre-sieved soil, and the sample was stirred in an Erlenmeyer flask for 30 min before standing for an hour. The supernatant was then collected under sterile circumstances and 10 mL of the Erlenmeyer flask's top was centrifuged at 1000× *g* for 10 min. There were five iterations of this cycle. The resulting whole supernatant was filtered to remove suspended soil debris with a 70 mm nylon sieve (Becton Dickinson, Spain).

4.5.3. Community-Level Physiological Profiling (CLPP) of Water and Soil Samples

TA's effects on the metabolism of microbial communities from water and soil and, specifically, alterations in the use of 31 distinct carbon sources, as previously described, were evaluated using the Biolog® EcoPlates (Tiselab S.L., Barcelona, Spain) [44,146].

To determine the toxicity of TA on the communities of microorganisms from soil and water, respectively, the following dilutions of TA were prepared for the ecotoxicity test: 0.2, 2, 20, and 200 mg/L in a final volume of 150 mL in the wells of a Biolog® EcoPlates with prefiltered river water, or the supernatant obtained from the soil sample. The dilutions' ultimate pH ranged from 6 to 7. Three replicates of each concentration were tested. All manipulations were carried out in a flow chamber in a sterile environment. The plates underwent a 6-day sterile incubation period in the darkness at 25 °C.

With the help of Gen5TM data analysis software and a Synergy H1 Microplate Reader (BIO-TEK, Vermont, USA), the OD (wavelength 590 nm) of each well was measured immediately upon inoculation and once per day for 144 h. Thus, the decrease in tetrazolium violet redox was used to measure the rate of utilization of the carbon sources. The average well color development (AWCD) was calculated for every one of the 3 replicates of the 96-well plate, as previously described by [147], as follows in Equation (1):

$$AWCD = S(AbsW - AbsC)/31 \quad (1)$$

where AbsW is the absorbance (or optical density) of each well with the carbon source and AbsC is the absorbance of the control well without it.

To study the different consumption levels of metabolites by the water and soil microorganisms, these were grouped as Weber et al. [148] previously described. After that, the AWCD was determined for each group according to Equation (1).

AWCD values are given as the average of the replicates ± standard deviation.

4.5.4. Genetic Sequencing of River and Soil Microorganisms

Through genetic sequencing, the taxonomic composition and the predominate taxa of these microbial communities were examined to better understand the impact of this natural compound on their growth and metabolism.

The Genomics Unit Cantoblanco, Science Park, performed the genetic sequencing of water and soil microbes (Madrid, Spain). After being previously homogenized in PBS, the samples' bacterial genomic DNA was extracted using G-spin columns from 200 mL aliquots (INTRON Biotechnology, Seongnam, Kyonggi-do, Repulic of Korea). DNA concentration was assessed using Thermo Fischer's Quant-IT PicoGreen reagent. As previously stated, DNA samples were utilized to amplify the V3-V4 region of the 16S ribosomal RNA (rRNA) gene [44,149,150].

Individual amplicon libraries were analyzed on an Agilent Bioanalyzer 2100, and the concentration was determined using real-time PCR (Kapa Biosystems). DNA samples were sequenced using an Illumina MiSeq instrument in accordance with a 2 × 300 procedure. Utilizing current Base Space applications, reads were quality-filtered using Illumina standard values, demultiplexed, and fastq files were mapped to the GreenGenes database (16S Metagenomics, Illumina, San Diego, USA).

In the run, all 145,498 reads for soil microorganisms and all 85,525 reads for water microorganisms passed the quality filtering with a perfect score.

4.6. Eisenia Foetida Assays

We purchased adult *Eisenia foetida* organisms from Todo Verde's composters (Zaragoza, Spain). Earthworms were conditioned in a sphagnum peat substrate from the Spanish Flowers Company (Zaragoza, Spain) for 15 days prior to testing. They were maintained in a stable environment at 18–25 °C, a pH of 7.8–8, and a humidity level of 80–85%.

Adult earthworms that were older than 60 days were selected. They all had a clitellum and similar size, and weighed between 300 and 600 mg. The OECD 207 (1984) [117] protocol was followed for the toxicity tests in a similar manner to that previously reported [44,78].

Commercial black peat (Verdecora vivarium, Spain), kaolinic clay, and quartzitic sand were combined in the following proportions to create the artificial soil, which was created in accordance with OECD 207 standard: 7:2:1 [44,78]. Deionized water was used to alter the substrate's moisture content in an amount equal to 35 to 45% of the dry weight of the soil. An amount of 600 mg of this synthetic soil was placed in 1 L capacity polypropylene containers with perforated lids to prevent moisture loss.

Each container included ten earthworms, and 120 mL of the TA dilutions 0.2, 2, 20, 200, and 2000 mg/L was added. The same method was used to create negative controls without TA. Each concentration was examined three times. A regulated environment of 20 ± 2 °C, 80–85% relative humidity, and 400–800 lx of light was used to maintain the containers. Earthworm mortality was assessed 14 days after treatment.

The EC_{50} and EC_{10} (the effective concentrations of TA resulting in 50% and 10%, respectively, of worm survival) values and their Confidence Intervals (CIs) were obtained from the dose–response curves for *E. faetida* using the Xlstat software cited before.

4.7. Allium cepa Assay

Allium cepa (variety Stuttgarter Riesen de 14/21) bulbs were purchased from Fitoagrícola Company (Spain) and kept in a dry environment between 10 and 20 °C in the dark until use to prevent the growth of fungi. Before the test, the young bulbs were peeled. Damage to the root ring was prevented with this operation.

The protocol developed by Fiskesjö (1993) [120] was followed to conduct *A. cepa* acute toxicity tests, consisting of the measurement of root elongation after 72 h of exposure to the test substance [44]. Because it has a suitable amount of calcium and magnesium ions for the plant to grow properly [44,151], mineral water from Aguas de San Martín de Veri S.A. (https://www.veri.es/es/el-producto, accessed 18 September 2022) from Spain was used as the growing medium for the bulbs, which were put in 15 mL tubes. Twelve replicates of each concentration (0.2, 2, 20, 100, and 500 mg/L) were used in ecotoxicological studies. The only component of the negative control was water. The bulbs were grown for 72 h at 25 °C in a dark room. Every 24 h, the test solutions were renewed.

The EC_{50} and EC_{10} (the effective concentrations of TA resulting in 50% and 10%, respectively, of root growth) values and their Confidence Intervals (CIs) were obtained from the dose–response curves for *A. cepa* using the Xlstat software cited before.

4.8. Statistics and Graphical Representation

Using the Xlstat software cited before, logit logistic regression was applied to create the dose–response curves for *D. magna* mobility, *E. foetida* survival, *A. cepa* root elongation, and periphyton communities' photosynthetic yield in order to determine the appropriate EC_{50} and EC_{10} values [44]. A chi-squared test was used to statistically evaluate dose–response models. Also, with the Xlstat software cited before, Student's *t*-test on two

independent samples and the variance association between the AWCD values of the three replicates were calculated to determine significance.

5. Conclusions

Our results show, for the first time, that TA can have an impact on aquatic organisms, especially for isolated nekton bacteria such as *V. fischeri* or invertebrates like *D. magna*. However, the river microbial communities of nekton and periphyton seem to be able to protect themselves or balance the effects that this product can produce on individual organisms, even biodegrading it. Earthworms also seem to have mechanisms to, at least, not suffer lethal effects when exposed to high concentrations of TA. However, this compound can present phytotoxicity in plants of economic interest, such as *A. cepa*, and produce a significant impact on both the growth and metabolic profiles of edaphic microbial communities. These microorganisms are the basis of trophic chains, so their alteration could have a significant impact on soil quality, especially at high doses that are not currently present in the environment. However, at the present time, this product is already being marketed for multiple commercial uses and its consumption is likely to increase in the coming years, so this study can shed light on the possible toxicity effects that it can cause in aquatic and terrestrial environments. Likewise, these results emphasize that products of natural origin must be studied from the point of view of their ecotoxicity and be subjected to the same supervision as synthetic ones so that they can become a more ecofriendly alternative in an industry that is increasingly committed to sustainability.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12234041/s1, Figure S1: Dose–response curve for periphyton test after 2 h exposure to tannic acid (TA). Photosynthetic values are given as a ratio with respect to the control, and each dose was measured in triplicate. Red line is the model, and dashed lines are the inferior and superior confidence limits (95%); Figure S2: Dose–response curve for *Eisenia foetida* test after 14-day exposure to tannic acid. Curves are the average of 3 replicates. Red line is the model; Table S1: Physico-chemical parameters of water and soil samples; Table S2: Diatom cell count and identification of periphyton communities; Table S3: Water samples' taxonomy; Table S4: Soil samples' taxonomy.

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5.4. Capítulo 4. Estudio ecotoxicológico del nerol en indicadores no diana de suelo y agua y su impacto en comunidades fluviales y edáficas.

Artículo en revisión en Environmental Toxicology and Pharmacology (Q1, FI: 4,2)

- Resumen:

NE es un terpenoide volátil de origen vegetal, utilizado comercialmente en perfumes, cosméticos, e industria alimentaria, cuyo interés ha crecido en los últimos años, entre otras, por sus propiedades antimicrobianas. Sin embargo, al igual que sucede con la mayoría de PN, se disponen de muy pocos estudios que analicen su ecotoxicidad.

Para tratar de ampliar el conocimiento en este campo, en este estudio se expusieron concentraciones de NE a una selección de bioindicadores estándar acuáticos (*D. magna* y *A. fischeri*) terrestres (*A. cepa* y *E. fetida*), así como a comunidades microbianas naturales del necton fluvial y edáficas, mediante las placas BIOLOG Ecoplate™, complementadas con la secuenciación del gen ARNr 16S para su caracterización taxonómica.

Nuestros resultados muestran una toxicidad aguda moderada, con efectos que se pueden considerarse tóxicos sobre *A. fischeri* de acuerdo con los criterios de la ECHA (European Chemicals Agency (ECHA), 2008), y poco tóxicos para el resto de los bioindicadores individuales que seguirían el siguiente orden de mayor a menor toxicidad: *A. fischeri* (CE_{50} = 9,53 mg/L) > *D. magna* (CE_{50} = 26,96 mg/L) > *A. cepa* (CE_{50} = 46,22 mg/L) > *E. fetida* (CL_{50} = 68,92 mg/kg). Sin embargo, las comunidades microbianas tanto edáficas como fluviales, mostraron una mayor resistencia a la exposición a NE, observándose leves cambios en el perfil metabólico a concentraciones de ≥100 mg/L, e inhibición completa a 1000 mg/L. A concentraciones bajas (0,1–10 mg/L), se detectó un efecto hormético en las comunidades fluviales, con un incremento en el crecimiento y la actividad metabólica respecto al control. Esta mayor resistencia se debe probablemente a la diversidad genética y funcional de las muestras, capaces de adaptarse a la exposición a NE, y que incluyen especies capaces de degradarlo y utilizarlo como sustrato.

Estos resultados sugieren que, pese a los riesgos de ecotoxicidad aguda que puede plantear este PN, su biodegradabilidad por parte de las comunidades microbianas presentes en el medio ambiente reduce el potencial de acumulación y toxicidad medioambiental. NE puede ser, por tanto, un producto de uso seguro para el medio ambiente.

- Relación con la Tesis Doctoral:

Este estudio responde al cuarto objetivo de la Tesis, al proporcionar una evaluación completa de la ecotoxicidad del NE frente a organismos bioindicadores y comunidades microbianas complejas, siguiendo el mismo enfoque metodológico que para los ATBs y el AT. Esta comparativa directa permite analizar el impacto ambiental de NE y contribuye a

dilucidar si este producto natural, junto con el AT, representa una alternativa ambientalmente más segura para su uso combinado con ATBs. Esta comparación proporciona el paso necesario para valorar la viabilidad de emplear dichas sinergias como solución frente a los problemas asociados al uso de ATBs tradicionales. El estudio de NE, integrado en este bloque de la Tesis, permite avanzar en el ciclo de evaluación ambiental de los productos naturales seleccionados en las sinergias, dando respuesta al objetivo global del trabajo sobre alternativas ecocompatibles en la lucha contra la resistencia antimicrobiana

- Importancia de los resultados:

La escasa bibliografía disponible acerca del impacto medioambiental de NE hace necesario una evaluación de su ecotoxicidad. Este trabajo es uno de los primeros que analiza los efectos tóxicos de NE que al igual que en el caso del AT, utilizamos una batería de bioindicadores, tanto acuáticos (*D. magna* y *A. fischeri*) como terrestres (*A. cepa* y *E. fetida*). Del mismo modo, se investigó su efecto sobre comunidades microbianas fluviales y edáficas analizadas taxonómicamente, que reflejan de una forma más completa que los bioindicadores individuales el efecto de un PN sobre los ecosistemas. Los resultados reflejan que el NE es un producto ambientalmente seguro pues sobre el único indicador que presenta toxicidad significativa es sobre la bacteria *A. fischeri,* indicador estándar de ecotoxicidad *in vitro*. Sin embargo, las comunidades bacterianas naturales, tanto edáficas como del necton fluvial, muestran una notable resiliencia, favorecida por la diversidad taxonómica y funcional y su capacidad de biodegradación, reduciendo el riesgo de persistencia ambiental y de efectos adversos a largo plazo. Esta información es clave para posicionar al NE como un candidato prometedor para el desarrollo de alternativas antimicrobianas más seguras y sostenibles, permitiendo fundamentar decisiones sobre su utilización en sinergia con ATB comerciales en el contexto de la búsqueda de soluciones frente a su resistencia bacteriana y la reducción de su impacto ambiental.

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5.5. Capítulo 5. Estudio ecotoxicológico de combinaciones antimicrobianas sinérgicas de ácido tánico y nerol con antibióticos: ¿Una alternativa segura a los antibióticos comerciales?

Artículo pendiente de envío a revista

- Resumen:

Las combinaciones sinérgicas de PN y ATB comerciales vienen siendo propuestas como una posible solución a los problemas que generan los ATB, como las resistencias microbianas o su impacto medioambiental, ya que permiten una reducción importante en las dosis de estos fármacos. Sin embargo, es necesario conocer previamente las consecuencias que estas combinaciones sinérgicas pueden tener en los ecosistemas. Para ello, en este estudio se expusieron combinaciones sinérgicas de NE (SACNEs) y de AT (SACTAs) a distintos bioindicadores individuales de medios acuáticos, como *D. magna* o *A. fischeri*, y a comunidades microbianas fluviales obtenidas del río Gállego (Aragón). Estas comunidades microbianas fueron caracterizadas metabólica y taxonómicamente mediante la secuenciación del gen ARNr 16S.

Nuestros resultados muestran una mayor sensibilidad de los bioindicadores individuales, en especial para las combinaciones sinérgicas de NE respecto a las comunidades microbianas. Esta resiliencia de las comunidades microbianas puede deberse, entre otros factores, a la elevada diversidad taxonómica, que permite una mejor adaptación frente a un agente tóxico, y a la capacidad de algunos microorganismos para metabolizar estos PN y utilizarlos como fuente de carbono. A bajas concentraciones, se observó un efecto hormético en algunas combinaciones, como SACNE 1, con incrementos en el crecimiento microbiano respecto del control. El resto de las combinaciones presentan una inhibición por debajo de la del control. SACNE 3, SACTA 1, SACTA 2 presentan una inhibición menor (entre 16 – 32%) que su antibiótico a la concentración MIC sobre la bacteria correspondiente a cada sinergia. La SACNE 2 presenta una inhibición muy similar a la respectiva concentración MIC de su antibiótico (10%). SACTA 3 presenta una inhibición mucho mayor que su antibiótico a concentración MIC (71%). El perfil metabólico muestra que todas las combinaciones presentan una inhibición total del metabolismo, excepto SACNE 3, que muestra un ligero metabolismo de aminoácidos a las 144 h, y SACNE 1, que muestra un claro efecto hormético de los aminoácidos y los carbohidratos. Estos resultados subrayan la necesidad de evaluar cada combinación de forma específica antes de su aplicación como alternativa a los ATB convencionales.

- Relación con la Tesis Doctoral:

Tras determinar que AT y NE pueden generar combinaciones sinérgicas con algunos ATB comerciales, y una vez conocido el impacto medioambiental que suponen estos PN, el último capítulo de esta tesis, y también su parte central, implica evaluar la ecotoxicidad que pueden presentar estas combinaciones. El estudio de ecotoxicidad, utilizando los

mismos bioindicadores seleccionados para los ATB individuales (capítulo 1 de resultados), fue llevado a cabo con los dos PNs seleccionados (NE y AT, capítulos 2 y 3 de resultados), y ahora es ampliado a las combinaciones sinérgicas, permitiendo así una comparación adecuada, que permita responder al objetivo principal de este trabajo de si estas combinaciones PN + ATB suponen una ventaja real frente al uso de ATB aislados.

- Importancia de los resultados:

La importancia de este artículo radica en que aborda un vacío crucial en la literatura científica, dado que hasta la fecha no hay apenas literatura científica que evalúe el impacto ecotoxicológico de combinaciones sinérgicas entre antibióticos convencionales y productos naturales individuales, y ninguna sobre el AT y el NE en organismos fluviales no diana ni en comunidades microbianas de río. Esta ausencia de información representa una limitación significativa para la comprensión del riesgo ambiental asociado al uso extendido de antibióticos y sus alternativas potenciadas con compuestos de origen natural.

El trabajo recoge una aproximación integral, combinando diferentes bioensayos a distintos niveles con bioindicadores individuales y el análisis de comunidades bacterianas a nivel genético y metabólico, para obtener una visión profunda sobre los efectos de estas sinergias en los ecosistemas acuáticos.

La información generada proporciona una base indispensable para futuras investigaciones, tanto en ecotoxicología acuática como en el desarrollo de estrategias antimicrobianas sostenibles. El conocimiento obtenido puede servir como referencia para que estas combinaciones sinérgicas puedan dar el paso a ensayos preclínicos con futura aplicación médica o veterinaria, y a orientar nuevas líneas de investigación sobre el control de la resistencia bacteriana.

Ecotoxicity of Synergistic Antimicrobial Combinations of Nerol and Tannic Acid with Antibiotics on Non-Target Fluvial Organisms: an alternative to commercial antibiotics?

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Abstract

The continuous use of antibiotics, in addition to solving serious problems related to infections, has been the source of the problem of bacterial resistance, which has led to the search for alternatives. This study presents an investigation into the ecotoxicity of synergistic antimicrobial combinations of tannic acid (TA) and nerol (NE), called SACTAs and SACNEs, respectively, with commercial antibiotics, gentamicin (GTM), streptomycin (STM), chloramphenicol (CHL) and ampicillin (AMP) on non-target aquatic organisms: individuals bioindicators (*Aliivibrio fischeri* and *Daphnia magna*) and river-derived microbial communities. These bacterial communities have been genetically and metabolically characterised using Biolog EcoPlates™ and sequenced through 16S rRNA.

SACTAs (combinations of NE with CHL and AMP) and SACNES (combinations of NE with STM and GTM) exhibited a diverse range of toxic effects. SACTAs showed less toxicity than SACNEs. SACNE 3, with STM (250 µg/mL (NE) + 31.25 µg/mL (STM), was the most toxic to *D. magna*, and SACNE 2, with GTM (125 µg/mL (NE) + 3.78 µg/mL (GTM), was the most toxic to *A. fischeri*.

All combinations inhibit bacterial growth compared to the control, except for SACNE 1. When compared to the inhibition produced by the MIC of the antibiotic for the bacteria where the synergy was found, all combinations showed greater inhibition except SACNE 1. All combinations differ from their antibiotic at MIC concentration by a maximum of 32%, except for SACTA 3, which differs by 71%. When observing the metabolic profile all combinations show total inhibition of metabolism, except for SACNE 3, which shows a slight amino acid metabolism at 144 h, and SACNE 1, which shows a clear hormetic effect of amino acids and carbohydrates.

These results emphasize the potential ecotoxicological benefits of using combination therapies to lower antibiotic doses while maintaining their antimicrobial efficacy.

1. Introduction

Antibiotics (ATBs) have been a fundamental therapeutic tool in the fight against infections, saving millions of lives. However, the extensive use of these bioactive compounds, which are largely excreted by both humans and animals, has led to their presence in water bodies, particularly rivers (Balakrishna et al., 2017; Z. Li et al., 2020). For example, up to 80% of tetracyclines and 90% of erythromycin (ERY), along with their metabolites, are eliminated through urine and feces (Kumar et al., 2005; Sarmah et al., 2006). In the best-case scenario, these compounds enter water bodies through wastewater treatment plants, whose purification systems often fail to completely remove them from the water stream (Mutiyar and Mittal, 2014; Sabri et al., 2020). In other cases, they reach rivers through untreated discharges, surface runoff, or leaching (Kuang et al., 2020). It has been reported that thousands of tons of antimicrobials and their transformation products are released into the environment each year (Harnisz et al., 2015; Ji et al., 2012).

For instance, ampicillin (AMP) has been detected in the Yamuna River India, with maximum concentrations of 104.20 µg/L in influents and 13.75 µg/L in the river after wastewater treatment (Mutiyar and Mittal, 2014). Gentamicin (GTM) concentrations range from 0.4 to 7.6 µg/L in wastewater (Löffler and Ternes, 2003; Tahrani et al., 2016). Chloramphenicol (CHL) has been found in the Han River of Korea at concentrations of 0.031 µg/L (Choi et al., 2008), and in the Yangtze River of China between 0.14-49.91 ng/L (Feng et al., 2019).

The presence of these molecules and their byproducts (Sy et al., 2017) induces toxicity in non-target organisms, as reported in numerous studies (Kovalakova et al., 2020). Specifically, streptomycin (STM) (Le et al., 2023; Qian et al., 2012), GTM (Dias et al., 2015), and CHL (Seoane et al., 2014; Xiong et al., 2019) exhibit toxicity in cyanobacteria and green algae. Similarly, AMP shows toxic effects, although with high variability in response (Baselga-Cervera et al., 2019). Rotifers and microcrustaceans (Isidori et al., 2005) or fish larvae (Pindling et al., 2018) are also affected by GTM and STM, respectively. In addition to individual non-target organisms, GTM, STM, and especially CHL can impact complex riverine microbial. GTM and STM significantly disrupt riverine bacterial communities and alter metabolic functions. CHL exerts a strong inhibitory effect on bacterial growth and community respiration (Pino-Otín et al., 2023a).

As rivers carry substantial loads of ATBs, resistance to these drugs could develop and spread, allowing ATB-resistant bacteria and resistance genes to disseminate into various environmental compartments of rivers worldwide through horizontal gene transfer (Singh et al., 2019). Over time, common bacterial infections become resistant to standard treatments, compromising the effectiveness of available ATBs and posing a global public health threat (Halawa et al., 2023). In 2016, the UN General Assembly identified antimicrobial resistance driven by the high consumption of ATBs as a critical threat and urged a collaborative approach through agencies such as the WHO, the Food and Agriculture Organization (FAO), and the World Organization for Animal Health (WOAH)

under the "One Health" framework, which integrates human, animal, and environmental health (Aslam et al., 2021).

Reducing ATB doses while maintaining their antimicrobial activity has emerged as a necessity. One promising approach is the use of synergistic combinations of commercial ATBs with naturally derived antimicrobials. This strategy can lower ATB doses, thereby reducing toxicity and the selective pressure that drives the emergence of resistance. Natural products (NPs) often target different molecular pathways than traditional ATBs, decreasing the likelihood of resistance development due to its multiple antibacterial mechanisms (Ayaz et al., 2019). Additionally, they typically exhibit lower persistence in aquatic ecosystems due to biodegradation by fluvial microbial communities (Gan et al., 2024).

Tannic Acid (TA) (*2,3,4,6-tetra-O-(3,4,5-trihydroxybenzoyl)-β-D-glucopyranose*) is a prominent plant-derived gallotannin belonging to the hydrolysable tannins group (Akiyama et al., 2001). Its notable sources include oak galls, particularly from species like *Quercus infectoria*, as well as the bark of chestnut (*Castanea sativa*) and oak (*Quercus* spp.), black tea leaves (*Camellia sinensis*), and the fruits and seeds of plants like grapes (*Vitis vinifera*) (Youness et al., 2021). NE, a volatile terpene also referred to as *(Z)-3,7-dimethylocta-2,6-dien-1-ol*, is a natural compound isolated from sources such as orange trees (*Citrus aurantium*), lemongrass (*Cymbopogon citratus*), hops (*Humulus lupulus*), and yeasts (*Saccharomyces cerevisiae*) (Li et al., 2024; Mukarram et al., 2021).

Both compounds exhibit remarkable bioactive properties, including notable antimicrobial activity (Kaczmarek, 2020; Wang et al., 2019b), which could explain their synergistic effects when combined with commercial ATBs (Lorca et al., 2024). Additionally, NE degrades at a moderate rate in aquatic ecosystems, particularly under sunlight or exposure to oxidants, and the EPA does not classify it as a potentially persistent or bioaccumulative molecule (Api et al., 2023). TA degradation in rivers can range from days to weeks depending on environmental conditions and the presence of specific microorganisms or catalysts (Ghosh and Bhadury, 2022b). Both compounds have been categorized as safe by international agencies: NE is recognized as a GRAS (Generally Recognized as Safe) flavor ingredient by the Flavor and Extract Manufacturers' Association (FEMA, https://www.femaflavor.org/flavor-library) and has been authorized by the FDA as a flavoring agent (21 CFR 172.515). TA is classified as safe by the FDA (Food and Drug Administration) in the United States, and by the EFSA in the European Union (European Food Safety Authority (EFSA), 2014) and it is already used in medical applications (Sun et al., 2022).

In previous studies (Lorca et al., 2024), three synergistic antimicrobial combinations of NE (SACNEs) were identified with GTM and streptomycin STM effective against *Salmonella enterica*, *Bacillus subtilis*, and *Streptococcus agalactiae*, as well as three combinations of Tannic Acid (SACTAs) with AMP and CHL effective against *Pasteurella aerogenes* and *Acinetobacter baumannii*. These six synergies enable a reduction in the ATB dose by 75% to 93.75% while maintaining their antimicrobial efficacy.

For these synergies to be alternatives to commercial ATBs, they must demonstrate that they are not more harmful to the environment. To our knowledge, their environmental impact, particularly on non-target riverine organisms or fluvial communities, remains unknown

This study seeks to fill this gap by employing an integrated approach that examines both individual species and communities to evaluate the ecotoxicity in aquatic environments of synergistic antimicrobial combinations of TA (SACTA) and NE (SACNE), capable of reducing the ATB dose while maintaining efficacy against clinically relevant strains.

Specifically, it aims to:

1. Evaluate, for the first time, the toxicity of three synergistic antimicrobial combinations of TA (SACTA) and three of NE (SACNE) on non-target aquatic organisms, *Aliivibrio fischeri* and *Daphnia magna*.

2. Conduct a novel assessment of the toxicity of these six synergistic antimicrobial combinations on riverine microbial communities, using 16S rRNA gene sequencing for analysis.

2. Material and Methods

2.1. Chemicals

The NPs, NE and TA, were purchased from Sigma-Aldrich. The ATBs GTM, STM and CHL were purchased from Acofarma, and AMP from Sigma-Aldrich. Characteristics and properties of NE, TA and ATBs used in this study are shown in table 1. Stock solutions were prepared using high purity distilled water (SIEMENS Ultra Clear™). To maintain appropriate experimental conditions, the pH of each stock solution was measured using a FiveEasy F20 pH meter. NaCl and DMSO (dimethyl sulfoxide) were purchased from Fisher Scientific. DMSO was used to solubilise both TA and NE. Solutions of the NPs were prepared using a final DMSO concentration of 5%, that does not affect bacterial growth in most of the strains tested (Ferrando et al., 2024), discarding those affected.

Six synergies were tested: three with TA (SACTA 1, 2 and 3) and three with NE (SACNE 1, 2 and 3). Information regarding the ATBs under study and NPs is shown in Table 1. The composition of SACTA and SACNE, the MICs of the individual components of the synergy, the MICs of the individual components, the bacteria affected by the synergy, and the percentage decrease in ATB and NP doses are shown in table 2.

Table 1.- Information about the antibiotics (ATB) and natural products (NP) assayed: abbreviation, CAS number, molecular weight (g/mol), family, supplier, purity (%), water solubility (mg/mL), pKa and Log $K_{o/w}$. Source: DrugBank and PubChem, 2025.

Compound	Abbreviation	CAS number	Molecular weight (g/mol)	Family	Supplier	Purity (p/p)	Water solubility (mg/mL)	pKa_1/pKa_2	Log $K_{o/w}$
Nerol	NE	106-25-2	154.2	Monoterpenes	Acofarma	99.0%	0.53	-	3.47
Tannic Acid	TA	1401-55-4	1701.2	Polyphenols		99.0%	>28	-	-
Gentamicin	GTM	1403-66-3	447.6	Aminoglycosides		≥97%	100	12.6/10.1	-1.88
Streptomycin	STM	57-92-1	581.6			≥97.0%	89	11.09/11.6	2.53
Chloramphenicol	CHL	56-75-7	323.1	Amphenicols		97.5%	1	8.7/-2.8	1.14
Ampicillin	AMP	69-53-4	394.4	Betalactams	Sigma-aldrich	≥90.0%	74	2.65/7.25	1.35

Table 2. Information on synergies, composition (µg/mL), individual Minimum Inhibitory Concentrations of antibiotics and natural products, the bacteria affected by the synergy and dose reduction in synergy of natural products and antibiotics (%).

Combination	Composition (µg/mL)	Concentration of NP (µg/mL)	Concentration of ATB (µg/mL)	MIC of NP alone (µg/mL)	MIC of ATB alone (µg/mL)	Bacterium	NP decrease (%)	Antibiotic decrease (%)
SACNE 1	NE 125 + STM 1,17	125	1,17	500	4,68	*Salmonella enterica*	75%	75%
SACNE 2	NE 125 + GTM 0,78	125	0,78	500	6,25	*Bacillus subtilis*	75%	87,5%
SACNE 3	NE 250 + STM 31,25	250	31,25	1000	125	*Streptococcus agalactiae*	75%	75%
SACTA 1	TA 46,87 + CHL 3,91	46,87	3,91	187,5	62,5	*Acinetobacter baumanii*	75%	93,75%
SACTA 2	TA 46,87 + AMP 7,81	46,87	7,81	187,5	125	*Acinetobacter baumanii*	75%	93,75%
SACTA 3	TA 40,62 + CHL 1	40,62	1	162,5	8	*Pasteurella aerogenes*	75%	87,5%

(1) **SACNE**: Synergic Antimicrobial Combination of **Nerol.** (2) **SACTA**: Synergic Antimicrobial Combination of **Tannic Acid.** (3) **MIC:** Minimum Inhibitory Concentration. (4) **NP:** Natural product. (5) **ATB:** Antibiotic.

2.2. Daphnia magna assay

The impact of NE, TA, and their synergistic combinations on *Daphnia magna* was evaluated using the standardized procedure described in the Daphtoxkit FTM magna (1996) (reference DM121219, Vidrafoc, Barcelona, Spain), following the OECD Guideline 202 (OECD, 2004). To ensure the integrity of the materials, the kit was kept in the dark at 5 °C until its use.

Resting eggs of *D. magna* were incubated for 72 hours at 22 °C under continuous illumination (6000 lx) in a TOXKIT model CH-0120D-AC/DC incubator (ECOTEST, Valencia, Spain). Once hatched, the neonates were fed for two hours with a vial containing *Spirulina* microalgae.

Subsequently, the organisms were exposed to various synergistic combinations expressed as percentages of SACNE or SACTA. The tested concentrations were 0.125%, 1.25%, 25%, 50% and 100% for SACNEs, and 2.5%, 25%, 50% 75% and 100% for SACTAs. DMSO served as the solvent for the natural compounds, with a maximum concentration of 5%.

The pH of the test medium was continuously monitored and kept between 7.0 and 7.5, following the protocol specifications. Each treatment included five replicates, each consisting of five neonates in 10 mL of test solution. Synthetic freshwater was used as the negative control. After a 48-hour exposure period under complete darkness, any *D. magna* individual that remained motionless for 15 seconds after gentle agitation was recorded as immobile, following OECD Guideline 202 recommendations. The immobilization data were then used to determine the EC_{50}, corresponding to the concentration causing 50% immobilization.

2.3. Aliivibrio fischeri assay

The acute toxicity of SACNEs and SACTAs on *Aliivibrio fischeri* was evaluated by measuring the inhibition of bacterial bioluminescence following exposure to a range of concentrations. The procedure adhered to the standardized ISO 11348-3 (2007) protocol. The strain used, *A. fischeri* NRRL-B-11,177, was supplied by Macherey-Nagel (ref. 945 006, Dueren, Germany).

For assay preparation, lyophilized *A. fischeri* cultures were rehydrated with the reactivation solution included in the test kit and held at 4 °C for five minutes to ensure full reactivation and cellular viability.

Stock solutions of SACNEs and SACTAs were prepared in 2% NaCl. The tested concentrations were 0.001%, 1%, 25%, 50% and 100% SACNEs, and 1%, 25%, 50%, 75% and 100% for SACTAs. The pH of all test solutions was maintained within the range of 6 – 8.

Each concentration was evaluated in four replicates, with separate tubes containing *A. fischeri* exposed individually to SACNEs or SACTAs. A control tube containing only the 2% NaCl solution served as the negative control.

Luminescence readings were obtained using a Biofix® Lumi-10 luminometer (Macherey-Nagel, Dueren, Germany). The baseline luminescence of 0.5 mL of bacterial suspension was first recorded, followed by the addition of 0.5 mL of the test solution to each vial. After a 30-minute exposure period, luminescence was measured again. Bioluminescence inhibition was expressed as a percentage reduction relative to the initial control measurement. EC_{50} values were calculated, corresponding to the concentration causing 50% inhibition of luminescence.

2.4 River Microorganisms Community Assay

2.4.1. River Samples

This study was designed with two main objectives: to perform a comprehensive genetic and chemical characterization and to conduct Biolog EcoPlates™ assays (Tiselab S.L., Barcelona, Spain).

Water samples were collected from the Gállego River in Zaragoza, Spain, in May 2024. Sampling and transport to the laboratory were carried out in accordance with the ISO 19458:2006 standard and under AENOR supervision, using sterile collection containers. During sampling, several in situ physicochemical parameters were recorded to characterize the river's water conditions. The measured water temperature was 17 °C, obtained with a precision Nahita thermometer (ICT S.L., La Rioja, Spain). The pH was 7.5, determined with a PanReac AppliChem A011435 meter (Darmstadt, Germany), and conductivity was 2.8 mS, measured using a Hanna HI8733 conductivity meter (Merck, Madrid, Spain).

A 5 L portion of river water was designated for genetic analysis. The sample was filtered using a 0.22 µm cellulose nitrate membrane (Sartorius, Göttingen, Germany) attached to a vacuum flask. The microorganisms retained on the filter were resuspended in 50 mL of sterile phosphate-buffered saline (PBS) (Teknovas S. A. U.) inside a Falcon tube. The suspension was then centrifuged at 5000 g for 10 minutes, producing a compact bacterial pellet at the bottom of the tube. This pellet was stored at −80 °C to preserve the integrity of the genetic material for subsequent sequencing.

An additional 1 L of river water was reserved for ecotoxicity testing. This volume was passed through a 70 µm nylon mesh sieve (Becton Dickinson, Madrid, Spain) and kept at

4 °C in complete darkness until further analysis to prevent any alteration of sample composition.

Finally, 2 L of river water were submitted to Laboratorios Valero Analítica (Zaragoza, Spain) for detailed physicochemical assessment. The complete results from that analysis are available in Supplementary Material 1.

2.4.2. Genetic Sequencing of River Microorganisms

The genetically processed material described in Section 2.4.1 underwent further molecular analysis using the Froilabo Trust −80 °C storage system. DNA extraction was carried out with the AllPrep® PowerViral® DNA/RNA Kit (Qiagen, Barcelona, Spain), following the manufacturer's protocol. The extracted nucleic acids were quantified by PicoGreen® fluorimetry, and 1.5 ng of DNA from each sample was used to amplify the V3–V4 region of the 16S rRNA gene.

Amplification of the V3–V4 region of the 16S rRNA gene was performed via polymerase chain reaction (PCR) over 21 cycles using Q5® Hot Start High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and 100 nM specific primers. The presence of amplicons of the expected size was confirmed by agarose gel electrophoresis, which verifies fragment size but not sequence specificity. Following this, the DNA products were diluted and subjected to a second PCR of 13 cycles using 400 nM primers from the Access Array Barcode Library for Illumina Sequencers (Fluidigm, CA, USA). This step enabled the incorporation of unique barcodes for each sample, thus completing the preparation of the Illumina sequencing library.

Following library construction, DNA fragments were analyzed and concentrated using the TapeStation system (Agilent, Madrid, Spain). Equimolar pooling was then carried out, and the combined sample was purified using AMPure magnetic beads. The pooled DNA was quantified via quantitative PCR (qPCR) using the Kapa-SYBR FAST qPCR Kit for LightCycler 480 (Sigma-Aldrich, Madrid, Spain) and an internal reference standard.

The final amplicon pool was denatured and loaded onto a flow cell at 10 pM concentration to initiate cluster generation. Sequencing was performed using the MiSeq Reagent Kit v3 in a 2 × 300 bp paired-end run on a MiSeq sequencer.

After sequencing, fastq files were generated through bcl2fastq processing, integrated into the Illumina workflow. Phylogenetic profiling was conducted using the 16S Metagenomics application (BaseSpace v1.1.0, Illumina, Madrid, Spain), and taxonomic classification was achieved using the Greengenes (13_5) reference database.

2.4.3. Community-Level Physiological Profiling (CLPP) of River Microorganisms

Biolog EcoPlate™ assays (Tiselab S.L., Barcelona, Spain) were applied to evaluate how SACNEs and SACTAs influence the metabolic performance of aquatic microbial communities. This approach allowed monitoring of alterations in the utilization patterns of 31 different carbon substrates, following previously established methodologies (Pino-Otin et al., 2023).

For the ecotoxicity evaluation, stock solutions of SACNEs and SACTAs were independently prepared at synergy (PN + ATB) and MIC levels, as well as at combined synergistic concentrations (see Table 2). All solutions were prepared in sterile water and dispensed into Biolog EcoPlate™ wells under aseptic conditions.

The metabolic response of the riverine microbial communities was assessed using the prefiltered river water described in Section 2.4.1. The pH of all test solutions was controlled within the range of 6 to 7. Each concentration was evaluated in triplicate under sterile conditions within a controlled laminar-flow environment.

Once prepared, the microplates were incubated for 7 days (168 hours) at 25 °C in darkness, maintaining sterility throughout the assay. Optical density (OD) readings at 590 nm were recorded immediately (Hybrid Multimode BioTekTM Synergy H1) after inoculation and every 24 hours thereafter.

Carbon source utilization rates were determined using a Synergy H1 microplate reader (BIO-TEK, Dallas, TX, USA) and analyzed with Gen5™ (v. 2.0) software. The assessment was based on the reduction of the tetrazolium violet redox indicator, following the methodology described by Pohland and Owen (2009).

2.5. Statistics and Graphic Representation

Dose-response curves for *D. magna* immobilization and *A. fischeri* bioluminescence were constructed using a logit logistic regression analysis, conducted using XLSTAT software (version 2014.5.03, Addinsoft 2024). This methodology also provided the EC_{50} values, along with their respective standard errors (SE). The statistical significance of the dose-response models was assessed using a chi-squared test.

The quantification of microbial activity for each Biolog EcoPlate® was performed using the Average Well Color Development (AWCD) method, following the procedures established by Garland and Mills, (1991) and referenced in previous studies (Pino-Otín et al., 2021). The results were visually represented using appropriate visualization techniques, and AWCD was calculated using Equation (1):

$$AWCD = \sum_{i=0}^{i=7}(OD_{t=xi} - OD_{t=x0}) \quad \text{Eq (1)}$$

Where ODi represents the optical density value from each well at a given time after subtracting the initial optical density $OD_{t=x0}$ from the optical density at that point ($OD_{t=xi}$) of that well. AWCD values among the three replicates were compared, and statistical significance was determined using a Student's T-test for two independent samples, conducted with XLSTAT software (version 2014.5.03). The coefficient of variation (CV) was used to assess the relative dispersion of absorbance data across the three replicates.

Subsequently, AWCD curves were fitted to a logistic model (Equation 2) to describe sigmoid microbial growth, as detailed in previous studies (Pino-Otin et al., 2019), using the Excel Solver (Microsoft 365) add-in:

$$AWCD = \frac{Cmax}{1+e^{b-rt}} \quad \text{(Eq. 2)}$$

where C_{max} represents the maximum attainable population density, *r* denotes the intrinsic rate of population growth, and *b* serves as a fitting parameter within the model.

3. Results

3.1. *D. magna* immobilization assay and *A. fischeri* bioluminescence assay

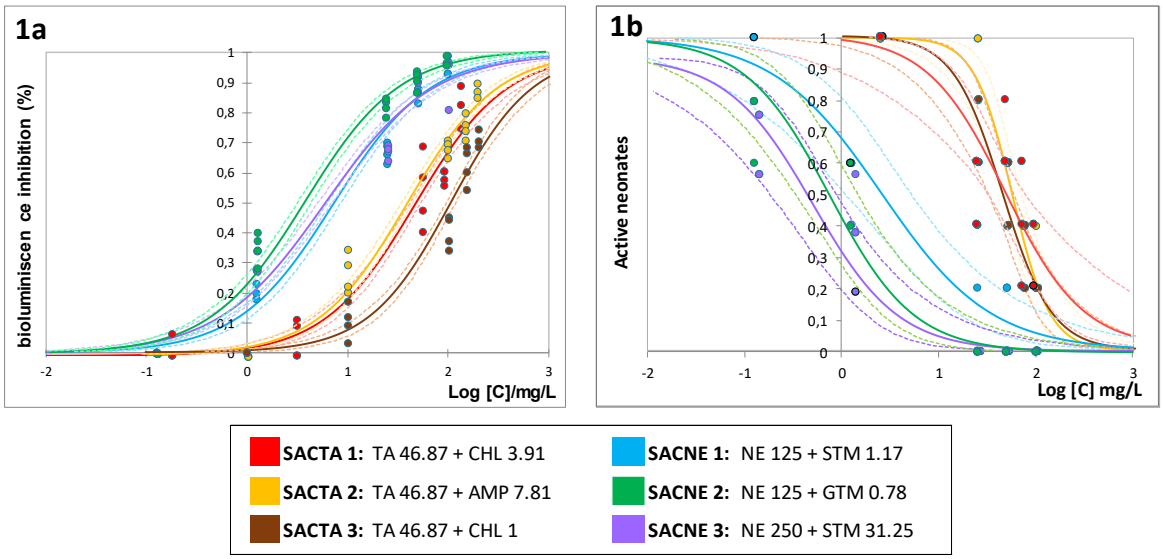

Figure 1. Dose-response curve for Daphnia magna (1a) after 48h of exposure and for Aliivibrio fischeri after 30 min (1b) of exposure to the six synergic combinations. Concentrations expressed in µg/mL. SACNE: Synergic Antimicrobial Combination of Nerol. SACTA: Synergic Antimicrobial Combination of Tannic Acid

EC_{50} values and toxicity curves were calculated as a percentage of the synergy concentration (SACTA or SACNE). These values and their confidence intervals are gathered in table 3 and can be compared in figure 1a (*D. magna)* and 1b (*A. fischeri)*.

For *D. magna* immobilization assay, EC_{50} values for NE combinations were 7.85% (3.31% – 16.10%) for SACNE 1, 3.70% (1.82% - 6.80%) for SACNE 2 and 0.43% (0.15% – 0.91%) for SACNE 3. For TA combinations, EC_{50} values were 87.15% (69.35% – 135.56%) for SACTA 1, 69.99% (59.92% – 82.33%) for SACTA 2 and 44.61% (24.39% – 82.31%) for SACNE 3.

For *A. fischeri*, on the other hand, EC_{50} values for NE combinations were 7.39% (6.31% – 8.58%) for SACNE 1, 3.76% (3.19% – 4.39%) for SACNE 2 and 5.84% (4.95% – 6.86%) for SACNE 3. For TA combinations, EC_{50} were 86.80% (78.27% – 95,86%) for SACTA 1, 38.76% (33.97% – 43.96%) for SACTA 2 and 106.30% (95.48% – 118.44%) for SACTA 3.

Concentration of the components of each synergic combination are shown in table 3:

Combination	EC_{50} *Daphnia magna* 24h (µg/mL)	EC_{50} *Aliivibrio fischeri* 30min (µg/mL)
SACNE 1	**9.81 (NE)** (4.14–20.12) + **0.09 (STM)** (0.04–0.19)	**9.24 (NE)** (7.89 – 10.74) + **0.09 (STM)** (0.07 – 0.10)
SACNE 2	**4.63 (NE)** (2.27–8.50) + **0.03 (GTM)** (0.01–0.05)	**4.70 (NE)** (2.84 – 5.50) + **0.03 (GTM)** (0.02 -0.04)
SACNE 3	**1.07 (NE)** (0.37-2.27) + **0.07 (STM)** (0.02-0.14)	**14.6 (NE)** (12.37 – 17.15) + **1.82 (STM)** (1.55 – 2.14)
SACTA 1	**40.85 (TA)** (32.51–63.54) + **3.41 (CHL)** (2.71–5.30)	**40.68 (TA)** (36.68 -44.92) + **3.39 (CHL)** (3.06 – 3.75)
SACTA 2	**32.80 (TA)** (28.08–38.43) + **5.47 (AMP)** (4.68-6.43)	**18.17 (TA)** (15.92 – 20.60) **+ 3.02 (AMP)** (2.65 – 3.43)
SACTA 3	**18.12 (TA)** (9.91–33.43) + **0.45 (CHL)** (0.24–0.92)	**43.17 (TA)** (38.82 – 48.11) **+ 1.06 (CHL)** (0.95 – 1.18)

Table 3. Concentrations of each component of the synergy with its confidence interval at the EC_{50} obtained in the *D. magna* and *A. fischeri* tests. Concentrations are expressed in µg/mL. (1) **SACNE**: Synergic Antimicrobial Combination of **Nerol.** (2) **SACTA**: Synergic Antimicrobial Combination of **Tannic Acid.** (3) **NE:** Nerol. (4) **TA:** Tannic Acid. (5) **STM:** Streptomycin. (6) **CHL:** Chloramphenicol. (7) **GTM:** Gentamicin. (8) **ERY:** Erythromycin.

3.2 Genetic Analysis of River Microbial Populations

The taxonomic classification of the microorganisms present in the river samples was carried out with the phylogenetic analysis of the 16S rRNA gene sequences, as illustrated in Figure 2, depicting the top taxa abundance of different taxonomic levels. The identification level is always above 90% until the species level, where it drops to 64.72%.

At the kingdom level, most of the microbe population was classified as bacteria (99.92%), the rest remaining unidentified. The dominant phyla were Proteobacteria (43.62%), Bacteroidetes (currently known as Bacteroidota) (31.90%) and Actinobacteria (currently known as Actinomycetota) (18.65%). Below 2% are Cyanobacteria, Firmicutes (currently known as Bacillota), Verrucomicrobia (currently known as Verrucomicrobiota) and Planctomycetes. 5.84% remained unidentified.

Among Proteobacteria, the main class was found to be Betaproetobacteria (26.92%), followed by Alphaproteobacteria (8.43%) and Gammaproteobacteria (5.19%). Flavobacteriia (19.52%) Cytophagia (5.42%) and Sphingobacteriia (6.47%) were the principal classes Bacteroidetes phylum, and Actinobacteria class the only one found (18.65%) for actinobacteria phylum. 9.40% remained unidentified.

The main order found in the Betaproteobacteria was Burkholderiales (25.07%). For Flavobacteriia, every read was classified as Flavobacteriales Order (19.52%), and the majority of the Actinobacteria Class was found to be Actinomycetales (18.15%). Shpingobacteriales belonging to the Sphingobacteriia was the other main order classified (6.47% of total reads). 25.36% of the sample remained unidentified.

For Betaproteobacteria, the main Family found was Comamonadaceae (82.71% of all Burkholderiales) and the Genus Limnohabitans (82.74% of all Comamonadaceae). Of this Genus, the main species found was *Limnohabitans parvus* (12.82% of total reads).

Of all Flavobacteriales Order, the main Family (16.93%) was Flavobacteriaceae, with 98.94% of those reads classified in the Flavobacterium family. However, no species were successfully classified in this Order. For the Order Actimomycetales, the main Family was found to be Microbacteriaceae (11.22%). 35.87% remained unidentified.

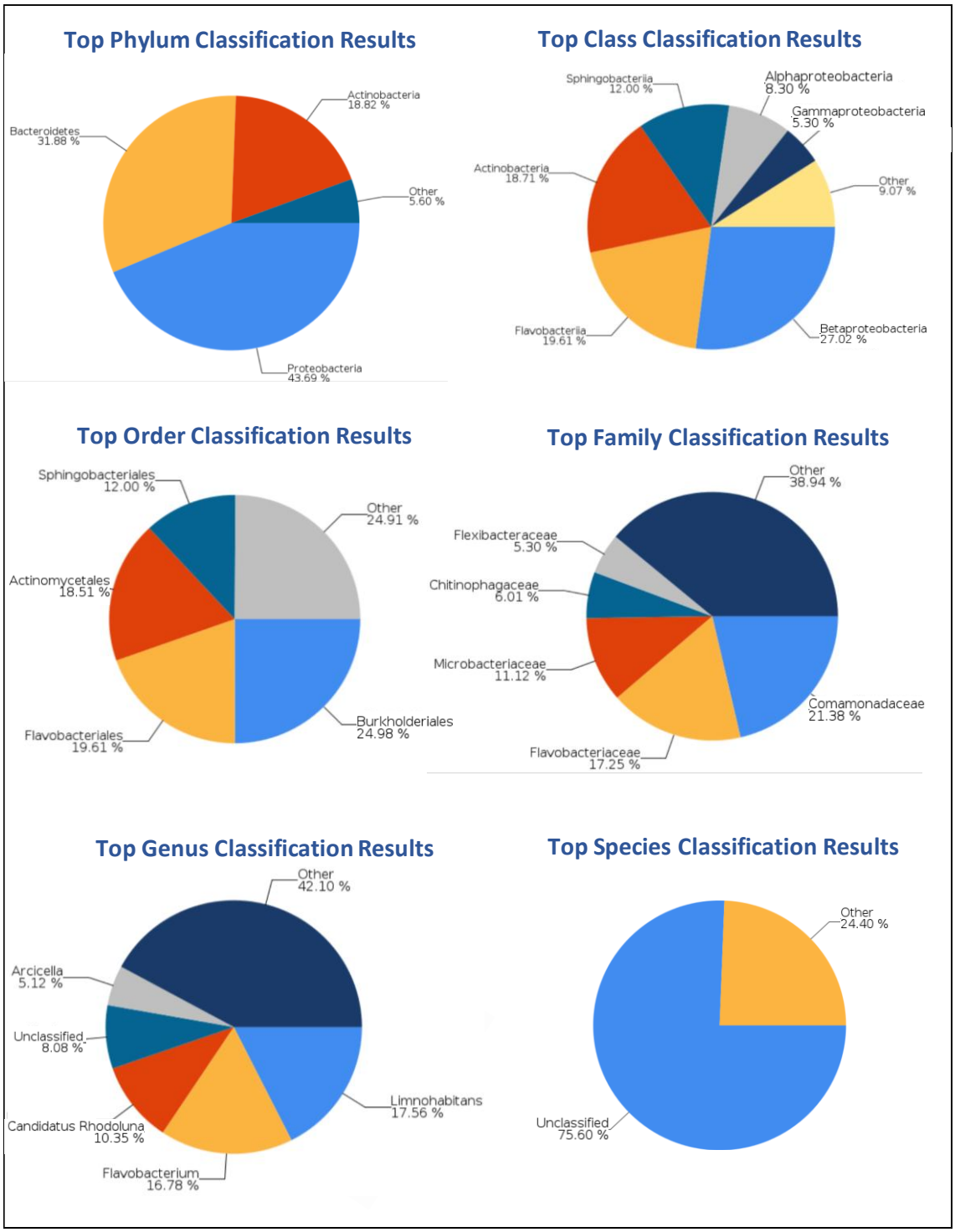

Figure 2. Taxonomic composition of a river bacterial community at multiple hierarchical levels, including kingdom, phylum, class, order, family, genus, and species, represented by relative abundance pie charts. Major groups and predominant taxa are shown for each classification level.

3.3. Average Well Color Development (AWCD) of Microbial Populations and Community-Level Physiological Profiling (CLPP)

In this section, figures 3 to 8 display the AWCD (Average Well Color Development) of the SACTAs and SACNES, their components at synergy concentration, the components at their MIC concentration, and a control. The parameters C_{max} (maximum AWCD value reached by each treatment during the experiment) and r (rate at which the AWCD value increases over time), the percentage decrease compared to the control, and the percentage comparison between the effect of the synergistic combination and the effect of the individual compounds are also shown. The metabolic profiles of the synergy, the components of the synergy at the concentration found in the synergy, and the components of the synergy at MIC concentration are also included.

AWCD curves for SACNE 1, showed in figure 3, and its individual constituents indicate a consistent positive deviation with respect to the control, reflecting increased bacterial metabolic activity in exposed samples. Only NE at MIC (500 µg/mL) shows marked growth inhibition compared to the control. At subinhibitory concentrations, STM and NE both stimulate metabolic growth above control levels, as evident from higher AWCD and C_{max} measurements, pointing to a stimulatory effect or lack of toxicity at these concentrations. The C_{max} for SACNE 1 reaches 1.09, exceeding the control value of 0.84. The combination exhibits 31% less inhibition of bacterial growth compared to control. If we compare the deviations from the control of MIC STM vs SACNE 1, bacterial growth is 26% lower for the synergic combination. Throughout the first 48 h, metabolic inhibition is observed, after which the metabolic capacity for carbohydrates, amino acids, carboxylic acids, and ketonic acids increases. NE at MIC remains the only condition with persistent metabolic inhibition beyond 48 h.

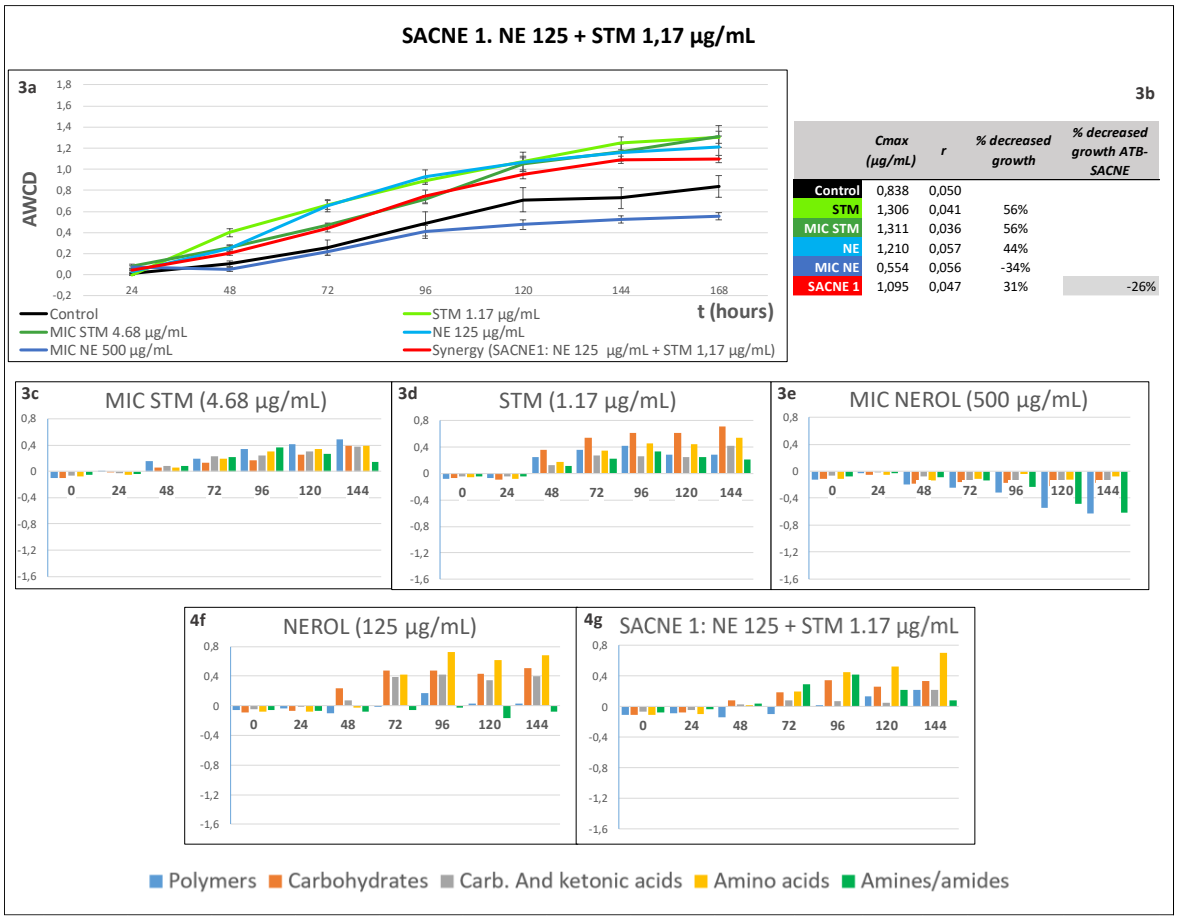

Figure 3. Metabolic activity and functional group utilization of bacterial communities exposed to different antimicrobial treatments. (a) AWCD (Average Well Color Development) curves represent overall metabolic activity for control, STM (streptomycin) at 1.17 µg/mL, MIC STM (Minimum Inhibitory Concentration, 4.68 µg/mL), NE (nerol) at 125 µg/mL, MIC NE (Minimum Inhibitory Concentration, 500 µg/mL), and the synergistic combination SACNE 1 (NE 125 µg/mL + STM 1.17 µg/mL). (b) Table with C_{max} (maximum AWCD reached), r (growth rate), percentage decreased growth compared to the control, and difference in the percentage decrease of the C_{max} of the antibiotic alone compared to the SACNE combination. (c–g) Functional group metabolic profiles over time (0-144 h) for polymers, carbohydrates, carboxylic and ketonic acids, amino acids, and amines/amides. The X-axis represents time (hours), and the Y-axis shows the deviation from the control.

AWCD curves of SACNE 2, shown in figure 4, indicated that only the GTM at synergy concentration 0.78 µg/mL stimulates bacterial metabolic activity above the control, as reflected by a higher C_{max} value (1.07 vs. 0.84 for control). NE at MIC concentration generates almost complete inhibition with a C_{max} of 0.06, while the rest of the tests show a C_{max} in the range of 0.46 - 0.55. When comparing the MIC of the ATB with SACNE 2, they show similar inhibition, with their C_{max} differing by only 10%. Within the metabolic profile, inhibition of all metabolic groups is observed, except for NE at a synergistic concentration, where amino acids, carbohydrates, and carboxylic and ketonic acids are the main metabolised groups.

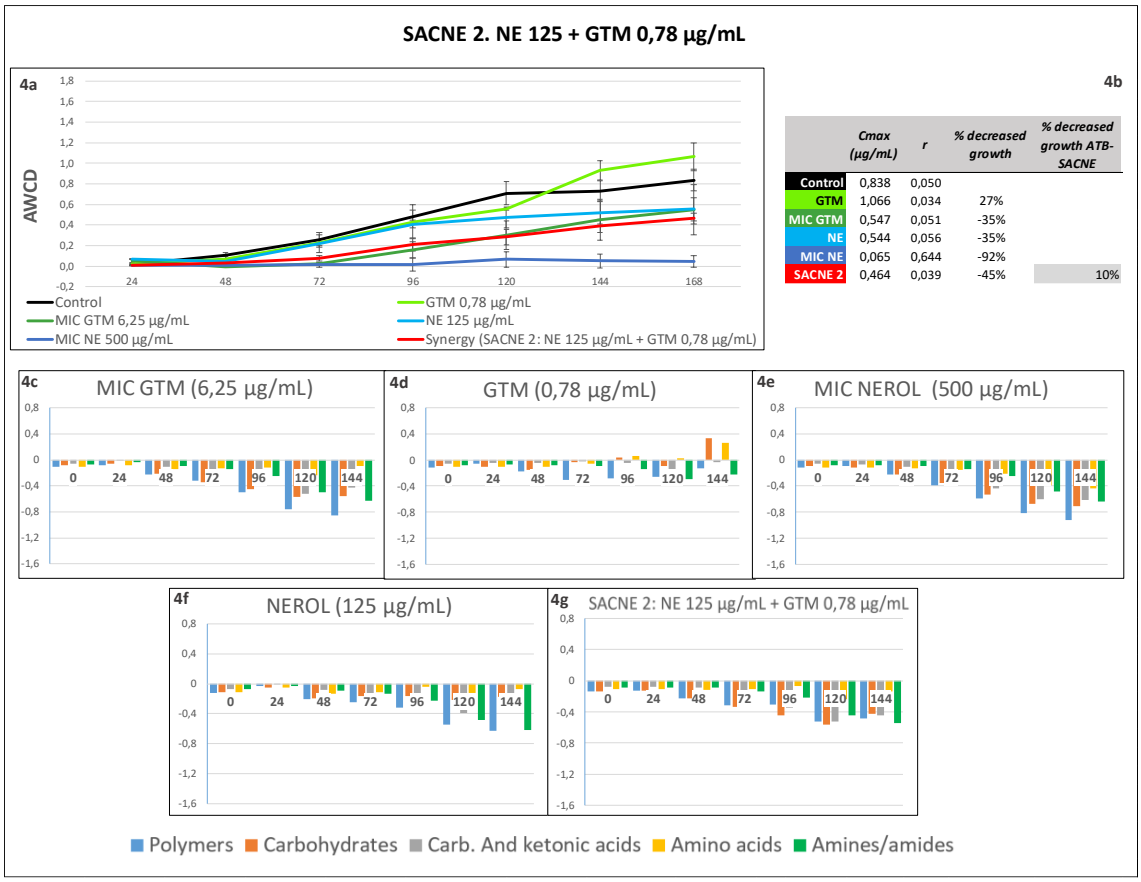

Figure 4. Metabolic activity and functional group profiling of bacterial communities exposed to different antimicrobial treatments. (a) AWCD (Average Well Color Development) curves represent overall metabolic activity for control, GTM (gentamicin) at 0.78 µg/mL, MIC GTM (Minimum Inhibitory Concentration, 6.25 µg/mL), NE (nerol) at 125 µg/mL, MIC NE (Minimum Inhibitory Concentration, 500 µg/mL), and the synergistic combination SACNE 2 (NE 125 µg/mL + GTM 0.78 µg/mL). (b) Table with C_{max} (maximum AWCD reached), r (growth rate), percentage decreased growth compared to the control, and difference in the percentage decrease of the C_{max} of the antibiotic alone compared to the SACNE combination. (c–g) Functional group metabolic profiles (polymers, carbohydrates, carboxylic and ketonic acids, amino acids, amines/amides) over time (0-144 h) for each treatment. The X-axis represents time (hours), and the Y-axis shows the deviation from the control.

AWCD curves shown in figure 5 indicate that only STM at MIC concentrations might present a slightly increased bacterial growth than the control. Furthermore, SACNE 3 presents a C_{max} close to the control and 31% higher than the MIC of STM at MIC concentration. NE at its MIC concentration achieves complete inhibition of bacterial growth. The metabolic profile shows complete inhibition of the metabolism of all functional groups except SACNE 3, where both amino acids and amines/amides are metabolised.

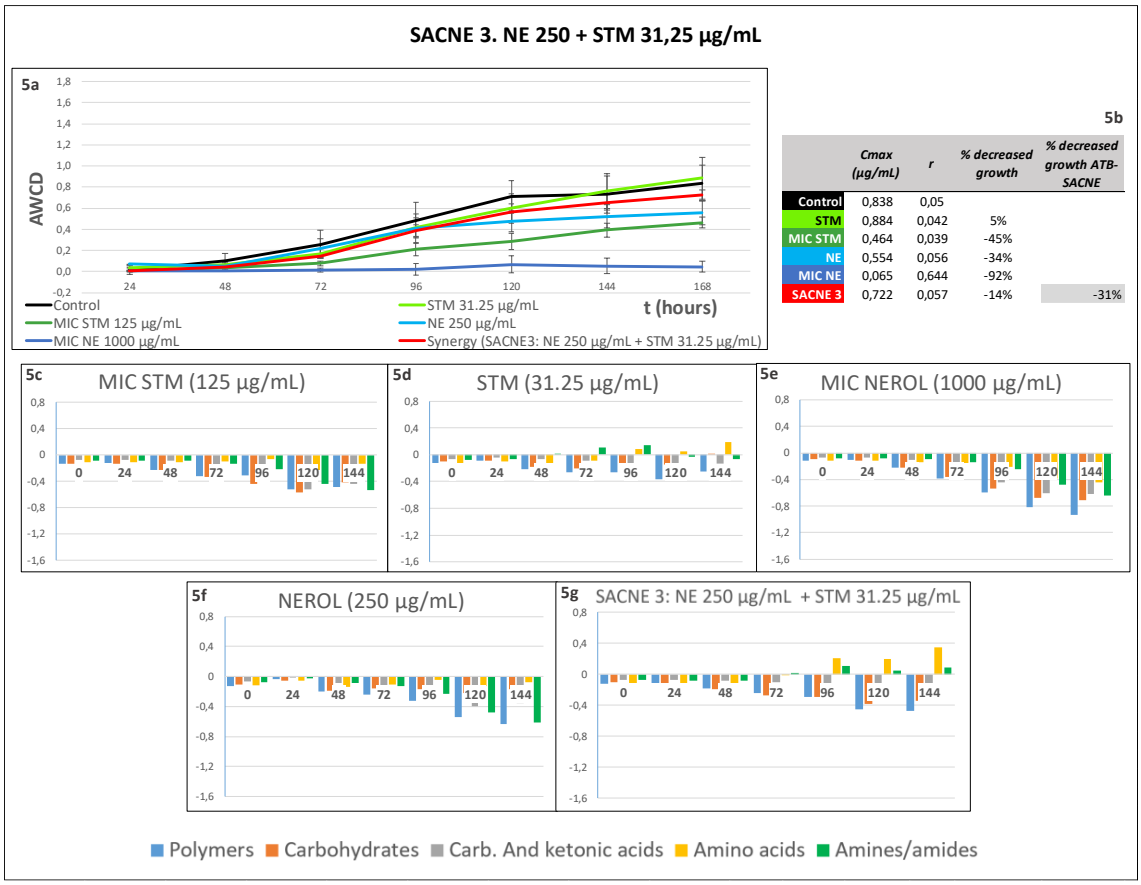

Figure 5. Metabolic activity and functional group utilization of bacterial communities exposed to different antimicrobial treatments. (a) AWCD (Average Well Color Development) curves represent overall metabolic activity for control, STM (streptomycin) at 31.25 µg/mL, MIC STM (Minimum Inhibitory Concentration, 125 µg/mL), NE (nerol) at 250 µg/mL, MIC NE (Minimum Inhibitory Concentration, 1000 µg/mL), and the synergistic combination SACNE 3 (NE 250 µg/mL + STM 31.25 µg/mL). (b) Table with C_{max} (maximum AWCD reached), r (growth rate), percentage decreased growth compared to the control, and difference in the percentage decrease of the C_{max} of the antibiotic alone compared to the SACNE combination. (c–g) Functional group metabolic profiles over time (0-144 h) for polymers, carbohydrates, carboxylic and ketonic acids, amino acids, and amines/amides. The X-axis represents time (hours), and the Y-axis shows the deviation from the control.

Figure 6 shows AWCD curves where TA at synergistic concentration has a much higher AWCD than the control with a C_{max} of 1.45, while CHL at its MIC concentration has an AWCD very similar to the control (1.07 vs 1.10). SACTA 1 has an AWCD 32% higher than the MIC of CHL, while the lowest C_{max} values are found in TA and CHL at their MIC concentration, with C_{max} values ranging from 0.28 to 0.40. Within the metabolic profile, the

inhibition of functional groups is complete in all cases except for TA at the synergy concentration, where the most metabolised groups are amino acids and polymers.

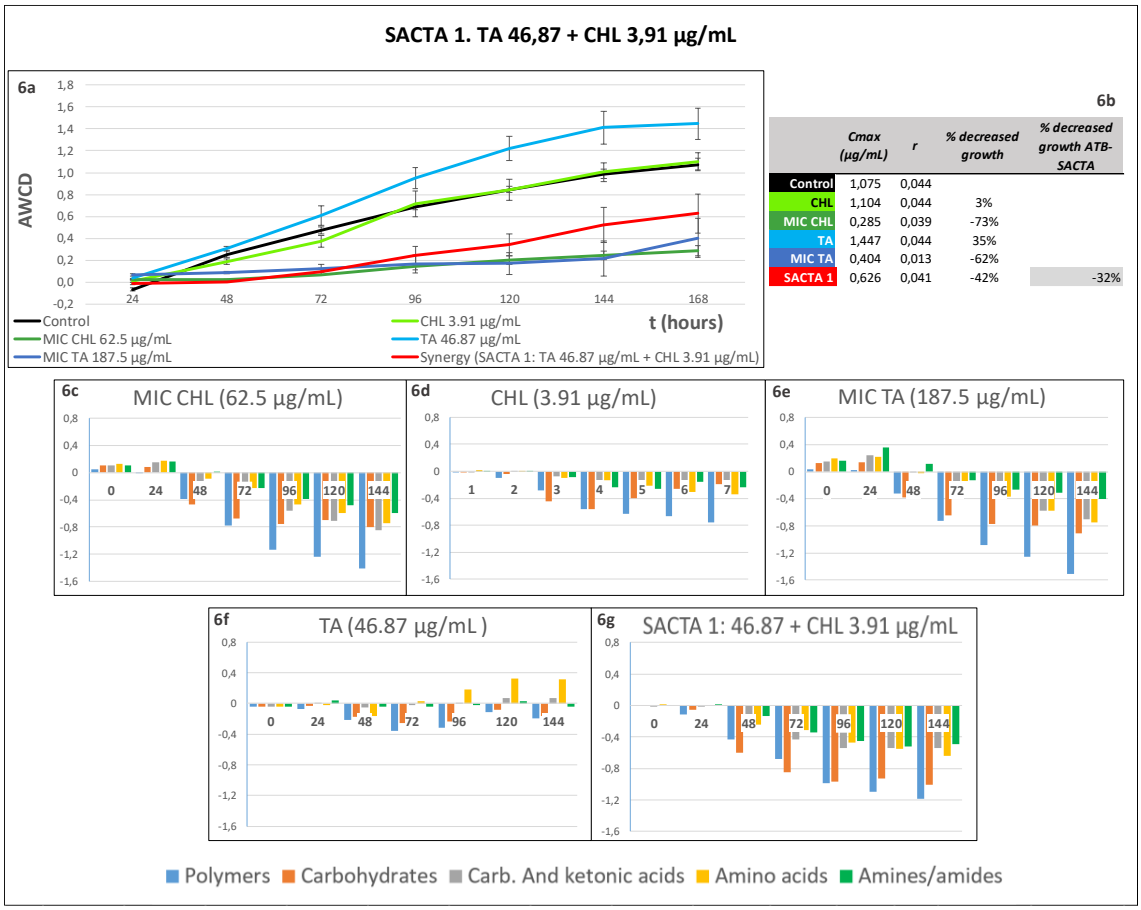

Figure 6. Metabolic activity and functional group utilization of bacterial communities exposed to different antimicrobial treatments. (a) AWCD (Average Well Color Development) curves represent overall metabolic activity for control, TA (Tannic Acid) at 46.87 µg/mL, CHL (chloramphenicol) at 3.91 µg/mL, MIC CHL (Minimum Inhibitory Concentration, 62.5 µg/mL), MIC TA (Minimum Inhibitory Concentration, 187.5 µg/mL), and the combination SACTA 1 (TA 46.87 µg/mL + CHL 3.91 µg/mL). (b) Table with C_{max} (maximum AWCD reached), r (growth rate), percentage decreased growth compared to the control, and difference in the percentage decrease of the C_{max} of the antibiotic alone compared to the SACTA combination. (c–g) Functional group metabolic profiles over time (0-144 h) for polymers, carbohydrates, carboxylic and ketonic acids, amino acids, and amines/amides. The X-axis represents time (hours), and the Y-axis shows the deviation from the control.

Figure 7 shows that both AMP and TA have a higher AWCD than the control, with C_{max} of 1.17 and 1.15, respectively. On the other hand, SACTA 2, TA and AMP at MIC concentrations show similar inhibition of bacterial growth (respective C_{max} values: 0.37, 0.40 and 0.20). When comparing the differences with respect to the ATB control at its MIC concentration with respect to SACTA 2, they show a similar effect, differing by 16%.

The metabolic profile of SACTA 2 shows complete inhibition of all functional groups after 96 hours. TA inhibits the metabolism of all functional groups except amino acids. AMP at synergistic concentrations completely inhibits metabolism. The MIC concentrations of the SACTA components have a hormetic effect, which is complete for AMP but disappears after 96 hours for TA.

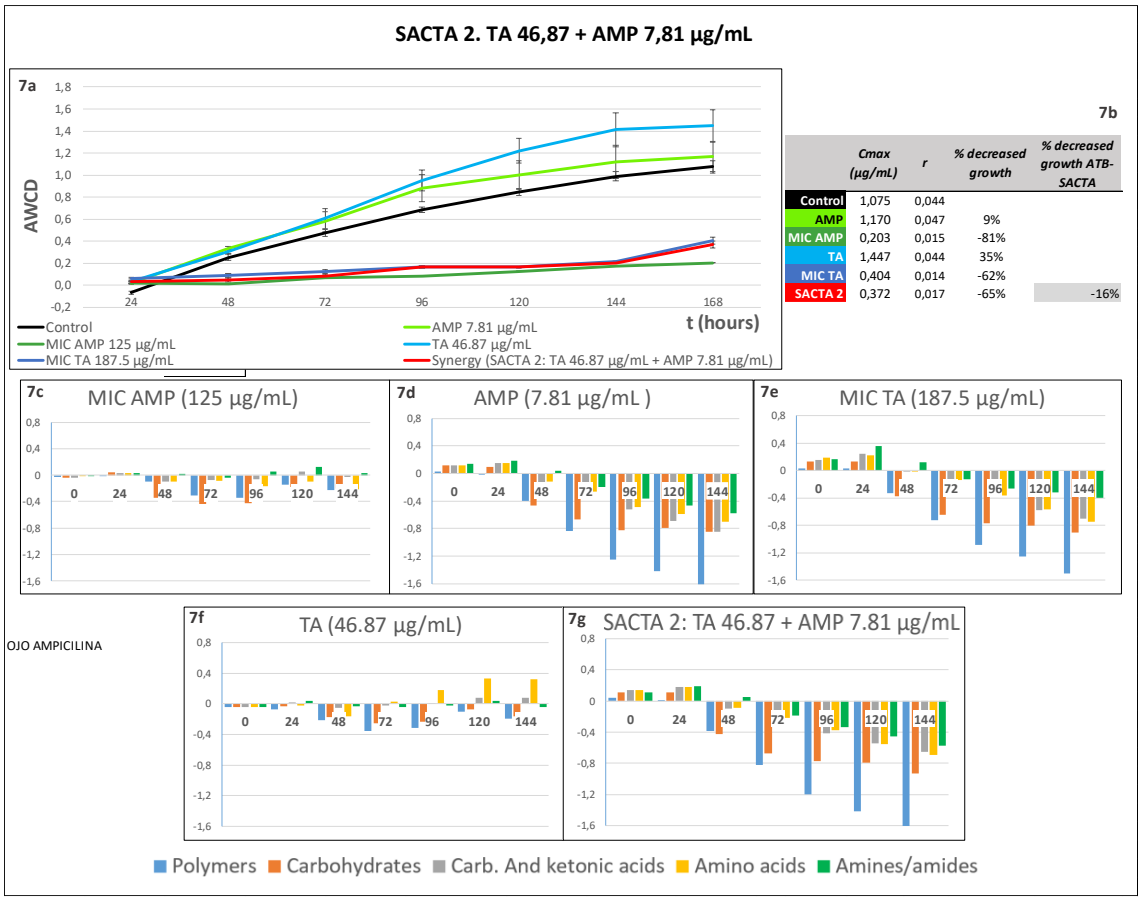

Figure 7. Metabolic activity and functional group utilization of bacterial communities exposed to different antimicrobial treatments. (a) AWCD (Average Well Color Development) curves represent overall metabolic activity for control, AMP (ampicillin) at 7.81 µg/mL, MIC AMP (Minimum Inhibitory Concentration, 125 µg/mL), TA (tannic acid) at 46.87 µg/mL, MIC TA (Minimum Inhibitory Concentration, 187.5 µg/mL), and the combination SACTA 2 (TA 46.87 µg/mL + AMP 7.81 µg/mL). (b) Table with C_{max} (maximum AWCD reached), r (growth rate), percentage decreased growth compared to the control, and difference in the percentage decrease of the C_{max} of the antibiotic alone compared to the SACTA combination. (c–g) Functional group metabolic profiles over time (0-144 h) for polymers, carbohydrates, carboxylic and ketonic acids, amino acids, and amines/amides. The X-axis represents time (hours), and the Y-axis shows the deviation from the control.

Figure 8 shows the effect of SACTA 3, which exhibits the greatest inhibition of all combinations, with a 73% inhibition compared to the control, even exceeding CHL at its MIC concentration by 71%. TA and CHL at their synergistic concentration have a hormetic effect, metabolising all functional groups in experiment with TA, while CHL only slightly inhibits amino acid metabolism. At MIC concentration, TA inhibits the metabolism of all

functional groups after 96 hours, while CHL has a slight hormetic effect on polymers, carbohydrates, and carboxylic and ketonic acids.

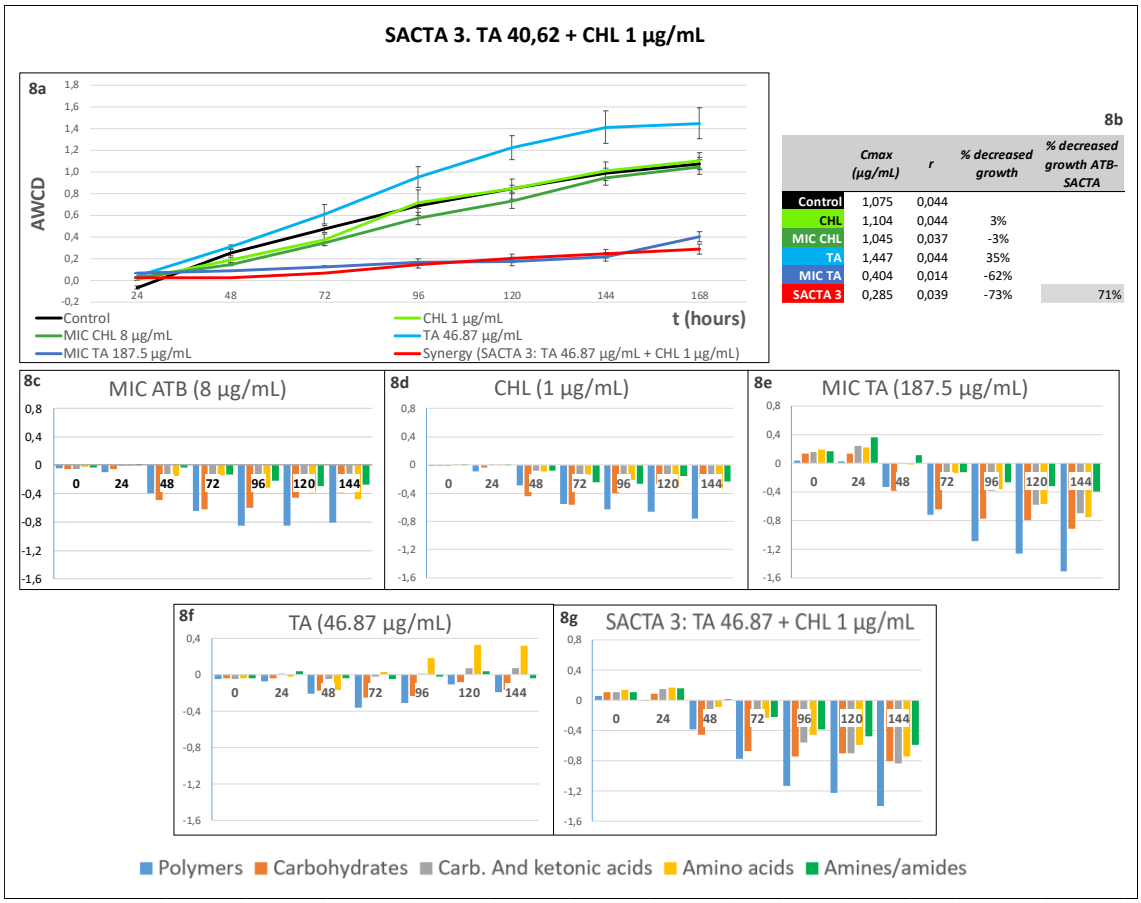

Figure 8. Metabolic activity and functional group utilization of bacterial communities exposed to different antimicrobial treatments. (a) AWCD (Average Well Color Development) curves display overall metabolic activity for control, CHL (chloramphenicol) at 1 µg/mL, MIC CHL (Minimum Inhibitory Concentration, 8 µg/mL), TA (tannic acid) at 46.87 µg/mL, MIC TA (Minimum Inhibitory Concentration, 187.5 µg/mL), and the combination SACTA 3 (TA 46.87 µg/mL + CHL 1 µg/mL). (b) Table with Cmax (maximum AWCD reached), r (growth rate), percentage decreased growth compared to the control, and difference in the percentage decrease of the Cmax of the antibiotic alone compared to the SACTA combination. (c–g) Functional group metabolic profiles over time (0-144 h) for polymers, carbohydrates, carboxylic and ketonic acids, amino acids, and amines/amides. The X-axis represents time (hours), and the Y-axis shows the deviation from the control.

In summary, of all the combinations studied, SACTA 3 has the greatest inhibitory effect, which is also greater than the MIC of the ATB in its combination (CHL). The combinations that have a similar effect to the ATB in their combination are SACNE 2 and SACTA 2, which differ from the control by only 10% and 16%, respectively. On the other hand, SACNE 1 shows a clear hormetic effect with bacterial growth above the control, and finally, SACNE 3 and SACTA 1 show higher growth than their ATBs at MIC concentration, but lower than the control sample.

4. Discussion

This study shows the impact of synergistic combinations of the natural products NE and TA with commercial antibiotics STM, GTM, CHL, and AMP. SACNEs show greater toxicity to *D. magna* than NE alone. When compared with the antibiotics involved, in synergy, a much lower concentration of antibiotics is needed to reach the EC_{50}. Looking at the results for *A. fischeri*, if we compare the EC_{50} values, we see that SACNE 3 was found to be significantly less toxic than NE, SACNE 2 was slightly more toxic, and SACNE 1 didn't show significant variations with respect to NE. Regarding ATBs, much lower concentrations were also required to achieve the EC_{50} than individual ATBs.

About SACTA, in D. magna, SACTA1 showed greater toxicity than TA, SACTA 2 showed similar toxicity, and SACTA 3 showed lower toxicity. In A. fischeri, when compared to TA, SACTA 1 and SACTA 3 have a lower TA concentration than their individual EC_{50}, while SACTA 2 has a similar concentration. The concentrations present in the synergies are also much lower in this case.

When observing the metabolic profile, a hormetic effect is observed for SACNE, while the other synergies show inhibition compared to the control. If we compare the synergies with their antibiotic at therapeutic concentration, we observe that SACNE 2 shows similar inhibition, SACNE 3, SACTA1 and SACTA 2 show lower inhibition ranging from 16-32%, and SACTA 3 shows 71% greater inhibition than its ATB. These essays provide, once again (Pino-Otín et al., 2023b, Lorca et al., 2025, submitted), a more comprehensive perspective than isolated bioindicators.

4.1 Comparative Results: Individual Organisms versus Microbial Communities

Therefore, synergistic combinations of ATBs with NP as NE and TA significantly reduce the concentrations of both compounds required to achieve toxicity endpoints, particularly in the case of ATBs. This effect underscores the enhancing role of NPs, facilitating antimicrobial action at lower doses while raising important questions about ecotoxicological safety across different biological levels—from individual bioindicators to complex microbial communities. Markedly different patterns emerging between individual bioindicators and complex microbial communities. In individual organism assays, NE-based synergies (SACNE) consistently exhibited higher toxicity than TA-based formulations (SACTA) across both test species. For *D. magna*, the toxicity ranking was SACNE 3 > SACNE 2 > SACNE 1 > SACTA 3 > SACTA 2 > SACTA 1, while for *A. fischeri* it was SACNE 2 > SACNE 3 > SACNE 1 > SACTA 2 > SACTA 1 > SACTA 3. These patterns align with the individual ecotoxicity profiles of the NPs: NE demonstrates clear toxicity to *A. fischeri* (EC_{50} lower than 100 mg/L), but a slight toxicity to *D. magna*, whereas TA is classified as slightly toxic (EC_{50} between 10 and 100 mg/L) in both organisms (ECHA, 2008).

However, when these findings are compared with responses of riverine bacterial communities, a contrast emerges. All SACTA combinations produced marked suppression of microbial population growth and metabolic capacity, particularly affecting polymer and carbohydrate utilization, whereas SACNE formulations exerted milder effects. Notably, SACNE 1 exhibited hormetic responses—approximately 31% higher maximum growth (C_{max}) and enhanced amino acid metabolism compared to controls (Figure 3). This apparent paradox—where NE is more toxic to individual organisms, but TA is more disruptive to communities—reflects fundamental differences in mechanisms of action and collective microbial defense strategies.

Although synergistic mixtures influenced growth dynamics and metabolic profiles of riverine communities, their overall impact appears to be lower than corresponding ATBs at MIC. Beyond SACNE 1, the combinations SACNE 3, SACTA 1, and SACTA 2 (containing STM, CHL, and AMP, respectively) caused measurable reductions in growth and carbon source utilization, but these decreases represented only 16–32% less inhibition compared with ATB alone at MIC. SACNE 2 displayed toxicity comparable to its MIC control, while SACTA 3 (TA 40.62 + CHL 1) exhibited markedly higher toxicity—approximately 71% greater inhibition than the ATB control.

4.2 Mechanisms of Action Explaining Differential Toxicity

The mechanistic basis for these differential effects can be attributed to the distinct physicochemical properties and modes of action of NE, TA, and the ATBs employed in these combinations. NE's amphiphilic nature and low molecular mass enable effective membrane penetration in isolated cells. In *D. magna*, particularly in SACNE 3 (NE 250 mg/L + STM 31.25 mg/L), NE may act directly on cell membranes, altering fluidity and permeability, facilitating ATB penetration through chitinous exoskeletons and gill membranes, and interfering with respiratory and osmoregulatory processes (Pino-Otín et al., 2022). Due to its low molecular weight solubility, NE accumulates at the air-water interface where *D. magna* filters food, increasing uptake and affecting mobility. STM, even at low doses, enhances this effect by inducing severe oxidative stress (Qian et al., 2012; Singh et al., 2015). In SACNE 2 (NE 125 mg/L + GTM 0.78 mg/L) and SACNE 1 (NE 125 mg/L + STM 1.17 mg/L), the membranolytic effect of NE persists, albeit with less intensity, and GTM and STM maintain their ability to induce cellular stress.

In *A. fischeri*, NE may cross outer membrane porins efficiently due to its amphiphilic nature and low molecular mass (Prajapati et al., 2021; Nikaido, 2003). Once in the periplasmic space, it can disrupt both outer and cytoplasmic membranes, compromising the integrity of the double membrane characteristic of Gram-negative bacteria (Guimarães et al., 2019; Kasthuri et al., 2022). In SACNE 2, GTM—facilitated by NE-induced permeability may increase—penetrate more efficiently and bind to the 30S ribosomal subunit, causing lethal errors in protein synthesis. This synergy is particularly potent. In SACNE 3, the membranolytic effect of NE could be so intense that it compromises cell viability before

STM reaches its site of action, while in SACNE 1 the balanced relationship between membrane damage and ribosomal inhibition allows for more efficient synergy.

In contrast, TA acts primarily through chelation of essential metal ions (Ca^{2+}, Mg^{2+}, Fe^{3+}), disrupting enzymatic processes and ionic homeostasis (Chen et al., 2019; Song et al., 2019). Its larger molecular size limits direct penetration in individual organisms, resulting in more gradual effects through protein precipitation and membrane alteration. In *D. magna*, TA's astringent nature affects filtering and digestive structures, inducing membrane disruption and oxidative stress, though the complementary effect of ATBs (CHL and AMP) remains limited, probably due to metabolic stress. Therefore, NE appears to exert direct membranolytic action and has greater bioavailability, making it more toxic to *D. magna* than TA, whose mechanism is more indirect and gradual.

In *A. fischeri*, TA's reduced capacity to pass through porins restricts it mainly to chelation of cations (Mg^{2+}, Ca^{2+}) in the periplasmic space and precipitation of membrane proteins, gradually altering permeability (Song et al., 2019). ATBs (AMP and CHL) encounter greater resistance to penetrating the partially altered outer membrane, which reduces overall toxicity. The internal toxicity order among SACTAs (SACTA 2 > SACTA 1 > SACTA 3) likely reflects differences in relative ATB doses and the ability of AMP and CHL to exploit TA-induced alterations (Figures 6 – 8).

However, within complex microbial communities, TA's metal-chelating activity probably interferes with nutrient exchange and symbiotic interactions, triggering cascading ecological effects across microbial trophic networks that compromise both structural integrity and functional stability (Huang et al., 2024; Lim et al., 2013). While NE's direct membranolytic mechanism is highly effective against isolated cells, its impact appears mitigated within structured biofilm or planktonic communities, where cell aggregation provides protection against external stressors. Microbial communities exhibit intrinsic resistance and resilience mechanisms absent in individual organisms, including cross-resistance development and reliance on detoxification metabolites secreted by neighboring species, generating collective protection against xenobiotics (Adamowicz et al., 2018).

The hormetic response in SACNE 1 likely relates to Proteobacteria taxa capable of metabolizing NE (Madyastha and Renganathan, 1983; Takahashi et al., 2018), supported by NE's high biodegradability (complete degradation within 15–28 days) and susceptibility to ultraviolet radiation and microbial metabolism (Api et al., 2023; Miller and Hawthorne, 2000). Several mechanisms explain the generally reduced community impact of synergies. Sub-MIC ATB concentrations impose lower degradation loads, allowing gradual replacement of sensitive taxa while maintaining functional diversity. Rapid biodegradation of coadjuvants can contribute to transient stress responses rather than full-scale community collapse. Tannins undergo slow degradation under natural conditions (Qiao et al., 2021), though temperature and light exposure accelerate this significantly—27% reduction over 14 days at 29°C versus 24°C (Earl et al., 2025) and up to 30% degradation after simulated 25-day solar exposure (Maie et al., 2008). Several bacterial species

metabolize tannic compounds (Chowdhury et al., 2004), potentially explaining lower impacts of synergistic mixtures versus ATBs alone at MIC.

Additionally, sub–MIC ATB levels can exert weaker selective pressure reducing resistant variant emergence (Scalbert, 1991) and helping sustain community growth and diversity essential for collective metabolic functionality (Bozo et al., 2007). In contrast, ATBs at MIC can cause rapid mortality among sensitive species, followed by resistant phenotype dominance occupying vacated niches—a "collapse-and-replacement" dynamic with severe consequences for community activity. Biofilm formation and collective efflux mechanisms protect populations against membranolytic compounds like NE and metal-chelating agents like TA (Mahizan et al., 2019; Ren et al., 2024). These cross-resistance mechanisms effectively lead to a reduction of membrane permeabilization and limit intracellular coadjuvant accumulation, modulating overall synergistic toxicity. Conversely, ATBs at MIC probably maintain sufficiently stable concentrations to overcome these defenses, inhibiting key processes like protein synthesis or cell wall transpeptidation (Czech et al., 2014). Some bacteria can utilize NPs as carbon sources or co-substrates, activating metabolic pathways that simultaneously transform or detoxify ATB components—a co-metabolism phenomenon less likely with ATBs alone at MIC due to absent co-substrates restricting community-level detoxification (Liu et al., 2024).

The pronounced toxicity of SACTA 3 in microbial communities may result from favorable bioavailability-stability balance, avoiding both rapid biodegradation and excessive extracellular protein precipitation (Olchowik-Grabarek et al., 2022), maintaining sustained TA levels enhancing outer and periplasmic membrane permeability. Under such conditions, CHL—a peptide bond synthesis inhibitor—more effectively reaches ribosomal targets, overcoming communal defenses and generating synergistic effects surpassing MIC toxicity (Qin et al., 2023).

4.3 Environmental Safety Assessment of Synergistic Combinations

Despite the increased intrinsic toxicity of synergies in individual bioassays compared to ATBs alone, comprehensive environmental safety assessment reveals highly favorable outcomes. For SACNE formulations on *D. magna*, ATB concentrations at EC_{50} (0.09 – 0.13 mg/L) are drastically reduced compared to individual EC_{50} values (STM: 772 mg/L; GTM: 840 mg/L) yet remain 150 – 870 times below clinical MICs (20 – 35 mg/L for STM; 5 – 10 mg/L for GTM). In *A. fischeri*, SACNE formulations require GTM and STM concentrations of 0.03 – 1.82 mg/L, well below individual EC_{50} values (>5000 mg/L) (Gan et al., 2025, submitted) and 11 – 1000 times lower than therapeutic MICs. The required NE dose (1 – 14.6 mg/L) remains well below its individual EC_{50} and, when combined, the safety margin is further widened while release of synthetic ATBs into the environment is limited.

For SACTA combinations, ATB concentrations in combined EC_{50} (0.45 – 5.47 mg/L for *D. magna*; 1.06 – 3.39 mg/L for *A. fischeri*) remain substantially below therapeutic MICs

(≈100 mg/L for AMP; 25 mg/L for CHL) and individual EC_{50} values (4324 mg/L for AMP; 285.62 mg/L for CHL) (Gan et al., 2025, submitted). Required TA concentrations (18.12 – 43.17 mg/L) remain within or close to its isolated EC_{50} range (32.04 mg/L), suggesting an additive rather than synergistic effect. Overall, these combined concentrations are 8 – 55 times lower than therapeutic peaks of CHL (≤25 mg/L) and 30 – 220 times lower than AMP (≈100 mg/L), indicating that synergies maintain considerable environmental safety margins compared to clinical levels.

The reduction of up to 90% in ATB concentrations minimizes exposure to subinhibitory levels that favor resistant strain selection and horizontal gene transfer. By maintaining bactericidal efficacy with lower doses, total ATB loads released into aquatic or terrestrial environments are reduced, limiting diffuse contamination and exposure of non-target communities to sub-doses that promote adaptive defense mechanisms. These benefits preserve therapeutic efficacy and mitigate resistance proliferation, rendering synergies more sustainable and ecologically safe than ATBs at MIC concentrations without compromising efficacy. Furthermore, the distinct mechanisms of NE and TA—targeting cell membranes and metal chelation pathways rather than well-defined molecular targets in conventional ATBs—further limit resistance development potential (Angelini, 2024).

Regarding environmental risk of NPs, concentrations in synergistic combinations are lower than or comparable to their individual EC_{50} values in water and in all cases well below levels detected in aquatic ecosystems. Environmental degradation enhances safety profiles: NE degrades in river environments in approximately 4.1 days (Miller and Hawthorne, 2000), while TA shows partial degradation of 30% after exposure equivalent to 25 days of sunlight (Maie et al., 2008), with microbial degradation potentially accelerating this process (Chowdhury et al., 2004).

Experiments involving natural fluvial microbial communities offer ecologically relevant complements to findings from single-organism bioassays. Upon exposure to the six synergistic formulations, these communities exhibited moderate declines in bacterial growth and metabolic activity, typically ranging between 10% and 30%, notably lower than inhibitory effects observed for ATBs at MIC concentrations. The only exception was SACTA 3, which produced up to 70% inhibition compared to its MIC concentration ATB. This finding is particularly significant, as complex microbial assemblages better represent natural environmental conditions and incorporate interspecific interactions, collective resistance mechanisms, and biodegradation processes absent in assays with isolated organisms.

The apparent contradiction between higher intrinsic toxicity in individual bioindicators and lower community impact reflects differing test system natures. D. magna and A. fischeri assays capture acute responses in isolated organisms under controlled conditions, whereas microbial communities possess collective resilience mechanisms buffering chemical stress through enzymatic biodegradation by specialized taxa, cross-protection among species, and compensatory replacement of sensitivity by tolerant populations.

To our knowledge, this is the first study evaluating ecotoxicity of ATB-NE and ATB-TA synergies. Converging evidence from individual bioindicators and complex assemblages supports a consistent conclusion: studied synergies are as safe or safer than ATBs at therapeutic MICs. Enhanced safety stems from substantial ATB burden reduction achieving antimicrobial efficacy, NP concentrations within environmentally safe ranges, and lower overall impact on metabolic and structural integrity of microbial communities forming aquatic ecosystem foundations.

Conclusions

In summary, the combined evidence from bioindicator assays and microbial community analyses supports that these synergies offer a balanced compromise between antimicrobial efficacy and ecological compatibility. They markedly reduce the ATB load required for therapeutic activity, maintain environmental concentrations of natural compounds within safe levels, and minimize disruptions to microbial community structure and function. However, the variability observed among combinations underscores the necessity for case-by-case evaluation before implementation, with future research extending investigations to additional environmental matrices and longer exposure periods to fully validate this approach's sustainability. These promising results warrant the advancement of these synergies into preclinical trials aimed at developing formulations that could serve as alternative pharmaceuticals to current commercial antibiotics in both human clinical and veterinary applications.

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4.4.3. Impacto ambiental de las sinergias con ácido tánico y nerol

Las sinergias de NE y AT con ATBs representan una estrategia prometedora que combina eficacia antimicrobiana y seguridad ambiental. Aunque estas combinaciones muestran mayor toxicidad intrínseca sobre *D. magna* y *A. fischeri* que los antibióticos individuales, los PN actúan como coadyuvantes que permiten alcanzar CL_{50} combinadas (0,03 – 5,47 mg/L) entre 100 y 10.000 veces inferiores a las de los ATB por separado. Crucialmente, las concentraciones de ATB necesarias se mantienen muy por debajo de las CMI terapéuticas experimentales (5 – 35 mg/L para aminoglucósidos; 0,6 – 8 mg/L para CHL y AMP), mientras que las dosis de PN (1 – 14,6 mg/L en el caso de NE y 18,12 – 43,17 mg/L en el caso de AT) permanecen cerca de sus CL_{50} individuales. La reducción de hasta un 90% en la dosis de ATB minimiza la presión selectiva sobre bacterias ambientales, reduce la aparición de resistencias y preserva la eficacia terapéutica a largo plazo. Los mecanismos de acción del NE y el AT, que tienen lugar a través de diferentes vías y no sobre dianas más definidas (como los ATB comerciales), va a contribuir también a reducir la aparición de resistencias (Angelini, 2024; Shin et al., 2018).

Los ensayos con comunidades bacterianas del necton fluvial proporcionan una perspectiva ecológicamente más relevante que complementa los resultados obtenidos con bioindicadores individuales. Cuando estas comunidades naturales complejas se exponen a las 6 sinergias estudiadas, se observa una disminución del crecimiento bacteriano y de la capacidad metabólica que oscila en algunos casos entre el 10 y el 30%, significativamente menor que el efecto producido por los ATB a sus concentraciones CMI, exceptuando la SACTA 3 que llega a niveles inhibitorios respeto de la CMI del ATB del 70%. Este hallazgo es particularmente revelador, ya que las comunidades microbianas naturales, como se ha discutido con anterioridad, reflejan mejor las condiciones ambientales reales, incluyendo interacciones interespecíficas, mecanismos de resistencia colectiva y procesos de biodegradación que no se capturan en ensayos con organismos individuales.

La aparente contradicción entre la mayor toxicidad intrínseca observada en bioindicadores individuales y el menor impacto sobre comunidades naturales se explica por las diferencias fundamentales en los sistemas de evaluación. Los ensayos con *D. magna* y *A. fischeri* miden respuestas agudas en organismos aislados bajo condiciones controladas, mientras que las comunidades microbianas poseen mecanismos de resistencia y resiliencia colectiva que les permiten amortiguar los efectos tóxicos. Estos incluyen la biodegradación de compuestos por bacterias especializadas, la protección cruzada entre especies y el reemplazo de especies sensibles frente a otras más resistentes.

En conjunto, la evidencia proveniente tanto de bioindicadores individuales como de comunidades microbianas naturales converge hacia una conclusión consistente: las sinergias estudiadas son igual o más seguras que los ATB utilizados a sus concentraciones CMI terapéuticas. Esta superioridad se fundamenta en 3 pilares: (1) la reducción drástica de la carga antibiótica necesaria, (2) el mantenimiento de concentraciones de PN en las

sinergias dentro de rangos que se muestran como seguros, y (3) el menor impacto sobre la funcionalidad de comunidades microbianas complejas que constituyen la base de los ecosistemas acuáticos.

Por tanto, estas sinergias se posicionan como una alternativa terapéutica ambientalmente responsable que concilia la efectividad antimicrobiana con la preservación de la integridad ecológica, ofreciendo una solución sostenible al dilema entre eficacia clínica y protección ambiental en el contexto de la creciente resistencia antibiótica.

7. CONCLUSIONES

7.1. Conclusiones del objetivo 1

Objetivo 1: determinar la ecotoxicidad de 8 antibióticos de uso común, seleccionados como base para la búsqueda de sinergias con productos naturales, utilizando comunidades microbianas no diana como bioindicadores.

1. El estudio ecotoxicológico de 8 antibióticos comerciales utilizados en la práctica clínica se realizó utilizando comunidades microbianas fluviales como indicadores que permiten obtener una visión ecosistémica más completa. La secuenciación 16S de las muestras permite, además, conocer la estructura de la comunidad microbiana de partida para poder interpretar los cambios metabólicos tras la exposición a los antibióticos.

2. Nuestros resultados demuestran que la presencia de estos 8 antibióticos utilizados en la práctica clínica produce efectos significativos en los ecosistemas fluviales. Incluso a concentraciones de 100 mg/L, se producen en ocasiones inhibiciones casi totales del crecimiento microbiano, y a dosis aún más bajas (0,1 mg/L), se observan alteraciones en la capacidad metabólica microbiana, especialmente en carbohidratos, ácidos carboxílicos y cetónicos, y aminas y amidas.

3. La exposición de las comunidades microbianas a estos antibióticos reflejó reducciones significativas del crecimiento y la actividad metabólica a 100 mg/L para todos los compuestos, especialmente en la utilización de polímeros, carbohidratos y ácidos carboxílicos y cetónicos. Cloranfenicol, eritromicina y gentamicina mostraron la toxicidad más acusada.

4. A concentraciones bajas (0,1 mg/L), algunos antibióticos estimularon levemente el crecimiento y la capacidad metabólica.

5. Las propiedades fisicoquímicas, en particular la lipofilia (log K_{OW}) y la acidez/basicidad (pKa), parecen ser los parámetros que influyen más decisivamente en la biodisponibilidad y toxicidad de estos antibióticos en comunidades microbianas acuáticas.

6. Aunque los efectos de toxicidad aguda son menos probables, el reiterado aporte de antibióticos a los cauces provoca una persistencia continua, lo que unido a las sinergias con otros contaminantes presentes puede desencadenar desequilibrios ecológicos con consecuencias complejas en estas comunidades, capaces de perturbar la cadena trófica y la salud ambiental.

7. De este modo, se justifica la necesidad de buscar alternativas a los antibióticos comerciales y se establece un marco comparativo sólido que permite

valorar y contrastar el grado de afectación microbiana, facilitando la identificación de alternativas o mitigaciones naturales frente a la contaminación por antibióticos.

7.2. Conclusiones del objetivo 2

Objetivo 2: Identificar y caracterizar la eficacia y la cinética de combinaciones sinérgicas entre los 8 antibióticos estudiados y los 2 productos naturales seleccionados (ácido tánico y nerol).

8. Se ha abordado la evaluación de la actividad antimicrobiana de 2 productos de origen natural, el ácido tánico y el nerol frente a 14 bacterias patógenas, incluyendo bacterias prioritarias según la Organización Mundial de la Salud (ESKAPE) lo que permitió la determinación de la concentración mínima inhibitoria en cada caso.

9. El ácido tánico mostró las concentraciones mínimas inhibitorias más bajas, alcanzando 162,5 mg/L frente a *Pasteurella aerogenes* y 187,5 mg/L frente a *Acinetobacter baumannii*. Por su parte, nerol mostró una concentración mínima inhibitoria de 500 mg/L contra *P. aerogenes* y *Salmonella enterica*. Estas concentraciones son bastante elevadas, por lo que estos productos utilizados individualmente no pueden considerarse unas buenas alternativas a los antibióticos comerciales.

10. Se abordó el estudio de la actividad antimicrobiana de combinaciones de estos productos naturales con los 8 antibióticos cuya ecotoxicidad se había estudiado previamente, pudiéndose obtener 10 combinaciones sinérgicas.

11. El nerol presentó 2 sinergias con estreptomicina (frente a *S. enterica*, *Bacillus subtilis* y *Streptococcus agalactiae*) y 2 con gentamicina (frente a *B. subtilis* y *S. agalactiae*), con reducciones del antibiótico de entre 75% y 87,5%.

12. El ácido tánico presentó un total de 6 sinergias, 3 con cloranfenicol (frente a *A. baumanii, S. agalactiae* y *P. aerogenes)*, 1 con ampicilina, 1 con eritromicina (ambas frente a *A. baumanii*) y 1 con estreptomicina (frente a *S. agalactiae*), con reducciones de concentración mínima inhibitoria entre 75% y 93,7%.

13. Además de las interacciones sinérgicas, se obtuvieron 38 interacciones de carácter aditivo, y ninguna de carácter antagónico.

14. Los estudios cinéticos de estas combinaciones sinérgicas mostraron una inhibición completa del crecimiento bacteriano a lo largo del tiempo, sugiriendo que los productos naturales potencian el efecto de los antibióticos facilitando su acceso a las dianas celulares o previniendo la resistencia bacteriana.

7.3. Conclusiones de los objetivos 3 y 4

Objetivos 3 y 4: Evaluar la ecotoxicidad del ácido tánico y el nerol en organismos y comunidades microbianas no diana.

15. Se ha estudiado por primera vez la ecotoxicidad del ácido tánico y del nerol de forma integral, contemplando indicadores de diferentes niveles tróficos y diferentes ecosistemas (fluvial y edáfico), con bioindicadores individuales y comunidades.

16. Este trabajo constituye el primer análisis integral de la ecotoxicidad del ácido tánico puro sobre *Allium cepa*, reportando por primera vez efectos fitotóxicos sobre la germinación de sus semillas y la elongación radicular, y uno de los escasos estudios sobre nerol a nivel ecotoxicológico. La escasez de literatura previa sobre estos productos naturales individuales, frente a extractos vegetales complejos o microbiota específica, subraya la originalidad científica de los resultados obtenidos.

17. Ambos productos naturales son poco o nada tóxicos frente a los bioindicadores acuáticos y edáficos estudiados, con la excepción del nerol, que presenta toxicidad *sobre Aliivibrio fischeri*.

18. Ambos productos naturales son poco tóxicos sobre comunidades microbianas complejas fluviales y edáficas, con la excepción del ácido tánico, que resulta muy tóxico en comunidades microbianas de suelo, produciendo una disminución en el crecimiento bacteriano y en la capacidad metabólica ya a concentraciones de 0,2 mg/L.

Respecto al ácido tánico:

19. La ecotoxicidad aguda sobre indicadores acuáticos como *A. fischeri* y *Daphnia magna* es baja (valores de Concentración Efectiva$_{50}$ de 22 y 32 mg/L, respectivamente).

20. No se han encontrado efectos de ecotoxicidad aguda sobre sobre indicadores edáficos como la lombriz de tierra *Eisenia fetida* (Concentración Letal$_{50}$ >2000 mg/L), y una toxicidad muy baja sobre plantas como *A. cepa* (>134 mg/L).

21. Las comunidades complejas formadas por bacterias y hongos del perifiton fluvial no muestran efectos de toxicidad tras la exposición al ácido tánico, no detectándose cambios en su capacidad fotosintética.

22. Las comunidades microbianas del necton fluvial presentan una toxicidad muy baja, con afectación en el crecimiento de la población y la capacidad de metabolizar sobre todo aminoácidos y aminas/aminas a partir de 200 mg/L.

23. Solo las comunidades microbianas edáficas muestran una sensibilidad muy destacada a la exposición del ácido tánico, mostrando una disminución en el crecimiento de la población y en su capacidad metabólica, especialmente de aminas/amidas y polímeros, a concentraciones bajas (0,2 mg/L), lo que se consideraría como efecto *muy tóxico*, de acuerdo con los estándares de la ECHA.

Respecto al nerol:

24. Nuestros resultados muestran una toxicidad aguda de mayor a menor toxicidad, en el siguiente orden: *A. fischeri* > *D. magna* > *A. cepa* > *E. fetida,* con valores que pueden considerarse *tóxicos* para *A. fischeri* y *poco tóxicos* para el resto de bioindicadores.

25. Las comunidades bacterianas, tanto del necton fluvial como edáficas, muestran una notable resiliencia, posiblemente favorecida por la diversidad taxonómica y funcional y su capacidad de biodegradación de este compuesto natural.

7.4. Conclusiones del objetivo 5

Objetivo 5: Evaluar la ecotoxicidad de las combinaciones sinérgicas identificadas entre los antibióticos y los productos naturales (ácido tánico y nerol) sobre organismos bioindicadores y comunidades microbianas no diana.

Ecotoxicidad sobre organismos bioindicadores:

26. En porcentaje, las Concentraciones Efectivas$_{50}$ de las combinaciones indican de mayor a menor toxicidad el siguiente orden para *D. magna*: SACNE 3 > SACNE 2 > SACNE 1 > SACTA 3 > SACTA 2 > SACTA 1. Para *A. fischeri*, el orden fue SACNE 2 > SACNE 3 > SACNE 1 > SACTA 2 > SACTA 1 > SACTA 3, poniendo de manifiesto que las combinaciones con nerol son más toxicas, tanto para *D. magna* como para *A. fischeri*.

27. Las sinergias de nerol con estreptomicina o gentamicina y de ácido tánico con ampicilina o cloranfenicol muestran, para los mismos bioindicadores, CE$_{50}$ combinadas para el antibiótico (0,03 – 5,47 mg/L) entre 100 y 10000 veces inferiores a las Concentraciones Letales$_{50}$ de los antibióticos por separado (772 mg/L para estreptomicina, 840 mg/L para gentamicina, 4324 mg/L para ampicilina y 285 mg/L

para cloranfenicol), evidenciando el efecto coadyuvante del producto natural sobre el antibiótico.

28. A pesar de esta mayor toxicidad intrínseca de la combinación, las concentraciones de antibiótico en las sinergias se sitúan por debajo de sus CMI terapéuticas, lo que garantiza un amplio margen de seguridad ambiental.

29. Las dosis de producto natural requeridas en las sinergias (1 – 14,6 mg/L para nerol y 18,12 – 43,17 mg/L para ácido tánico) se mantienen por debajo o en torno a sus Concentraciones Letales$_{50}$ individuales, garantizando un perfil tóxico benigno para el medio acuático.

30. La reducción de hasta un 90% en las dosis de antibiótico necesarias minimiza la presión selectiva sobre bacterias ambientales, reduciendo la aparición de resistencias y la transferencia horizontal de genes.

Ecotoxicidad sobre comunidades fluviales:

31. Todas las sinergias que contienen ácido tánico (SACTAs) provocan una disminución del crecimiento de las poblaciones fluviales respecto al control y una potente disminución de su capacidad de metabólica, especialmente de polímeros y carbohidratos. Sin embargo, las SACNEs, (sinergias con nerol) producen un menor efecto sobre estos parámetros. SACNE 1, incluso, destaca por ser la única que presenta un efecto hormético.

32. La mayor toxicidad del ácido tánico sobre comunidades microbianas refleja su capacidad para disrumpir las complejas interacciones ecológicas y metabólicas que sostienen estas comunidades, mientras que el nerol es más eficaz contra organismos individuales, quizás gracias a su acción membranolítica directa.

33. El impacto en el crecimiento y el perfil metabólico de las sinergias del nerol y ácido tánico con los antibióticos seleccionados es en general menor que el del antibiótico a su concentración mínima inhibitoria. Sin embargo, observamos diferencias en función del antibiótico y el producto natural que constituyen cada sinergia.

34. La combinación SACNE 1 (con estreptomicina) no solo no es tóxica para las comunidades microbianas del necton fluvial, sino que ejerce un efecto hormético en el crecimiento y en la capacidad metabólica sobre todos los metabolitos, especialmente el de aminoácidos.

35. Las combinaciones SACNE 3, las SACTA 1 y SACTA 2 (con estreptomicina en el primer caso, y cloranfenicol en los dos restantes), aunque producen una disminución en el crecimiento de la comunidad microbiana y disminuyen también

su capacidad de metabolizar grupos como polímeros y carbohidratos, muestran un efecto sensiblemente menor que el que produce el antibiótico de cada sinergia a su concentración mínima inhibitoria (entre un 16 y 32% menos).

36. Sin embargo, la combinación SACNE 2 presenta una toxicidad muy similar a la concentración mínima inhibitoria del antibiótico.

37. Únicamente la combinación SACTA 3 es mucho más tóxica que el antibiótico, cloranfenicol, a su concentración mínima inhibitoria, un 71% más tóxica.

7.5. Conclusiones generales

Este trabajo doctoral demuestra que las combinaciones sinérgicas identificadas entre ácido tánico o nerol y antibióticos comerciales constituyen una alternativa terapéutica a explorar que cumple con el objetivo principal de reducir el consumo de antimicrobianos y minimizar su ecotoxicidad ambiental. Las 10 sinergias caracterizadas permiten disminuir las concentraciones de antibiótico entre un 75% y un 93,7% manteniendo la eficacia antimicrobiana frente a patógenos prioritarios, incluidas bacterias del grupo ESKAPE. Desde la perspectiva ambiental, la mayoría de las combinaciones presentan una ecotoxicidad sobre comunidades microbianas fluviales significativamente inferior (16 – 37% menos) a la de los antibióticos administrados a sus concentraciones terapéuticas individuales (CMI), con excepciones específicas que justifican la evaluación caso por caso. Esta drástica reducción en la dosis de antibiótico no solo disminuye la carga contaminante directa en ecosistemas acuáticos, sino que minimiza la presión selectiva sobre bacterias ambientales, reduciendo indirectamente el desarrollo y diseminación de resistencias antimicrobianas. Los perfiles toxicológicos favorables del ácido tánico y nerol, reconocidos por agencias reguladoras internacionales (EPA, FDA, EFSA), junto con su capacidad de potenciar antibióticos mediante múltiples mecanismos, posicionan estas sinergias como estrategias prometedoras para aplicaciones clínicas y veterinarias bajo el marco del Reglamento UE 2019/6, contribuyendo así a los objetivos globales de reducción del uso de antimicrobianos y protección de la salud ambiental y humana bajo el enfoque *One Health*.

7.6. Limitaciones del estudio y futuras líneas de investigación

En el presente estudio se aborda el problema del impacto medioambiental de los antibióticos y las resistencias bacterianas. Para ello, se ha evaluado la ecotoxicidad de 8 antibióticos comerciales, se han obtenido combinaciones sinérgicas entre antibióticos y nerol y ácido tánico, se ha evaluado ecotoxicológicamente ambos productos naturales, y

finalmente se han evaluado ecotoxicológicamente las sinergias con bioindicadores acuáticos y comunidades bacterianas acuáticas.

A pesar de los resultados prometedores que presentan las combinaciones sinérgicas obtenidas, es necesario ampliar el campo de investigación a aquellos ámbitos que, por disponibilidad de recursos técnicos y temporales, no abarca esta Tesis.

Es necesario realizar una evaluación ecotoxicológica de las sinergias también con bioindicadores individuales de suelo y comunidades bacterianas edáficas que desempeñan funciones clave en la fertilidad del suelo, el ciclado de nutrientes y la resiliencia ecológica para permitir una comparativa todavía más completa entre efectos de antibióticos individuales, productos naturales individuales y sinergias.

Además, la selección de bioindicadores puede ampliarse aún más, ya que no se abarcan organismos comúnmente utilizados, como algas o cianobacterias (*M. aeruginosa* o *P. subcapitata*) comunidades de perifiton en todos los casos.

El *endpoint* de los ensayos de ecotoxicidad ha sido principalmente la toxicidad aguda, aunque en las comunidades microbianas se ha trabajado con periodos temporales de hasta una semana. Se podría ampliar el periodo de estudio de efectos crónicos o acumulativos que podrían derivarse de exposiciones prolongadas a bajas concentraciones durante más tiempo. Esta limitación es especialmente relevante en el caso de los antibióticos, cuya presencia en el medio ambiente suele ser persistente y continua, y cuya interacción con otros contaminantes puede generar efectos sinérgicos o antagónicos difíciles de predecir.

La evaluación de la ecotoxicidad de estas combinaciones no incluye un estudio detallado de la estabilidad que pueden presentar tanto en aguas como en suelos. Este factor es un aspecto fundamental del impacto medioambiental de una sustancia, y la interacción de dos componentes puede modificarla de formas no previstas.

Aunque se ha realizado una caracterización taxonómica y funcional de las comunidades microbianas mediante secuenciación genética y ensayos metabólicos (BIOLOG EcoPlate™), no se ha abordado el análisis de genes de resistencia antimicrobiana (RAM) ni la posible transferencia horizontal de estos genes en presencia de combinaciones sinérgicas. Este aspecto es crucial para valorar el impacto real de las sinergias en la evolución de resistencias bacterianas, especialmente en entornos como las estaciones depuradoras de aguas residuales (EDAR).

La selección de productos naturales se ha limitado a 2 compuestos (ácido tánico y nerol), elegidos por su solubilidad, actividad antimicrobiana y capacidad sinérgica. Sin embargo, existen numerosos productos naturales con propiedades antimicrobianas descritas en la literatura (como carvacrol, timol, eugenol o cinamaldehído), cuya inclusión podría ampliar el espectro de sinergias posibles y permitir una comparación más robusta entre familias químicas.

Del mismo modo, la selección de antibióticos se ha centrado en 8 moléculas representativas, pero no incluye antibióticos de última generación ni aquellos con

mecanismos de acción alternativos, como los inhibidores de síntesis de ADN o los péptidos antimicrobianos.

En cuanto a las cepas bacterianas utilizadas, aunque se ha trabajado con un conjunto diverso de patógenos prioritarios según la OMS, no se ha evaluado la eficacia de las sinergias frente a biofilms bacterianos, que representan una forma de resistencia especialmente relevante en infecciones crónicas y en ambientes hospitalarios.

Finalmente, no se presenta un plan que culmine con el desarrollo de un producto farmacéutico, como cremas, geles u otras preparaciones, a partir de las sinergias obtenidas.

A partir de estas limitaciones, se pueden proponer las siguientes líneas de investigación a desarrollar en el futuro y que permitirían ampliar y profundizar en los resultados obtenidos:

1. Evaluación ecotoxicológica en ecosistemas terrestres

Realizar ensayos sistemáticos en suelos agrícolas y forestales, utilizando comunidades microbianas edáficas como bioindicadores, permitiría valorar el impacto de las sinergias en procesos como la mineralización, la nitrificación y la degradación de materia orgánica.

2. Estudios de toxicidad crónica y multigeneracional

Implementar ensayos de exposición prolongada a concentraciones subletales de antibióticos y PN, tanto en bioindicadores individuales como en comunidades microbianas, permitiría detectar efectos acumulativos, adaptaciones fisiológicas y posibles alteraciones en la estructura comunitaria.

3. Análisis genético de genes de resistencia y transferencia horizontal

Incorporar técnicas de metagenómica y PCR cuantitativa para detectar genes de resistencia (RAM) y evaluar su expresión en presencia de sinergias antibióticas, especialmente en ambientes como EDAR, ríos urbanos o suelos fertilizados con purines.

4. Ampliación del repertorio de productos naturales

Explorar nuevas combinaciones sinérgicas utilizando productos naturales de distintas familias químicas, con especial atención a aquellos con baja toxicidad ambiental y alta biodegradabilidad, como los flavonoides, alcaloides y lignanos.

5. Estudios sobre biofilms y mecanismos de virulencia

Evaluar el efecto de las sinergias sobre la formación de biofilms, la producción de toxinas y otros factores de virulencia bacteriana, mediante ensayos específicos y microscopía confocal.

6. Modelización ambiental y evaluación de riesgo

Desarrollar modelos matemáticos que integren datos de ecotoxicidad, persistencia ambiental, degradación y dispersión de los compuestos, para estimar el riesgo ecológico de las sinergias en distintos escenarios de uso (hospitalario, agrícola o industrial).

7. Formulación galénica de combinaciones sinérgicas ATB – PN

Desarrollar formulaciones farmacéuticas estables que integren antibióticos comerciales con productos naturales sinérgicos (como ácido tánico o nerol), optimizando su biodisponibilidad, solubilidad y perfil farmacocinético en función de su posible aplicación clínica. Esto incluiría estudios de compatibilidad, liberación controlada y encapsulación en sistemas como nanopartículas, liposomas o hidrogeles. Esto es un paso necesario para una sistematización y comparabilidad adecuadas de los estudios posteriores.

8. Ensayos preclínicos de eficacia y seguridad in vivo

Evaluar la eficacia antimicrobiana de las combinaciones sinérgicas en modelos animales de infección, comparando su rendimiento frente a antibióticos convencionales. Paralelamente, realizar estudios toxicológicos para determinar la seguridad sistémica, genotoxicidad y efectos adversos de los productos naturales en combinación, tanto en líneas celulares como en organismos.

9. Estudios de farmacodinámica y farmacocinética combinada

Analizar cómo interactúan los antibióticos y los productos naturales a nivel de liberación, absorción, distribución, metabolismo y excreción (LADME), así como su comportamiento en tejidos infectados. Esto permitiría ajustar las dosis y establecer regímenes terapéuticos eficaces con menor carga antibiótica.

10. Desarrollo de productos tópicos para infecciones cutáneas o mucosas

Formular cremas, geles o soluciones para uso tópico con las combinaciones sinérgicas obtenidas, especialmente dirigidos a infecciones por bacterias

multirresistentes en piel, heridas o mucosas. Estos productos podrían tener ventajas en términos de menores efectos adversos y menor inducción de resistencias.

11. Registro y validación como medicamento o producto sanitario

Iniciar el proceso de validación regulatoria para el registro de estas combinaciones como medicamentos o productos sanitarios, incluyendo estudios de estabilidad, escalado industrial, cumplimiento de normativas EMA/FDA y diseño de ensayos clínicos fase I–III.

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9. MÉRITOS

9.1. Otros artículos publicados durante esta Tesis

1. ***Hydroquinone Ecotoxicity: Unveiling Risks in Soil and River Ecosystems with Insights into Microbial Resilience***

   Revista: TOXICS, 12, 115. Cuartil: Q1. Factor de Impacto: 4.1

   Autores: Antonio Valenzuela, Diego Ballestero, Cristina Gan, Guillermo Lorca, Elisa Langa y María Rosa Pino-Otín

   Fecha de publicación: 29 de enero 2024

   DOI: 10.3390/toxics12020115

2. ***Enhancing Commercial Antibiotics with Trans-Cinnamaldehyde in Gram-Positive and Gram-Negative Bacteria: An* In Vitro *Approach***

   Revista: PLANTS, 13, 192. Cuartil: Q1. Factor de Impacto: 4.1

   Autores: Autores: Natalia Ferrando, María Rosa Pino-Otín, Diego Ballestero, Guillermo Lorca, Eva María Terrado y Elisa Langa

   Fecha de publicación: 30 de noviembre 2024

   DOI: 10.3390/plants13020192

3. ***Ecotoxicity of five veterinary antibiotics on indicator organisms and water and soil communities***

   Revista: ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY, 274, 116185. Cuartil: Q1. Factor de Impacto: 6.1

   Autores: María Rosa Pino-Otín, Antonio Valenzuela, Cristina Gan, Guillermo Lorca, Natalia Ferrando, Elisa Langa y Diego Ballestero

   Fecha de publicación: 14 de marzo 2024

   DOI: 10.1016/j.ecoenv.2024.116185

9.2. Asistencia a Congresos

1. **9th Ibero-American Congress on Contamination and Environmental Toxicology. 2 de diciembre, 2021.**

   Presentación: Oral

   Título: Ecotoxicity of Eight Widely Used Antibiotics on River Microbiota

   Autores: Guillermo Lorca, Cristina Gan, Natalia Ferrando, Cristina Gan, Eva Terrado, Elisa Langa, Diego Ballestero, María Rosa Pino-Otín.

2. **9th Ibero-American Congress on Contamination and Environmental Toxicology. 2 de diciembre, 2021.**

   Presentación: Oral

   Título: Impact of Eight Commonly Used Antibiotics on the Physiological Profile of the Soil Microbiota

   Autores: Natalia Ferrando, Cristina Gan, Guillermo Lorca, Diego Ballestero, Elisa Langa, Eva Terrado, María Rosa Pino-Otín.

3. **Society of Environmental Toxicology and Chemistry, SETAC Latin America 14th Biennial Meeting, 21 de septiembre, 2021.**

   Presentación: Oral

   Título: Effects of Satureja Montana L. Hydrolate on Freshwater Benthic Algal Communities and Its Microbiological Impact

   Autores: Guillermo Lorca, María Rosa Pino-Otín, Elisa Langa, Eva Terrado, Jonatan Val, Juliana Navarro, Diego Ballestero

4. **SETAC Europe 32nd Annual Meeting. 15 – 19 de mayo, 2022.**

   Presentación: Póster

Título: Effects of the Tannic Acid on the Physiological Profile of River Microbiota

Autores: Guillermo Lorca, Cristina Gan, Natalia Ferrando, Antonio Valenzuela, Eva Terrado, Elisa Langa, Diego Ballestero, María Rosa Pino-Otín.

5. **SETAC Europe 32nd Annual Meeting. 15–19 de mayo, 2022.**

Presentación: Póster

Título: Comparative Study of the Ecotoxicity in Water and Soils of 4 Hydrolates and 2 Natural Products of Plant Origin

Autores: María Rosa Pino-Otín, Diego Ballestero, Cristina Gan, Natalia Ferrando, Guillermo Lorca, Antonio Valenzuela, Eva Terrado, Elisa Langa, Ana Mainar.

6. **SETAC Europe 32nd Annual Meeting. 15–19 de mayo, 2022.**

Presentación: Póster

Título: Comparing the Effects of Tannic Acid and Critronellol on the Physiological Profile of Soil Microbiota

Autores: Natalia Ferrando, Cristina Gan, Guillermo Lorca, Antonio Valenzuela, María Rosa Pino-Otín, D. Ballestero, Eva Terrado, Elisa Langa.

7. **SETAC Europe 32nd Annual Meeting. 15–19 de mayo, 2022**.

Presentación: Póster

Título: Ecotocixity of Three Widely Used Antibiotics on Non-Target Soil and Water Organisms

Autores: Cristina Gan, Antonio Valenzuela, Guillermo Lorca, Natalia Ferrando, Eva Terrado, Elisa Langa, María Rosa Pino-Otín, Diego Ballestero

10. ÍNDICE DE ABREVIATURAS

AE – Aceite Esencial

AMO –Amoxicilina

AMP – Ampicilina

ATB – Antibiótico AT – Ácido Tánico

ATCC – American Type Culture Collection (Colección Americana de Cepas Tipo)

AUC – Area Under the Curve (Área Bajo la Curva)

AWCD – Average Well Color Development (Desarrollo medio del color de los pocillos)

BHI – Brain Heart Infusion

C_{max} – Concentración Máxima

CMI – Concentración Mínima Inhibitoria

CMB – Concentración Mínima Bactericida

CE – Concentración Efectiva

UFC – Unidades Formadoras de Colonias

CHL – Cloranfenicol

CL – Concentración Letal

CLPP – Community Level Physiologycal Profiling (Perfil fisiológico a nivel de comunidad)

CN – Caldo Nutritivo

DDH – Dosis Diarias por Habitante

DMSO – Dimetilsulfóxido

DO – Densidad Óptica

ECHA – European Chemicals Agency (Agencia Europea de Sustancias y Mezclas Químicas)

EDAR – Estación Depuradora de Aguas Residuales

ECDC – European Centre for Disease Control (Centro Europeo para la Prevención y Control de Enfermedades)

EDAR – Estación de Depuración de Aguas Residuales

EHEC – Eterohemorragic *Escherichia coli* (*E.coli* enterohemorrágica)

EIEC – Enteroinvasive *Escherichia coli (E. coli* enteroinvasiva*)*

EMA – European Medicines Agency (Agencia Europea del Medicamento)

EPA – Environmental Protection Agency (Agencia de Protección Medioambiental)

EPEC – Enteropathogenic *Escherichia coli* (*E. coli* enteropatogénica)

ERY – Eritromicina

ESVAC – European Surveillance of Veterinary Antimicrobial Consumption (Vigilancia Europea del Consumo de Antibióticos Veterinarios)

FEMA – Federal Emergency Management Agency (Agencia Federal de Gestión de Emergencias)

FIC – Fractional Inhibitory Concentration (Concentración Inhibitoria Fraccional)

GTM – Gentamicina

IC – Intervalo de Confianza

LOQ – Limit of Quantification (Límite de cuantificación)

MOPS – 3-ácido sulfónico propílico de morfolina

NE – Nerol

NOEC – No Observed Effect Concentration (Concentración sin Efecto Observado)

OECD – Organización para la Cooperación y el Desarrollo Económico

OMS / WHO – Organización Mundial de la Salud / World Health Organization

UNEP – Programa de las Naciones Unidas para el Medioambiente

PCU – Population Correction Unit (Unidad de Corrección de Población)

PEN – Penicilina

PN – Producto Natural

PBS – Phosphate Buffered Saline (Tampón Fosfato Salino)

PCR – Polymerase Chain Reaction (Reacción en Cadena de la Polimerasa)

PRAN – Plan Nacional frente a la Resistencia a los Antibióticos

RAM – Resistencias Antimicrobianas

ROS – Reactive Oxygen Species (Especies Reactivas de Oxígeno)

SACNE – Combinación Sinérgica Antibiótica de Nerol

SACTA – Combinación Sinérgica Antibiótica de Ácido Tánico

SARM – *S. aureus* resistente a meticilina

SASM – *S. aureus* sensible a meticilina

STM – Estreptomicina

SR – Substrate Richness (Riqueza del sustrato)

TC – Tetraciclina

TSA – Tryptone Soy Agar

11. ANEXOS

11.1. Análisis fisicoquímico de la muestra de agua fluvial del Río Gállego (mayo 2021)

Parámetro	Unidad	Resultado
Oxidabilidad	*mg/L*	*1.6 %*
Sólidos en suspensión	*mg/L*	*< 4 ± 20 %*
Sólidos disueltos	*mg/L*	*1600 ± 20% %*
Calcio	*mg/L Ca*	*144 ± 10%*
Magnesio	*mg/L Mg*	*9.7 ± 15%*
Sodio	*mg/LNa*	*278 ± 16%*
Potasio	*mg/L K*	*2.39 ± 15%*
Amonio	*mg/L NH_4*	*0.04*
Alcalinidad	*mg/L $CACO_3$*	*640 ± 15%*
Sulfatos	*mg/L SO_4*	*29.8 ± 10%*
Croruros	*mg/L Cl*	*61.5 ± 10%*
Nitratos	*mg/L NO_3*	*< 2.29 ± 10%*
Nitritos	*mg/L NO_2*	*1.44 ± 20%*
Fluoruros	*mg/L F*	*< 0.5 ± 14%*
Bromuros	*mg/L Br*	*< 0.20*

11.2. Determinación de CMI para los 8 antibióticos comerciales (mg/L)

Bacteria	PEN	AMP	AMO	GTM	STM	ERY	TC	CHL
Escherichia coli	-	*7,80*	*7,8*	*12,5*	*9,37*	*300*	*0,78*	*7,81*
Salmonella enterica	-	*16,62*	*62,5*	*0,78*	*4,68*	*37,5*	*1,17*	*3,91*
Klebsiella pneumoniae	-	*62,5*	*250*	*0,78*	*9,37*	*18,75*	*1,17*	*7,81*
Serratia marcescens	-	*6,25*	*125*	*5*	*0,40*	*250*	*62,5*	*62,5*
Proteus mirabilis	-	*0,078*	*0,62*	-	*12,5*	-	-	*3,12*
Pseudomona aeruginosa	-	-	*949*	*5*	*72,25*	-	*30*	*>125*
Klebsiella aerogenes		*>250*	*>949*	*6,25*	*1,56*	*75*	*1,9*	*3,91*
Acinetobacter baumannii	*500*	*125*	*125*	*12,50*	*75*	*10*	*0,78*	*62,5*
Bacillus subtilis	-	-	*0,31*	*6,25*	*6,25*	-	*5*	*1,56*
Staphylococcus aureus	*1.25*	*0,15*	*0,625*	*50*	*50*	*0,62*	*0,31*	*7,5*
Enterococcus faecalis	*>1042*	*62,5*	*0,15*	*25*	*6,25*	*6,25*	*12,5*	*100*
Streptococcus agalactiae	*0.156*	*0,15*	*16,625*	*25*	*125*	*<0,4*	*0,19*	*14*
Pasteurella aerogenes	*>500*	-	*>500*	*15,625*	*25*	*>500*	*3,125*	*8*

11.3. Determinación de CMI y CMB para nerol y ácido tánico (mg/L)

Bacteria	Nerol			Ácido tánico		
	MIC	MBC	MIC/ MBC	MIC	MBC	MIC/ MBC
Escherichia coli	*2000*	*2000*	*1*	*>2600*	*>2600*	-
Salmonella enterica	*500*	*500*	*1*	*>2600*	*>2600*	-
Klebsiella pneumoniae	*1000*	*1000*	*1*	*>1387*	*>1387*	-
Serratia marcescens	*>1000*	*>1000*	-	*>2600*	*>2600*	-
Proteus mirabilis	-	-	-	-	-	-
Pseudomona aeruginosa	*>1000*	*>1000*	-	*>2600*	*>2600*	-
Klebsiella aerogenes	*2000*	*2000*	*1*	*>5200*	*>5200*	-
Acinetobacter baumannii	*1000*	*1000*	*1*	*187,5*	*>187,5*	*>1*
Bacillus subtilis	*500*	*>1000*	*>1*	*>3570*	*>3570*	-
Staphylococcus aureus	*1000*	*1000*	*1*	*325*	*>325*	*>1*
Enterococcus faecalis	*1000*	*1000*	*1*	*650*	*650*	*1*
Streptococcus agalactiae	*1000*	*500*	*1*	*320*	*>320*	*>1*
Pasteurella aerogenes	*500*	*>500*	*>1*	*162,5*	*>325*	-
Candida albicans	*1000*	*1000*	*1*	*1800*	*>1800*	*>1*

11.4. Prueba de toxicidad del disolvente DMSO (% v/v)

Microorganismo	Concentración Mínima Inhibitoria de DMSO
Escherichia coli	*10%*
Salmonella enterica	*2,5%*
Klebsiella pneumoniae	*2,5%*
Serratia marcescens	*5%*
Proteus mirabilis	-
Pseudomona aeruginosa	*5%*
Klebsiella aerogenes	*2,5%*
Acinetobacter baumannii	*5%*
Bacillus subtilis	*2,5%*
Staphylococcus aureus	*10%*
Enterococcus faecalis	*5%*
Streptococcus agalactiae	*5%*
Pasteurella aerogenes	*5%*
Candida albicans	*5%*

11.5. Recuento de células diatomeas e identificación de comunidades de perifiton

Identificación taxonómica		Densidad (individuos/mL)
No diatomeas		
Cyanobacteria	*Chroococcus* sp.	2
	Leptolyngbya sp.	53
	Oscillatoria sp.	16
	Phormidium sp.	29
	Pleurocapsa sp.	5
	Pseudanabaena sp.	2
Rhodophyta	*Bangia* sp.	52
Chlorophyceae	*Draparnaldia* sp.	15
	Oedogonium sp.	102
	Pediastrum boryanum	4
	Scenedesmus spp.	45
Ulvophyceae	*Cladophora* sp.	15
	Gongrosira sp.	5
	Ulothrix sp.	247
Conjugatophyceae	*Spirogyra* sp.	4
Diatomeas		
Bacillariophyceae	*Achnanthidium eutrophilum* (Lange-Bertalot)Lange-Bertalot	62
	Achnanthidium minutissimum (Kütz.) Czarnecki f. anormale	2
	Achnanthidium minutissimum (Kützing) Czarnecki	310
	Achnanthidium pyrenaicum (Hustedt) Kobayasi	493
	Achnanthidium straubianum (Lange-Bertalot)Lange-Bertalot	4
	Amphora copulata (Kütz) Schoeman & Archibald	6
	Amphora inariensis Krammer	2
	Amphora pediculus (Kützing) Grunow	81
	Cocconeis euglypta Ehrenberg	4
	Cocconeis euglyptoides (Geitler) Lange-Bertalot	2
	Cocconeis pediculus Ehrenberg	10
	Cyclotella meneghiniana Kützing	10
	Diatoma moniliformis (Kützing) Williams ssp. moniliformis	2
	Encyonema silesiacum (Bleisch in Rabh.) D.G. Mann	2
	Eunotia sinuosa Hustedt	2
	Gomphonema saprophilum (Lange-Bertalot & Reichardt) Abarca Jahn Zimmermann & Enke	33
	Halamphora veneta (Kützing) Levkov	19

	Navicula antonii Lange-Bertalot	6
	Navicula cryptotenella Lange-Bertalot	4
	Nitzschia amphibia Grunow	27
	Nitzschia aurariae Cholnoky	2
	Nitzschia dissipata (Kützing) Grunow ssp.dissipata	10
	Nitzschia fonticola Grunow in Cleve et Möller	19
	Nitzschia frustulum (Kützing) Grunow	6
	Nitzschia inconspicua Grunow	121
	Nitzschia microcephala Grunow in Cleve & Moller	8
	Nitzschia palea (Kützing) W.Smith	2
	Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot	85
	Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot	12
	Rhoicosphenia abbreviata (C. Agardh) Lange-Bertalot	2
	Staurosira venter (Ehrenberg) Cleve & Moeller	62
	Surirella brebissonii var.*kuetzingii* Krammer et Lange-Bertalot	2

11.6. Análisis fisicoquímico de la muestra de agua fluvial del río Gállego y de la muestra de suelo (octubre 2022)

Parámetro del agua	Unidad	Resultado
Conductividad	*mg/L*	*2320,0*
Oxidabilidad	*mg/L*	*2,3*
pH	*-*	*7,8*
Sólidos en suspensión	*mg/L*	*6*
Sólidos disueltos	*mg/L*	*1925*
Calcio	*mg/L Ca*	*235*
Magnesio	*mg/L Mg*	*38,1*
Sodio	*mg/LNa*	*415*
Potasio	*mg/L K*	*6,08*
Amonio	*mg/L NH_4*	*<0.1*
Fosfatos	*mg/L PO_4*	*<0.2*
Sulfatos	*mg/L SO_4*	*415*
Croruros	*mg/L Cl*	*618*
Nitratos	*mg/L NO_3*	*17,7*
Nitritos	*mg/L NO_2*	*<0.05*

Parámetro del suelo	Unidad	Resultado
Contenido en arcilla	*%*	*12,3*
Contenido en arena	*%*	*51,5*
Contenido en limo	*%*	*37,1*
K (mg/L)*	*mg/L*	*100,2*
*P Olsen** (mg/Kg)*	*mg/kg*	*10,2*
*Ca meq 100 *****		*23,6*
EC1:5 (dS/m)	*dS/m*	*0,6*
Nitrógeno total	*%*	*0,1*
CaCO3 (%)	*%*	*42,5*
pH	*-*	*8,1*

**potasio disponible*
*** fósforo disponible*
**** calcio intercambiable*