Universidad de Concepción

Facultad de Ciencias Ambientales
Programa de Doctorado en Ciencias Ambientales mención Sistemas Acuáticas Continentales

Título

Evaluación de la presencia de nanopartículas de TIO_2 (NPs TiO_2) en la cuenca del río Biobío y sus efectos sobre indicadores biológicos modelos

Tesis para optar al grado de

Doctor en Ciencias Ambientales con mención en Sistemas Acuáticos Continentales

GESTER GISSELA GUTIÉRREZ NEIRA

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Dr. José María Monserrat

Miembro de comité de tesis–Evaluador externo
Instituto de Ciencias Biológicas (ICB)
Universidad Federal de Río Grande
Rio Grande, Brasil.

DEDICATORIA

A la memoria de mi padre, su legado ha moldeado a la mujer, profesional y persona que soy hoy, este logro también te pertenece.

Gester Gutiérrez Neira

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Antecedentes Académicos

Doctora (c) en Ciencias ambientales con mención en sistema acuáticos continentales. Facultad de Ciencias de la Universidad de Concepción, Chile.	**2020 – 2025**
Diplomada en Gestión y Análisis del Ambiente. Facultad de Ciencias Ambientales y Centro EULA-CHILE, Universidad de Concepción, Chile.	**2014**
Químico Ambiental Facultad de Ciencias de la Universidad Católica de la Santísima Concepción, Chile.	**2015**

Línea de investigación principal | Contaminación, Tecnología y Remediación del Agua.

Esta línea de investigación se fundamenta en estudios realizados en el río Biobío entre 2020 y 2022, donde se llevaron a cabo monitoreos de campo para la cuantificación y caracterización de nanopartículas de TiO_2 en aguas superficiales. El objetivo principal es generar conocimiento científico sobre la presencia de estos contaminantes en ecosistemas acuáticos, proporcionando evidencia de su ocurrencia y potencial impacto ambiental. Permitiendo abrir nuevas líneas de investigación orientadas al desarrollo de tecnologías innovadoras para la mitigación, remediación y recuperación de cuerpos de agua afectados por contaminantes emergentes. Asimismo, esta investigación contribuye al diseño de estrategias para la gestión sostenible y circular del recurso hídrico, promoviendo el uso de soluciones basadas en principios de química verde y nanotecnología ambiental.

Línea de Investigación Secundaria | Cambio Global, Sociedad y Agua.

Esta línea de investigación se sustenta en los estudios realizados en el laboratorio, en el cual se evalúa el impacto ecotoxicológico de las nanopartículas de dióxido de titanio (NPs TiO_2). analizando alteraciones en los mecanismos fotosintéticos de productores primarios mediante la expresión génica de biomarcadores clave, que evidencian estrés oxidativo y afectación en la eficiencia fotosintética, junto con esto se evalúa la transferencia trófica de la toxicidad, a organismos mayores a través de feeding assays. El objetivo de esta línea de investigación es evaluar los efectos subletales y las posibles alteraciones ecológicas inducidas por nanocontaminantes en los ecosistemas acuáticos. A través de estos estudios, se busca generar conocimiento científico riguroso que contribuya a la comprensión de su impacto ambiental y facilite la toma de decisiones informadas. Asimismo, se pretende sensibilizar tanto a la comunidad científica como al público en general sobre la responsabilidad antropogénica en la contaminación por nanopartículas, promoviendo estrategias para su monitoreo y gestión sostenible.

Becas adjudicadas

▪ Extensión de Beca por COVID-19, 2024. Otorgado por la Agencia Nacional de Investigación y Desarrollo de Chile (ANID).

▪ Beca ANID Gastos de Operaciones, 2022-2023. Beca otorgada por la Agencia Nacional de Investigación y Desarrollo para la ejecución de los gastos asociados al proyecto de tesis.

▪ Beca ANID Doctorado Nacional, 2020. Beca de Estudios de Doctorado en Chile, año académico 2020-2024. Otorgado por la Agencia Nacional de Investigación y Desarrollo de Chile (ANID). Número de beca nacional doctorado 21200069.

Productividad científica Durante la permanencia en el programa de doctorado

Gutiérrez, Gester G., Perfetti-Bolaño, A., Meléndrez, M., Pozo, K., Corsi, I., Barra, R.O., Urrutia, R., 2024. First evidence of anthropogenic TiO_2 nanoparticles occurrence in Chilean rivers. Environmental Advances 16, 100536. https://doi.org/10.1016/j.envadv.2024.100536.

Gutiérrez, Gester G., Rivas-Valdés, F., Benavente, B. P., Olivares, R., Hepp, M. I., Barra, R. O., & Urrutia, R. (2025). Toxicological Responses of Photosynthetic Genes in *Chlorella vulgaris* Exposed to Environmentally Relevant Concentrations of TiO_2 Nanoparticles. International Journal of Molecular Sciences, 26(21), 10271. **https://doi.org/10.3390/ijms262110271**

Gutiérrez Gester G., Manuel Meléndrez., Barra R., Francisca Valenzuela-Aguayo, Urrutia R. (2025). Synthetic Nanoparticles in Aquatic Systems: Hidden Ecotoxicity and the Sustainability Challenge. Environmental Science: Nano - Royal Society of Chemistry (**enviado**).

Proyectos

Directora Alterna – Proyecto FONDEF IDeA I+D 2025 (ID25I10458)
Desarrollo de sensor portátil basado en electroforesis capilar para la detección rápida de microorganismos en aguas (2025 – 2027). Liderazgo científico y técnico en el diseño y validación de tecnología para detección de patógenos en matrices acuáticas. Coordinación con equipos interdisciplinarios y vinculación con la industria para pilotaje y transferencia tecnológica. Contribución a la formulación de estrategias para la evaluación de riesgo y sostenibilidad del prototipo.

Tesista – Proyecto de Tesis Doctoral
Evaluación de la presencia de NPs TiO_2 en la cuenca del río Biobío y sus efectos sobre indicadores biológicos modelos **(2020-2025)**. **Tutores**: Dr. Roberto Urrutia, Dr. Ricardo. Barra. **Evaluadores Externos**: Dra. Gladys Vidal, Dra. Ilaria Corsi y Dr. Manuel Meléndrez.

Adjudicación Becas ANID Doctorado Nacional, 2020. Beca de Estudios de Doctorado en Chile, año académico 2020-2024. Otorgado por la Agencia Nacional de Investigación y Desarrollo de Chile (ANID). Número de beca nacional doctorado 21200069.

Beca ANID Gastos de Operaciones, 2022-2023. Beca otorgada por la Agencia Nacional de Investigación y Desarrollo para la ejecución de los gastos asociados al proyecto de tesis.

Extensión de Beca por COVID-19, 2024. Otorgado por la Agencia Nacional de Investigación y Desarrollo de Chile (ANID).

Colaboradora – . FONDEF ID20i10040
Desarrollo de filamentos de impresión 3D en base a materiales compuestos para aplicaciones biomédicas. FONDEF ID20i10040 (2020-2022). Evaluación de riesgo en productores primarios como microalgas *Selenastrum capricornutum*. Investigador responsables: Manuel Meléndrez. 2022, Concepción, Bio Bio, Chile.

Colaboradora
Evidencia de efectos subletales en *Daphnia magna* expuesta a sucesivos shock tóxicos de licor negro Evidencia de efectos subletales en *Daphnia magna* expuesta a sucesivos shock tóxicos de licor negro. Investigador responsables: Soledad Chamorro. 2015, Concepción, Bio Bio, Chile.

Congresos, Charlas y Prensa

Congresos

Coautora: Kaolin Workshop, 18th International Clay Conference (AIPEA): Quezada G. García K. Gutiérrez G. Rozas R. Toledo P. "Kaolin Modeling and Simulation". 13 de julio de 2025, Dublín, Irlanda.

Expositor: SETAC Latinoamérica 15ª Reunión Bienal (2023): Gutiérrez, G., Olivares R., Corsi I., Barra R.,Urrutia R. "Toxic effects on the photosynthetic mechanism of *Chlorella vulgaris* (microalgae) caused by relevant environmental concentrations of TiO_2 nanoparticles. 17-20 de septiembre de 2023. Montevideo, Uruguay.

Expositor: XVI Congreso sociedad Chilena de limnología (2022): Gutiérrez, G., Corsi I., Barra R., Urrutia. "Nanopartículas y su presencia en cuerpos de agua superficial". 11 al 14 de Octubre, Coyhaique. Chile.

Póster: 10º Congreso Ibérico y 7ª Iberoamericano de Contaminación y Toxicología Ambiental:Chamorro, S. Hernández, L. Gutiérrez, G. Morales, G. y Vidal, G. (2015). Evidencia de efectos subletales en *Daphnia magna* expuesta a sucesivos shock tóxicos de licor negro. 14 al 17 de Julio, Vila Real. Portugal.

Póster: VIII Congreso Latinoamericano de Ciencias Ambientales y IX Congreso Chileno de Física y Química Ambiental: Gutiérrez, G. Medina, P. Chamorro, S. (2015). "Evaluación de efectos agudos y crónicos de compuestos farmacéuticos: Paracetamol, Ibuprofeno y Furosemida en *Arbacia spatuligera*, *Daphnia magna* y *Selenastrum capricornutum*". 5 al 9 de Octubre, Pucón-Chile.

Charlas

Charla Explora Biobío: Charla científica en colegio Ranquihue. Lugar comuna de Tirúa. Noviembre 2023. Rol de Docente, Se llevo una charla a alumnos de 7º y 8º año en la Comuna de Tirúa, Escuela Ranquihue, con el título "El camino a la ciencia".

Charla Explora Biobío: Charla científica en colegio Patricio Lynch. Lugar comuna de Penco. Noviembre 2023. Rol de Docente, Se llevo una charla a alumnos de toda edad de enseñanza básica en un contexto de feria científica, con el título "¿Cuándo hablamos de contaminación?"

Charla Explora Biobío: Charla científica en colegio Salesianos. Lugar comuna de Concepción. Noviembre 2023. Rol de Docente, Se llevo una charla a alumnos de enseñanza Media en un contexto de congreso científico, con el título "¿Cuándo hablamos de contaminación?"

Charla "Evaluación de toxicidad de compuestos tóxicos sobre el medio ambiente". Año 2015. en Rebeca Matte Bello E-564, Concepción VIII. Realizado bajo el marco de la XII Semana Nacional de la Ciencia y la Tecnología dentro de la actividad "1000 Científicos 1000 aulas.

Evaluador científico en UNEP (Internacional)

Revisor Invitado de la UNEP (United Nations Environment Programme), para la revisión de la séptima edición de la evaluación global de Perspectivas del medio ambiente mundial (GEO-7). (Periodo 2024-2025).

Evaluador científico en INN (nacional)

Revisor técnico de Norma Chilena 409/1 para su actualización en instituto nacional de normalización (INN). (Periodo 2025).

Prensa

Gutiérrez, G. (2025, 29 octubre). Nanopartículas bajo la lupa: estudio revela efectos tóxicos del dióxido de titanio en microalgas de agua dulce. Revista EcoCiencias. Recuperado de:

https://revistaecociencias.cl/2025/10/29/nanoparticulas-bajo-la-lupa-estudio-revela-efectos-toxicos-del-dioxido-de-titanio-en-microalgas-de-agua-dulce/

Gutiérrez, G. (2025, 30 octubre). Nanopartículas de dióxido de titanio afectan genes de microalgas: estudio alerta sobre vacío normativo. El Desconcierto. Recuperado de:

https://eldesconcierto.cl/2025/10/30/nanoparticulas-de-dioxido-de-titanio-afectan-genes-de-microalgas-estudio-alerta-sobre-vacio-normativo

Gutiérrez, G. (2025, 29 octubre). Investigación estudia respuestas toxicológicas de microalga expuesta a nanopartículas de dióxido de titanio. Portal Innova. Recuperado de:

https://portalinnova.cl/investigacion-estudia-respuestas-toxicologicas-de-microalga-expuesta-a-nanoparticulas-de-dioxido-de-titanio/

Gutiérrez, G. (2025, octubre). Científica UCSC investiga los efectos de cremas en microorganismos acuáticos. Diario La Estrella, sección Actualidad. **Reportaje impreso**.

Dirección de tesis y seminarios

Profesor Co-Tutor de investigación:

Seminario titulado “Evaluación del efecto ambiental de las nanopartículas de dióxido de Titanio (TiO_2 NPs) y Plata (Ag NPs) sobre los productores primarios en sistemas acuáticos continentales” autora: Srita. Cynthia Salcedo, Alumna de Ingeniería Ambiental, Facultad de ciencias Ambientales, Universidad de Concepción, Campus Concepción. (I Semestre 2023).

Tesis titulada “Aproximación a la evaluación del riesgo ecológico por nanopartículas de cobre en cuerpos de agua dulce” Autor: Sr. Joaquín Cisternas Alumno de Ingeniería Ambiental, Facultad de ciencias Ambientales, Universidad de Concepción, Campus Concepción. (II Semestre 2024).

Seminario titulado “Análisis Ecotoxicológico de la Correlación de Metales Pesados y Coliformes en Sedimentos del Humedal Boca Maule de Coronel” autora: Srita. Brenda Figueroa, Alumna de Química Ambiental, Facultad de Ciencias, Universidad de la Santísima Concepción, Campus Concepción. (II Semestre 2024)

Revisor en Curso CS1021C Habilitación Profesional de la Carrera de Biología Marina:

Memoria titulado “Descripción de los estados tempranos del desarrollo embrionario de *Arbacia spatuligera* y el efecto de la modificación de la temperatura de incubación” autor: Sr. Óscar Bravo, Alumno de Biología Marina, Facultad de Ciencias, Universidad de la Santísima Concepción, Campus Concepción. (II Semestre 2024)

Memoria titulado “Detección de Metales pesados y niveles de expresión de Metalotioneínas en chorito (*Mytilus chilensis*, Hupé 1854) de caleta del gran Concepción” autor: Sr. Cristóbal Opazo, Alumno de Biología Marina, Facultad de Ciencias, Universidad de la Santísima Concepción, Campus Concepción. (II Semestre 2024).

Antecedentes Laborales

1) Dirección en Traza Verde. (2024-2025)

Directora de proyectos

- Asesorías a empresas e instituciones sobre temas y Normativa medioambientales.
- Análisis Químicos y Biológicos, de acuerdo con cumplimientos de Resolución de Calificación Ambiental.
- Investigación y difusión

Logro:

Adjudicación licitación con empresas CMPC Laja y CMPC Santa Fé, para asesorías de análisis toxicológicos históricos para presentación antes superintendencia de medioambiente.

Adjudicación licitación en mercado público: Análisis y evaluación de riesgo ecológico a través de compra ágil: 1057545-126-COT25, con Hospital de la Unión, Región de los ríos.

Centro de Ciencias Ambientales EULA-Chile, Universidad de Concepción.
(2015-2024)

Encargado de Laboratorio microbiología y subrogante de bioensayos.

- Colaboradora en análisis y procesos de: Acreditación de laboratorios Bioensayos con evaluaciones Ecotoxicológicas con especies: *Daphnia magna, Chlorella vulgaris, Selenastrum capricornutum, Isochrysis galbana, Artemia salina*. Noviembre de 2015 - 2024, Concepción, Bio Bio, Chile.

- Encargada en análisis y procesos de: Acreditación de laboratorio de Microbiología con ensayos de coliformes Fecales y totales en agua superficial y residuos industriales líquidos. Noviembre de 2015 - 2024, Concepción, Bio Bio, Chile.

- Encargada en Análisis y proceso de: Acreditación de laboratorio de Fitoplancton con ensayos de Clorofila a y total en agua superficial. Noviembre de 2015 - 2024 (9 años), Concepción, Bio Bio, Chile.

Logro:

- Se certificaron análisis bajo NCH/ISO 17025:2017: Ante INN e instituciones internacionales como IAS:

  Norma Chilena 2083:1999 - Bioensayo de toxicidad aguda mediante la determinación de la inhibición de la movilidad de *Daphnia magna* o *Daphnia pulex* (Crustacea, Cladocera), Instituto Nacional de Normalización INN. Expedición: 2015-2023. International Accreditation Service, Inc. (IAS) expedición 2018-2024. **(Primer laboratorio a nivel nacional acreditado entre los años 2018-2023)**

  Norma Chilena 2706:2002 - Calidad de agua - Bioensayo de inhibición de crecimiento de algas de agua dulce con *Selenastrum capricornutum (Raphidocelis subcapitata)* International Accreditation Service, Inc. (IAS). Expedición: 2023-2024. **(Primer laboratorio a nivel nacional acreditado entre los años 2023-2024)**

  Norma Chilena 2313 Aguas residuales - Métodos de análisis - Parte 22 y 23: **Determinación de coliformes fecales** en medio EC y A-1. International Acreditation Service (IAS) Expedición: 2019-2024.

Certificaciones

Gestión Ambiental

Consultor Certificado para la realización de Declaraciones y Estudios de Impacto Ambiental, otorgado por el Servicio de Evaluación de Ambiental **(2024)**.

Las nanopartículas se han vuelto parte de nuestra vida cotidiana. Se encuentran en productos como bloqueadores solares, cosméticos, pinturas o medicamentos, y aunque nos aportan beneficios tecnológicos, su destino final en el medio ambiente aún es poco conocido.

Esta tesis doctoral investigó la presencia y los efectos de la nanopartícula de dióxido de titanio (NPs TiO_2), en el río Biobío, un ecosistema de gran relevancia ecológica y económica para el centro sur de Chile.

Los monitoreos realizados mostraron concentraciones de nanopartículas entre 17,6 y 22,9 µg/L, especialmente aguas abajo de plantas de tratamiento de aguas servidas, lo que evidencia que los sistemas actuales no logran eliminarlas completamente. A través de microscopía electrónica se confirmó que las partículas encontradas tenían tamaños entre 10 y 200 nanómetros y correspondían a las formas Rutilo y Anatasa, típicas de aplicaciones industriales.

Para comprender cómo estas nanopartículas afectan la vida acuática, se realizaron ensayos con dos especies clave en la red trófica: La microalga *Chlorella vulgaris*, representativa de los productores primarios y el microcrustáceo *Daphnia magna*, un consumidor primario esencial en el equilibrio ecológico de las aguas continentales.

En las microalgas se observó un fenómeno conocido como respuesta hormética, donde bajas concentraciones de contaminante estimulan el crecimiento y concentraciones mayores generan efectos adversos. A 17,6 µg/L se detectó un aumento en la densidad celular tras 72 horas de exposición, acompañado de cambios en la expresión de genes relacionados con la fotosíntesis, lo que sugiere mecanismos de adaptación para mantener la producción de energía bajo estrés por NPs TiO_2.

Los ensayos con *Daphnia magna* revelaron un patrón diferente. No se detectó toxicidad aguda (48 h), pero sí efectos crónicos cuando las nanopartículas se incorporaron a la dieta mediante microalgas contaminadas ("feeding assay"). En este caso, la reproducción disminuyó significativamente a partir de 17,6 µg/L, con un NOAEC de 8,8 µg/L, un LOAEC de 17,6 µg/L y una EC_{50} de 17,8 µg/L, valores que coinciden con las concentraciones medidas en el río Biobío.

Esto significa que, aunque el contaminante no mata directamente a los organismos, reduce su capacidad de reproducirse, lo que puede alterar la dinámica poblacional y el equilibrio ecológico a largo plazo. El hallazgo confirma que la vía de exposición trófica (por alimento) es mucho más crítica que la exposición directa en el agua, ya que permite la bioacumulación de nanopartículas dentro de los organismos.

La revisión bibliográfica incluida en esta tesis analizó más de un centenar de estudios recientes sobre ecotoxicología de nanopartículas, identificando una tendencia común: los efectos no siempre siguen una relación simple dosis-respuesta y dependen fuertemente del tipo de partícula, su tamaño, forma y las condiciones del medio. Además, destacó la necesidad urgente de actualizar las normativas ambientales para incorporar metodologías modernas de evaluación, incluyendo análisis moleculares y bioensayos multitróficos.

En conjunto, los resultados muestran que las NPs TiO_2 pueden actuar como disruptores de procesos metabólicos esenciales en organismos acuáticos, afectando tanto a productores como a consumidores primarios. Dado que las concentraciones que causan efectos se encuentran dentro del rango ambiental observado, su presencia representa un riesgo real para la estabilidad de los ecosistemas del río Biobío.

Este trabajo entrega evidencia científica local que puede contribuir a mejorar la gestión ambiental y el diseño de políticas públicas sobre contaminantes emergentes, promoviendo la adopción de tecnologías más eficaces para el tratamiento de aguas y el monitoreo de nanopartículas. Comprender cómo estos materiales interactúan con la vida acuática es fundamental para proteger nuestros ríos y asegurar un uso sostenible del agua, un recurso vital para las comunidades y la biodiversidad del Biobío.

FINANCIAMIENTOS

AGENCIA NACIONAL DE INVESTIGACIÓN Y DESARROLLO (ANID)
Programa Formación de Capital Humano
Becas Doctorado nacional, Chile
Folio N° 21200069

Centro de Recursos Hídricos para la Agricultura y la Minería (CRHIAM)
Universidad de Concepción, Chile

ÍNDICE

El aumento en la producción y uso de nanomateriales ha generado creciente preocupación respecto a su liberación y comportamiento en ecosistemas acuáticos. Entre ellos, las nanopartículas de dióxido de titanio (NPs TiO_2) destacan por su amplia aplicación en productos industriales y de consumo, como bloqueadores solares, cosméticos y pinturas, lo que incrementa su probabilidad de ingreso a cuerpos de agua a través de descargas urbanas e industriales. Sin embargo, aún existen importantes incertidumbres sobre su presencia ambiental, su comportamiento en matrices naturales y sus efectos ecotoxicológicos en organismos acuáticos.

En este contexto, el objetivo de esta investigación fue evaluar la presencia, caracterización y efectos ecotoxicológicos de NPs TiO_2 en el río Biobío (Chile), integrando monitoreo ambiental, análisis fisicoquímico y evaluación biológica en organismos modelo de distintos niveles tróficos.

El estudio incluyó un monitoreo ambiental realizado durante 2022 en cuatro puntos representativos del río Biobío (Alto Biobío, Santa Bárbara, Hualqui y Hualpén), considerando diferentes estaciones del año. Se recolectaron muestras de agua superficial que fueron analizadas mediante espectrometría de masas con plasma acoplado inductivamente (ICP-MS) para determinar concentraciones elementales asociadas a TiO_2. La detección y caracterización morfológica de nanopartículas se realizó mediante microscopía electrónica de transmisión (TEM) y microscopía electrónica de barrido en modo de presión variable (VP-SEM) acoplada a espectroscopía de dispersión de energía (EDS). En paralelo, se desarrollaron bioensayos ecotoxicológicos con la microalga *Chlorella vulgaris*, siguiendo la guía OECD TG 201, y con el microcrustáceo *Daphnia magna*, mediante ensayos agudos y crónicos de acuerdo con la norma ISO 10706. Además, se evaluaron respuestas moleculares mediante RT-qPCR en genes asociados al metabolismo fotosintético (*atpB, psaA, psaB, psaD, psbA y rbcL*), utilizando 18S como gen de referencia.

Las nanopartículas de TiO_2 fueron detectadas en todas las estaciones de muestreo, con concentraciones entre 17,6 y 22,9 µg/L, principalmente aguas abajo de plantas de tratamiento de aguas servidas. La microscopía electrónica confirmó tamaños entre 10 y 206 nm y la presencia de fases cristalinas de anatasa y rutilo, indicando un origen mixto natural y antropogénico.

En *Chlorella vulgaris*, la exposición a concentraciones ambientalmente relevantes (1,1–17,6 µg/L) mostró una respuesta de crecimiento hormética, con estimulación a concentraciones intermedias y ausencia de inhibición en la dosis más alta. A nivel molecular, *rbcL* fue significativamente reprimido a 1,1–4,4 µg/L, mientras que *psaA* y *psaD* se sobreexpresaron a 8,8–17,6 µg/L, lo que indica un refuerzo compensatorio del fotosistema I. Estas trayectorias transcripcionales divergentes demuestran que los puntos finales moleculares revelan efectos subletales que no son evidentes únicamente a partir del recuento celular. En conjunto, estos resultados destacan el potencial de los genes relacionados con la fotosíntesis como biomarcadores tempranos para detectar estrés inducido por nanopartículas en productores primarios acuáticos.

En *Daphnia magna*, no se observó toxicidad aguda, pero sí efectos crónicos significativos por vía trófica, con una NOAEC de 8,8 µg/L, LOAEC de 17,6 µg/L y EC_{50} de 17,8 µg/L ($R^2 = 0,916$). Estos valores coinciden con las concentraciones ambientales detectadas en el río, indicando un riesgo ecológico real bajo condiciones naturales de exposición.

La revisión bibliográfica incluida en esta tesis integró más de cien estudios recientes, identificando estrés oxidativo, genotoxicidad y alteraciones reproductivas como mecanismos comunes de toxicidad, y destacando la necesidad de incorporar enfoques moleculares y tróficos integrados en la evaluación de riesgo ambiental.

En conjunto, los resultados demuestran que las nanopartículas de TiO_2 están presentes en el río Biobío y pueden alterar procesos biológicos esenciales, desde la regulación fotosintética en productores primarios hasta la reproducción de consumidores clave. Este trabajo aporta evidencia científica relevante para el monitoreo ambiental, la evaluación de riesgo ecológico y el desarrollo de marcos regulatorios orientados a la gestión de nanomateriales en ecosistemas de agua dulce en Chile.

ABSTRACT

The increasing production and widespread use of engineered nanomaterials have raised growing concerns regarding their release, environmental behavior, and potential ecological impacts in aquatic ecosystems. Among them, titanium dioxide nanoparticles (TiO_2 NPs) are one of the most extensively produced nanomaterials worldwide due to their broad applications in cosmetics, paints, food additives, and industrial products. As a consequence, these nanoparticles are frequently introduced into freshwater systems through wastewater effluents and urban discharges. Despite their extensive use, significant uncertainties remain regarding their occurrence in natural environments, their physicochemical behavior in aquatic matrices, and their ecotoxicological effects on aquatic organisms.

The objective of this study was to evaluate the presence, characterization, and ecotoxicological effects of TiO_2 nanoparticles in the Biobío River (Chile), integrating environmental monitoring, physicochemical analysis, and biological assessment across different trophic levels.

Environmental monitoring was conducted during 2022 at four representative sites along the Biobío River basin (Alto Biobío, Santa Bárbara, Hualqui, and Hualpén), covering seasonal variations. Surface water samples were collected and analyzed for elemental concentrations using inductively coupled plasma mass spectrometry (ICP-MS). Nanoparticle detection and morphological characterization were performed using transmission electron microscopy (TEM) and variable pressure scanning electron microscopy (VP-SEM) coupled with energy dispersive spectroscopy (EDS).

Ecotoxicological effects were assessed using bioassays with organisms representing different trophic levels. Growth inhibition assays were performed with the freshwater microalga *Chlorella vulgaris* following OECD guideline TG 201. Additionally, acute and chronic toxicity bioassays were conducted using the freshwater microcrustacean *Daphnia magna* according to ISO 10706 protocols. Molecular responses were further evaluated through RT-qPCR analysis of key photosynthesis-related genes (*atpB, psaA, psaB, psaD, psbA, and rbcL*), using the 18S rRNA gene as a reference for normalization.

TiO_2 nanoparticles were detected across all sampling locations, with concentrations ranging between 17.6 and 22.9 µg L^{-1}, particularly downstream of wastewater treatment plants. Electron microscopy confirmed nanoparticle sizes ranging from 10 to 206 nm and the presence of anatase and rutile crystalline phases, suggesting a mixed natural and anthropogenic origin.

Exposure experiments with *Chlorella vulgaris* at environmentally relevant concentrations (1.1–17.6 µg L^{-1}) revealed a hormetic growth response, characterized by stimulation at intermediate concentrations and no inhibition at the highest tested concentration. At the molecular level, the photosynthetic gene rbcL was significantly repressed at lower concentrations (1.1–4.4 µg L^{-1}), whereas psaA and psaD were upregulated at higher concentrations (8.8–17.6 µg L^{-1}), suggesting a compensatory reinforcement of photosystem I activity. These divergent transcriptional patterns demonstrate that molecular endpoints can reveal sub-lethal stress responses that are not detectable through traditional cell density measurements.

In *Daphnia magna*, no acute toxicity was observed; however, significant chronic effects were detected through trophic exposure pathways. Chronic toxicity parameters showed a NOAEC of 8.8 µg L^{-1}, a LOAEC of 17.6 µg L^{-1}, and an EC_{50} of 17.8 µg L^{-1} ($R^2 = 0.916$). Notably, these effect thresholds overlap with the environmental concentrations detected in the Biobío River, indicating a potential ecological risk under natural exposure conditions.

The literature review included in this thesis synthesized more than one hundred recent studies and identified oxidative stress, genotoxicity, and reproductive impairment as the most commonly reported mechanisms associated with nanoparticle exposure. The analysis also highlighted the importance of integrating molecular biomarkers and trophic interactions into environmental risk assessment frameworks.

Overall, the results demonstrate that TiO_2 nanoparticles are present in the Biobío River and can affect key biological processes ranging from photosynthetic regulation in primary producers to reproductive performance in aquatic consumers. This research contributes valuable scientific evidence for environmental monitoring, ecological risk assessment, and the development of regulatory frameworks addressing the environmental management of nanomaterials in freshwater ecosystems.

CAPÍTULO 1

El termino de nanopartículas evolucionó a partir de la investigación sobre partículas ultrafinas que poseen hasta aproximadamente 100 nm en cualquiera de las dimensiones de su estructura molecular, a esta escala las nanopartículas poseen diferentes propiedades relacionadas a su forma y tamaño, dentro de estas se destacan razón superficie/volumen, carga iónica, formación de aglomeraciones y entro otros (Cao, 2004; Sendra et al., 2017b), que les permiten interactuar con el medioambiente y moléculas biológicas que son esenciales para la vida (Prajitha et al., 2019). Las nanopartículas tienen dos tipos de orígenes, ya sea natural o sintéticas (Irfan Ijaz & Bukhari, 2020).

La necesidad de producir nanopartículas, es el mejoramiento de productos utilizados por la población de forma masiva, ya sean como insumos para productos de tipo: farmacéuticos, médicos, de transporte, alimenticios, incluso su utilización dentro de los procesos industriales, permitiendo la eliminación de contaminantes regulados relacionadas a la minería y plantas de tratamientos de aguas servidas (Hao et al., 2020; Jin et al., 2020; Nourafkan et al., 2018). En general, cuando las nanopartículas son utilizadas como transporte por adsorción de principios activos o como fotocatalizadores, estos son creados con el objetivo de hacer productos más eficientes y con liberación controlada (Kumari et al., 2010). Sin embargo, el control llega solo hasta su aplicación, dejando interrogantes respecto a que pasa con el continuo ciclo de vida del compuesto hasta cuando se convierte en residuo y llega a fuentes naturales que no fueron consideradas luego de su uso. Estudios indican, que las nanopartículas una vez transformadas en nanocontaminantes, pueden seguir activos, transformándose en un potencial peligro para el medioambiente, como ejemplo de esto se encuentran las nanopartículas usadas en productos de uso personal y alimenticios tales como nanopartículas de dióxido de titanio (NPs TiO_2).

Las NPs TiO_2 tiene diferentes fases cristalinas, de las cuales las más utilizadas y estables son: anatasa y rutilo (Yuenyongsuwan et al., 2018), estas se incluyen en productos alimenticios y de cuidado personal bajo los nombre de Blanco de Titanio, Blanco 6, CI Pigment White 6, CI-77891, E 171 y P25 (Adali, 2020). Las NPs TiO_2 tienen la característica de que al estar expuestas a la luz ultravioleta producen electrones libres, que por efecto sinérgico y en cadena reducen otros protones o se enlazan con la superficie de otros compuestos disponibles en medio acuoso (Martínez Martínez, 2019; Mitra et al., 2008). Desde los años 60 la administración de drogas y alimento de los EE.UU el año 2015 aprobó el uso de TiO_2 en los alimentos con niveles de hasta el 1% y en otros productos como protectores solares y cosméticos con filtro solar hasta un 25% de concentración del total del producto (FDA, 2022). Durante la producción de un producto que contiene TiO_2, muchas veces estos no están en un tamaño <100nm, sin embargo, al pasar por los diferentes procesos, el TiO_2 podría alcanzar tamaños nanométrico por efecto de síntesis descendente (top-down) no intencional, formando mezclas de formas nanopartículadas en los nanocompuestos y materiales a granel (Cao, 2004; Labille et al., 2005).

En América los principales propulsores de nanoproductos son Estados Unidos, México, Brasil, Argentina y Chile (Pardo-Guerra, 2011), siendo algunas de las materias primas exportadas por Chile el dióxido de titanio (https://www.nanotecchile.com/productos/nanoparticulas-y-aditivos/). En cuanto a importación, en el país se encuentras distintos productos de cosméticos y de aseo personal que contienen nanopartículas como maquillajes, bb-creams, protectores solares, y dentífricos producidos por diferentes laboratorios internacionales como *La Roche posey*, *Lancome*, *Vichy*, *L'Oreal*, *Dentaid*, entre muchos más. En los últimos años la investigación sobre nanomateriales en Chile ha ido creciendo, focalizándose 100% en estudios relacionada a la producción de estos (CEDENNA, 2020), sin embargo, las evaluaciones de riesgos ambientales aún están al margen de este crecimiento, considerando que se utilizan nanoproductos desde mucho antes, por la importación y producción de estos en países más avanzados como lo indica Parado-Guerra (2011). La etapa de sinterización, producción y aplicación, continua dejando grandes interrogantes respecto al impacto que tienen los nanomateriales en el ambiente, cuando estos son transferidos a todos los compartimentos ambientales de manera difusa o directa (Mitrano & Nowack, 2017). Sin embargo, existen estudios que avanzan en estas temáticas y advierten el peligro que suponen las nanopartículas, por ejemplo en un estudio realizado al sur de Tailandia, se reportó la presencia de nanopartículas en la atmosfera debido al uso de aerosoles que lo contenían (Chomanee et al., 2020). Gottschalk y Nowack (2011) en su revisión bibliográfica definen que las principales fuentes ambientales de nanopartículas luego de su aplicación, son las relacionados a la eliminación de los residuos antropogénicos, como plantas de incineración de residuos, rellenos sanitarios y las plantas de tratamiento de aguas servidas (PTAS) (Gondikas et al., 2018). En relación con esto último, en las PTAS se pueden concentrar nanopartículas en los lodos y re-suspender en el agua. Un estudio realizado por Westerhoff *et al*. (2011), detectó TiO_2NPs en efluentes de 10 plantas de tratamientos de aguas residuales del estado de Arizona, E.E.U.U, en concentraciones que oscilan entre 2 y 20 µg L^{-1}, con un tamaño entre 4-30 nm de diámetro y formas esféricas (Westerhoff et al., 2011). Por otra parte, Bäuerlein *el al*. (2017) también cuantificó en el orden de µg/L diferentes tipos de nanopartículas, advirtiendo que los tratamientos con lodo activado no son suficientes para eliminar el 100% de estos nanocontaminantes, tranformandose en un potencial peligro para los sistemas acuático no objetivo (Bäuerlein et al., 2017).

También estudios experimentales de partición han indicado que las nanopartículas se eliminan en gran medida de una matriz de aguas residuales durante el tratamiento secundario por sorción en lodos de las PTAS (Musee et al., 2014; Park et al., 2013). Por otra parte, las nanopartículas que son absorbidos en los lodos pueden llegar al entorno natural si los lodos se utilizan como fertilizante en la agricultura. Las nanopartículas también pueden sufrir una transformación durante el tratamiento de aguas residuales, por ejemplo, otras nanopartículas metálicas como las nanopartículas de plata (NP-Ag), están presentes principalmente en el efluente y en el lodo como Ag_2S (Kaegi et al., 2011). Estudios muestran que la desagregación parcial de las nanopartículas puede ser fomentada por eventos naturales como variaciones estacionales en las corrientes hidrológicas, mediante la mezcla de dos corrientes acuosas de diferente calidad de agua como lo son las aguas residuales con una corriente de agua superficial. La formación de agregados más pequeños debido a la desagregación podría conducir a una re-suspensión y una mayor movilidad de las nanopartículas dentro de la columna de agua (Troester et al., 2016), permitiéndoles interactuar con los diferentes organismos que estén presentes. Las nanopartículas tienen la capacidad de anclarse, penetrar y acumularse en la membrana celular, cambiando sus propiedades como: colapso de las bombas de protones, inhibición de la síntesis de ADN, generación de especies reactivas de oxígeno (ROS), inactivación de proteínas y enzimas, desnaturalización de ribosomas y degradación de moléculas de lipopolisacáridos (Hu et al., 2015; Moreno-Garrido et al., 2015; Xiang et al., 2018).

Algunas investigaciones asociadas a estas temáticas se realizan en condiciones controladas, probando que las nanopartículas causan daño biológico. Ejemplo de esto es la investigación de Pretti *el al*. (2014) en donde a través de un ensayo agudo determinó que las nanopartículas interrumpen la pared celular de la microalga *Dunaliella tertiolecta* provocando la pérdida de flagelos e inflamación celular (Pretti et al., 2014). Por otro lado la presencia de NPS dentro de la célula de microalgas puede modificar la expresión genética, ejemplo de esto es la disminución de transcripciones vinculadas a la fotosíntesis en presencia de NPs TiO_2 en *Chlamydomonas reinhardtii*, en donde también se ven afectadas las proteínas, especies reactivas de oxígeno (ROS) y alteraciones de actividad enzimática relacionadas a la hidrólisis dentro de la célula, evidenciando los múltiples efectos ecotoxicológicos de las nanopartículas (da Costa et al., 2016; Perreault et al., 2012; Simon et al., 2013). Considerando que las ROS son moduladores cruciales de las funciones celulares y que a bajas concentraciones participan en la señalización celular, la inducción de la respuesta mitogénica y la defensa contra agentes infecciosos, mientras que por el contrario una elevada concentración puede alterar la función celular normal y promover el daño irreversible a lípidos, ácidos nucleicos y a proteínas celulares (Carvajal Carvajal, 2019), es importante considerar marcadores biológicos relacionados a las ROS para poder evidenciar daños crónicos como alerta temprana ante los posibles nanocontaminantes en el ambiente. En otros estudio se registran efectos sobre la membrana de los cloroplastos causados por NP-TiO_2, aquí las nanopartículas al ingresar a las células, se agregan sobre la membrana de cloroplasto inhibiendo la síntesis de ATP y NADPH, provocando una deficiencia energética para la división y desarrollo de las células, disminuyendo la densidad de microalgas en los cultivos (Middepogu et al., 2018). Este efecto es relevante si consideramos que la fotosíntesis es el mecanismo esencial para la vida, ya que las microalgas se encuentran en el primer eslabón de la red trófica captando energía solar y transformándola para ser utilizada como fuente de glúcidos y otros componentes orgánicos para el sustento de sí misma y otros organismos superiores, además otra función importante de estos organismos es el aporte de O_2 atmosférico que estos producen de manera simultánea en el proceso de la fotosíntesis (Cheloni et al., 2016; Chen et al., 2018; Lehninger et al., 2002; Saison et al., 2010; von Moos et al., 2015). Estudios han demostrados que las NPs TiO_2 también afectan las estructuras externas de las diatomeas, formando agregados en los medios de cultivo de *Phaeodactylum tricornutum*, atrapando y envolviendo las células de modo que provoca una inhibición significativa de los rendimientos cuánticos del fotosistema II. Simultáneamente se observó que el contenido de clorofila a, azúcares solubles y las actividades de las enzimas antioxidantes **superóxido dismutasa (SOD)** y **peroxidasa (POD)** aumentaron significativamente. concluyendo que las especies presentan una defensa activa contra el estrés de las nanopartículas (Deng et al., 2017). En cuanto a las cianobacterias, las nanopartículas sobre expresan las ROS bajo luz visible, lo que causan daños a las ficobiliproteínas y ficobilina, pigmentos fotosintético-principales en estos organismos, siendo el principal motivo del mal funcionamiento del sistema fotosintético, inhibiendo así el crecimiento de las células (Fan et al., 2019). Es importante señalar que los organismos fotosintéticos no absorben la radiación en las mismas longitudes de onda del espectro visible, debido a diferencias en los pigmentos implicados en los mecanismos fotoquímicos (Geider et al., 2001). En este sentido, resulta particularmente relevante analizar cómo se ven potenciadas las interacciones de las nanopartículas fotocatalizadoras en ambientes con una alta variabilidad en las condiciones de radiación, considerando la vulnerabilidad de los ecosistemas desde el nivel de los productores primarios fotosintéticos.

En cuanto a organismos mayores, estudios in vitro indican que la unión superficial entre NPs TiO_2 duplican los efectos tóxicos relacionados a la generación de ROS, en organismos como *Daphnia magna* y *Oryzias latipes* (S. Li et al., 2014). Bhagat *el al*. (2020), especifica en su revisión que a la membrana que protege los embriones de pez cebra luego de su fecundación, pueden ingresar nanopartículas a la fase embrionaria a través de sus poros de tamaño 0,5 a 0,7 µm de diámetro, debido al intercambio de agua, oxígeno y nutrientes siendo las nanopartículas confundidas por alimento (Bhagat et al., 2020).

Los estudios mencionados anteriormente, demuestran que las nanopartículas causan riesgos ecotoxicológicos desde los organismos fotosintéticos a organismos complejos, Sin embargo, no se integran las condiciones ambientales reales, considerando que la toxicidad de las nanopartículas está determinada por el medio en que se encuentren. Y que de seguro luego de ser eliminadas por las PTAS este es distinto del medio en el que fue sintetizado, pudiendo transformar de este modo sus propiedades iniciales (Labille et al., 2005), por efecto de aglomeración, fotosensibilidad o adsorción con otras moléculas presentes en el medio (Banerjee & Roychoudhury, 2019). Es por esto por lo que la Agencia de Protección Ambiental de E.E.U.U (EPA) ha tenido poco éxito en la búsqueda de nuevas reglas para brindar protección suficiente contra la contaminación de nanopartículas emergentes. Por otra parte, la FDA, no hace una referencia categórica a la nanotecnología como segura o dañina, pero planea considerar las características específicas de productos individuales, publicando documentos de orientación para alentar a los fabricantes a consultar con la agencia antes de llevar sus productos al mercado sin embargo esto no es un requisito (Singh, 2016). Esto debería ser considerando como una alerta temprana ya que la evaluación de riesgos a la salud pública y ambiental siguen siendo áreas desatendidas, existiendo lagunas de información que no permiten el desarrollo de los correctos modelos de evaluación de riesgos para nanopartículas, sobre todo en países latinoamericanos emergentes en estas temáticas por lo que Chile no está ajeno a este problema medioambiental.

Chile poseen en su mayoría cuencas hidrográficas que descienden desde la cordillera de los Andes en el Este desembocando en el mar en el Oeste, las cuencas cumplen un sinfín de servicios ecosistémicos y que se han visto afectados de algún modo por el cambio climático. La región del Biobío posee una red de ríos que contribuyen a la cuenca hidrográfica y que posee un alto valor socioeconómica para la región y el país. El río Biobío, proporciona diferentes servicios ecosistémicos dentro de los que se destacan extracción de agua para riego en la industria de la agricultura, forestal, abastecimiento de agua potable, generación de energía hidroeléctrica, entre otros (Díaz et al., 2018). La cuenca del Biobío en la actualidad proporciona agua potable a 1,2 millones de personas y que a su vez eliminan las aguas domiciliarias a través de las principales plantas de tratamientos de aguas servidas, cerrando un ciclo de uso y re-uso de este recurso. Considerando la importancia de cuantificar el ciclo de vida de las nanopartículas, hasta hoy no existe un seguimiento respecto al consumo de nanoproductos ni cuánto de estos están ingresando a los cuerpos acuáticos, ni en qué forma, considerando lo mencionado en secciones anteriores los tipos de nanoproductos utilizados en Chile. En la región se han realizado estudios que cuantifican contaminantes emergentes y que evidencian el hecho de que las PTAS no poseen la tecnología suficiente para eliminar contaminantes que no son regulados en las actuales normativas de calidad de agua (Rozas et al., 2016), siendo aún más preocupante la falta de información respecto a las nanopartículas (NPs) que estarían ingresando a los sistemas acuático y amenazando diferentes especies con un alto valor de endemismo presentes en la cuenca del Biobío.

Por lo tanto, considerando la relevancia ecológica del río Biobío, la alta presión antrópica sobre su cuenca y la falta de información respecto a la presencia, comportamiento y efectos de las nanopartículas en estos ecosistemas, resulta fundamental investigar su ocurrencia y posible impacto sobre los productores primarios acuáticos. En este contexto, y con el propósito de orientar el desarrollo de la investigación, en el Capítulo 2 del presente estudio se plantean las siguientes hipótesis de trabajo:

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CAPÍTULO 2

HIPÓTESIS

H 1: Debido al uso masivo de productos que contienen nanopartículas de TiO_2, y que no son removidas por los sistemas de tratamiento de aguas servidas, se espera que estén presentes en mayor abundancia en la parte baja del río Biobío, especialmente después de las descargas de las plantas de tratamiento.

H 2: La exposición a concentraciones ambientales de nanopartículas de TiO_2 altera los mecanismos fotosintéticos de *Chlorella vulgaris*, generando efectos ecotoxicológicos en los productores primarios, afectando potencialmente la estabilidad de la cadena trófica en ecosistemas acuáticos.

OBJETIVO GENERAL

Evaluar la presencia de nanopartículas de TiO_2 en el río Biobío y sus potenciales efectos ecotoxicológicos sobre productores primarios presentes en los ecosistemas acuáticos.

OBJETIVOS ESPECÍFICOS

- Determinar la presencia de nanopartículas de TiO_2 en agua superficial a lo largo del río Biobío.

- Determinar la toxicidad de nanopartículas de TiO_2 sobre microalgas a distintos niveles celular y molecular.

- Evaluar los efectos indirectos sobre organismos que dependen energéticamente de los productores primarios.

CAPÍTULO 3

Synthetic Nanoparticles in Aquatic Systems: Hidden Ecotoxicity and the Sustainability Challenge

Review

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Highlights

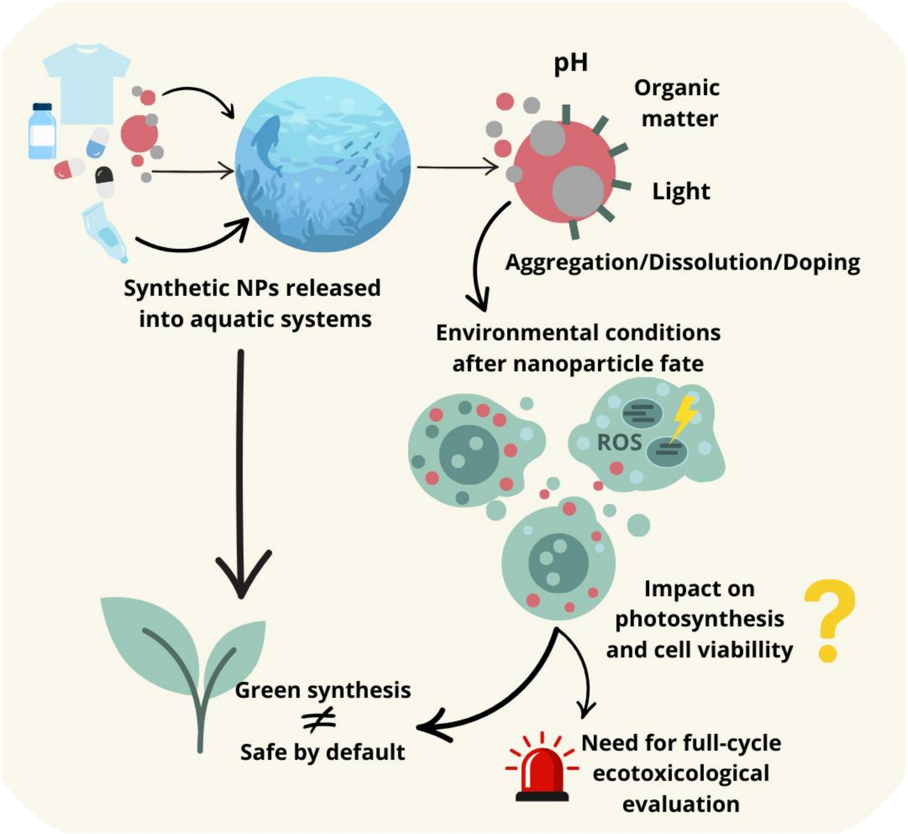
Synthetic nanoparticles undergo environmental transformations that alter their stability and toxicity in aquatic ecosystems.

The effects on primary producers depend on surface chemistry, size, and solubility of nanoparticles, influencing photosynthesis and growth.

Current ecotoxicity studies often underestimate real impacts due to simplified experimental models and conditions that do not accurately represent natural environments.

Eco-design and green synthesis of nanoparticles represent key strategies to minimize environmental impacts and improve their sustainability in various applications.

Graphical Abstract



ABSTRACT

Synthetic nanoparticles (NPs) have emerged as versatile materials in a range of industrial and biomedical applications. However, their release into aquatic systems raises growing concern due to their unique physicochemical transformations and ecotoxicological impacts. This review critically explores how nanoparticle properties—such as crystallinity, size, surface reactivity, and light sensitivity—influence their environmental behavior, bioavailability, and toxicity, particularly toward primary producers like microalgae. We highlight recent findings on the mechanisms of nanoparticle-induced photosynthetic inhibition and oxidative stress at the cellular and molecular levels. A key contribution of this review is the introduction of the "Green Synthesis Paradox", a concept that challenges the assumed environmental safety of green-synthesized nanomaterials by demonstrating their potential hidden toxicity in real ecosystems. We argue that sustainability in nanotechnology must be assessed across the full life cycle of nanoproducts, from synthesis to environmental fate. By integrating ecotoxicological perspectives into the design and regulation of nanomaterials, this review calls for a paradigm shift toward responsible innovation in nanoscience.

Keywords: Nanoparticles, Aquatic ecosystems, Toxicity, Primary producers, Environmental transformations, Regulation, Green synthesis.

3.1. INTRODUCTION.

Nanoparticles (NPs) are nanometer-sized particles of natural or synthetic origin that have revolutionized numerous research fields. The wide variety of available nanomaterials and their continuous expansion, with compositions that cover practically all the elements of the periodic table, have allowed the implementation of NPs in applications as diverse as drug delivery, biomedicine, improvement of services and products for the population, among others (Dincer et al., 2019; Hao et al., 2020; Stamatoiu et al., 2012).

Globally, nanotechnology has represented a significant technological leap, driving innovation in materials and industrial products (Anyaegbunam et al., 2024; Laxmi et al., 2023; Singh et al., 2023). However, in other parts of the world (e.g., Latin America), a gap persists between industrial development that uses nanotechnology and the regulation of associated environmental risks. The lack of an adequate regulatory framework and information gaps generate uncertainty regarding the possible impacts of nanoproducts on the environment (Pardo-Guerra, 2011).

Nanoparticles are engineered and manipulated at the molecular level, adjusting their physicochemical properties by processes such as high pressures, extreme temperatures, or doping with surfactants. This gives them unique characteristics such as high reactivity, photocatalytic properties, agglomeration ability, and surface areas with specific reaction sites (Jin et al., 2020; Nourafkan et al., 2018; Sendra et al., 2017b).

The aforementioned properties make nanoparticles especially attractive for their use; however, for a decade now, it has been evident that nanoparticles and the products containing them can have adverse effects on both human health and ecosystems, once they reach them as final disposal. Concentrations of nanoparticles have been detected in surface water bodies near sewage treatment plants, soils connected to landfills, and even in the atmosphere, as a result of the incineration and combustion of materials containing nanocomposites (Bäuerlein et al., 2017; Chomanee et al., 2020; Gottschalk et al., 2013; Queipo et al., 2015).

Currently, understanding the effects of nanoparticles on ecosystems is complex, since the innocuousness of these materials in conventional disposal processes, such as wastewater treatment, was not considered in their conception. In view of this problem, the present review aims to examine the physicochemical properties of nanoparticles, describing how these characteristics can influence their environmental toxicity, especially in aquatic ecosystems where their final destination was not contemplated from their initial synthesis.

3.2 CHARACTERISTICS OF SYNTHETIC NANOPARTICLES

3.2.1 Crystalline structure.

In nature, crystalline forms predominate because their atoms and molecules are arranged in three-dimensional repetitive patterns with well-defined symmetries, such as translation, reflection, and rotation. This arrangement, known as the crystal lattice, constitutes the fundamental principle in the structural basis of nanoparticles (Cao, 2004; Iadonisi et al., 2014). Given the variety of existing crystal configurations and shapes, these have been classified into 14 geometrical structures called Bravais lattices (Sengupta & Sarkar, 2015).

A key feature of nanoparticles is that the repetition of their basic structural unit provides a complete description of the final structure. This organizational capability makes nanoparticle engineering a versatile tool, as it allows their synthesis and controlled manipulation through adjustments in physical parameters such as temperature, light, and electromagnetic fields.

In addition, the crystal structure influences the functional properties of nanomaterials. The wave functions of the atoms in a nanocrystal retain, in part, the individual characteristics of the atoms, which determine their use and application in specific nanoproducts. This phenomenon is key to optimizing the physical and chemical properties of nanoparticles according to their industrial and environmental applications.

3.2.2 Size.

The size of nanoparticles has been defined as an intermediate scale between a single atom or molecule and up to approximately 100 nanometers (nm) in molecular diameter (Auffan et al., 2009). However, the International Organization for Standardization (ISO), through the technical committee TC 229, defines a nanomaterial as a material with at least one external dimension at the nanoscale (1-100 nm), or that presents an internal or surface structure at this same scale (ISO, 2015).

Size reduction at the nanoscale confers unique properties to materials, mainly derived from their large specific surface area and quantum effects. These properties include changes in melting point, optical, magnetic, and mechanical characteristics, which are not observed in larger materials (Casarett, 2008; Mirzaei et al., 2012). For example, at the nanometer scale, quantum confinement effects can give rise to atypical electronic and catalytic behaviors, enabling advanced applications in different fields.

A prominent example of these properties is the dispersion of cuboidal quantum dots in nematic liquid crystals, which results in faster electrical switching and anisotropic photoluminescence (Satapathy et al., 2019). Similarly, suspension of insulating nanoparticles such as TiO_2 and Si_3N_4 in liquid crystals significantly reduces the density of moving ions, transient currents, and threshold voltage (Chen et al., 2009).

On the other hand, adding semiconducting nanoparticles such as ZnO enhances not only the intensity of moving ions but also the electrical conductivity of nanocrystals (Jiang & Toshima, 2009; Pushpavathi et al., 2019). These findings reflect the importance of nanometer size in optimizing physical and chemical properties, expanding the range of applications of functional materials.

3.2.3 Electric and magnetic properties.

Due to their small size and high surface-to-volume ratio, nanoparticles possess unique electronic and magnetic properties. This allows them to interact with energy differently compared to larger materials. These changes originate from electronic structure modifications and increasing surface influence, making nanomaterials particularly sensitive and suitable for applications such as sensors (Nie et al., 2020).

Nanoparticles' small size and morphology cause specific effects in their interaction with electromagnetic energy. For example, gold and silver nanoparticles (40-100 nm in diameter) exhibit visible color changes, creating yellow or red hues due to the excitation of surface plasmon resonances (Tran & Nguyen, 2011).

On the other hand, ferromagnetic nanoparticles such as iron, nickel, cobalt, and certain oxides, with sizes of approximately 10 nm, exhibit superparamagnetism. This implies that they respond strongly to an external magnetic field without possessing a permanent magnetic moment, as thermal energy induces fluctuations in the magnetic dipole of each particle (Cole et al., 2011). These properties are applied in medical diagnostics, such as in contrast enhancement in magnetic resonance imaging and in magnetic hyperthermia therapies, where tumors are destroyed by inducing localized heat (Fortin et al., 2007).

These characteristics are inherent to metallic nanoparticles, especially noble metals, known for their excellent electrical and magnetic conductivity. These properties have enabled the development of advanced applications, such as biomarkers, chemical and biological sensing, optoelectronics, thermal phototherapy, biological imaging, DNA labeling, microscopy, and photoacoustic imaging (Čubová & Čuba, 2020).

3.2.4 Nanoparticles and surfactants.

Combinations between nanoparticles and surfactants are an essential foundation in industries such as extractive and pharmacy medicine. Zeta potential measurements can define the adsorption areas of specific components, whether biological molecules or contaminants, facilitating removal processes or controlled interaction (Nourafkan et al., 2018).

Surfactants play a key role in the synthesis of nanoparticles since they allow for the control of their formation by adjusting the concentration and type of surfactant used. Systematic investigations have shown that different surfactant structures can significantly influence nanoparticle size, morphology, and organization. For example, the formation of basic nanostructures with diameters between 20 and 80 nm has been observed, which can subsequently cluster into particles of final size between 1 and 5 µm, adopting mushroom, cube, and sphere shapes (Housaindokht & Pour, 2012; Trpkov et al., 2018; Xu et al., 2011). A relevant example is the use of sodium dodecylbenzenesulfonate, a surfactant that facilitates the synthesis of nano-spindles, nano-ellipsoids, nano-spheres, and nano-polyhedra of uniform sizes. These results are achieved by adjusting parameters such as reaction temperature, additive amount, and reagent concentrations (Kongsat et al., 2021).

The versatility of surfactants in the manipulation and synthesis of nanoparticles makes it possible to obtain materials with controlled properties, which broadens their applications in various industrial and technological fields.

3.3. PHYSICOCHEMICAL INTERACTION OF NANOPARTICLES IN THE AQUATIC ENVIRONMENT.

The presence, distribution, and persistence of NPs in aquatic systems depend on their colloidal stability. NPs can dissolve, aggregate, agglomerate, settle, or remain stable in the aqueous phase. Experimental partitioning studies indicate that a significant fraction of NPs is removed from wastewater during secondary treatment by sorption onto sewage treatment plant sludge (Musee et al., 2014; Park et al., 2013; Wang et al., 2012). However, these NPs adsorbed on sludge may return to the environment if these sludges are used as agricultural fertilizers, generating a potential risk of release into soils and water bodies.

During wastewater treatment processes, nanoparticles can also be chemically transformed. For example, silver nanoparticles (AgNPs) are mainly transformed into silver sulfide (Ag_2S) in both effluent and sludge (Kaegi et al., 2011).

Partial disaggregation of nanoparticles can be stimulated by natural events such as seasonal variations in hydrological currents, mixing of aqueous streams of different quality (e.g., wastewater and surface water), precipitation events, or local variations in shear stress within a moving fluid. This disaggregation can form smaller aggregates, which increases their resuspension and mobility within the water column, favoring their dispersal in the aquatic system (Troester et al., 2016).

In addition, NPs can enter water bodies through terrestrial runoff, reaching subterranean aquifers and eventually flowing into surface water streams. Kim et al. (2009) demonstrated that iron nanoparticles, stabilized with polyelectrolytes, maintained their original properties after 8 months of desorption in sand columns. This finding raises questions about the design of functional coatings that enhance the biodegradation potential of nanoparticles, thereby reducing their persistent effects in the aquatic environment (Park et al., 2013).

3.3.1 Speciation.

The speciation of nanoparticles in aquatic systems is determined by complex reactions and relationships, influenced by factors such as organic matter content, ligand or surfactant concentration, pH, and ionic strength of the medium (Luoma, 2008). Zhang et al. (2016) described the complex nature of the physicochemical behavior of silver nanoparticles (AgNPs) in aquatic environments, highlighting the need for further research to fully understand the dissolution kinetics of these nanoparticles. They noted that current assumptions based on Gibbs free energy and Ag^+ ion release fail to describe the actual fate of AgNPs in natural environments accurately.

In a different study, Yu et al. (2015) demonstrated that AgNPs, upon exposure to natural aqueous environments and sunlight, undergo oxidation and reduction simultaneously, leading to the release of Ag^+ ions and the formation of new AgNPs.

Metallic nanoparticles can exist in different oxidation states, which depend on the environment in which they are found. These states could lead to their destabilization, thus increasing the risk of contamination in aquatic environments (McGillicuddy et al., 2017; Reidy et al., 2013; Wijnhoven et al., 2009).

3.3.2 Gradients of pH and organic matter.

he colloidal stability and environmental behavior of nanoparticles in aquatic systems are strongly influenced by the hydrochemical conditions of the surrounding medium, particularly by the presence of natural organic matter and electrolyte composition. Natural organic matter can adsorb onto nanoparticle surfaces, modifying particle–particle interactions and affecting aggregation processes. This adsorption may enhance nanoparticle stability through electrostatic and steric repulsion mechanisms, thereby influencing their aggregation behavior, transport, and environmental fate in aquatic environments (Liu et al., 2013).

As organic matter layers do not extend significantly in solution, nanoparticles can interact directly with cells such as microalgae, especially at pH close to 7.5. This could explain the presence of toxicity in aquatic organisms (Quik et al., 2010).

Specific interactions between nanoparticles and organic matter have been observed in different studies. For example, carbon nanoparticles such as fullerenes and carbon nanotubes (Chen & Elimelech, 2007; Hyung et al., 2007), as well as TiO_2 and ZnO nanoparticles (Keller et al., 2010), have shown strong interaction with organic compounds.

In the case of AgNPs, the presence of organic matter has a positive effect by stabilizing their colloidal dispersion and preventing their dissolution. However, it can also induce their partial transformation into Ag_2S particles, which are more stable and less reactive (Zou et al., 2017).

3.3.3 Heteroagglomeration and homoagglomeration.

In aquatic systems, dissolved organic matter plays a key role in stabilizing residual nanoparticles. Homoagglomeration, i.e., aggregation between similar nanoparticles, is usually the predominant mechanism leading to their sedimentation. However, heteroagglomeration, which involves the deposition of nanoparticles on natural colloids and solid fractions of the environment, represents the most relevant process in terms of sedimentation (Quik et al., 2012).

In heteroagglomeration, aggregation and disaggregation processes can occur simultaneously. These phenomena are equally important in determining the behavior and fate of nanoparticles in the aquatic environment. Nevertheless, research on disaggregation processes is considerably less frequent compared to that focused on aggregation. Partial disaggregation can be induced by factors such as humic acid adsorption, a decrease in ionic strength, an increase in organic matter concentration, as well as by variations in temperature or exposure to sunlight (Loosli et al., 2014; Metreveli et al., 2015).

These processes are critical when assessing the potential for bioaccumulation in exposed organisms. Agglomerated nanoparticles may still be bioavailable to aquatic biota even if they increase in size. Filter-feeding organisms that process large volumes of water, using their cilia to trap particles, are particularly susceptible to nanoparticle ingestion. This phenomenon is observed even in species that filter particles ranging in size from 0.4 to 150 micrometers (Baun et al., 2008).

It is important to highlight that ingesting agglomerated nanoparticles with sizes greater than 100 nanometers does not eliminate the associated risks. The weak forces that hold particles together in homoagglomeration or heteroagglomeration processes can dissociate under changes in environmental conditions, releasing smaller nanoparticles. This process can even occur inside organisms, amplifying potential risks to exposed biota (Luo et al., 2025; Wang et al., 2024; Zeng et al., 2024).

3.3.4 Light.

The photosensitivity of nanoparticles is closely related to the activity of surface electrons, which can be excited upon interaction with ultraviolet (UV) or visible light. This phenomenon gives rise to the surface plasmon effect, characterized by molecular resonance that induces the generation of energetic electrons (also called hot electrons) and the energetic separation between holes and electrons. In a typical model, surface electrons absorb resonant photons with coincident energies, causing them to oscillate on the surface of metallic nanomaterials. This generates an enhanced local electromagnetic field and simultaneously excites a large number of energetic electrons (Linic et al., 2011; Narang et al., 2016).

Visible light-driven photocatalysis has become a prominent area of research due to its potential application in clean and renewable energies. Photosensitive nanoparticles, when activated by sunlight, are used as photocatalysts in air and water purification processes. This has generated significant interest in recent years due to their ability to harness solar energy (Naik et al., 2010). However, the low efficiency in utilizing this energy and the short diffusion length of the photogenerated electron-hole pairs limit the effectiveness of these photocatalytic processes (Soni et al., 2008).

Photosensitive nanoparticles are often doped with other molecules or elements to improve their efficiency. This procedure extends the range of light absorption and reduces the recombination of electron-hole pairs, optimizing their photocatalytic performance (Meng et al., 2012).

In aquatic environments, nanoparticles interacting with visible and UV light have a marked redox effect, especially when they interact with atoms of high electronegativity. This generates oxygen vacancies on the surface of the crystal lattice, further enhancing the oxidation-reduction processes and favoring nanoparticle reactivity (Qian et al., 2018; Wang et al., 2019).

3.4 NANOPARTICLES INTERACTIONS AT DIFFERENT LEVELS OF BIOLOGICAL ORGANIZATION.

Nanoparticles (NPs) have the ability to attach to and accumulate on cell membranes, altering their structural and functional properties. These interactions may lead to disruption of proton pumps, inhibition of DNA synthesis, generation of reactive oxygen species (ROS), inactivation of sulfur-containing proteins and enzymes, ribosome denaturation, and degradation of molecules such as lipopolysaccharides (Hu et al., 2018; Moreno-Garrido et al., 2015; Xiang et al., 2018).

Among the physicochemical factors influencing nanoecotoxicity, particle size plays a critical role. Smaller nanoparticles generally exhibit a greater capacity to penetrate cells and interact with biological structures, resulting in stronger biological effects and lower EC_{50} values (Ivask et al., 2014; Lei et al., 2016). For example, CeO_2 nanoparticles in their nanometric form have been reported to be significantly more toxic than their micrometric counterparts (Angel et al., 2015). Similarly, nanometric TiO_2 particles can induce stronger cytotoxic responses due to increased production of reactive oxygen species compared with bulk materials (Sendra et al., 2017a). Variations in nanoparticle size therefore modify their physicochemical properties and ultimately affect their interaction with organisms such as microalgae (Al-Awady et al., 2015; Lekamge et al., 2019; Sendra et al., 2017b).

In the case of iron nanoparticles, particles of approximately 20 nm have been reported to exhibit higher toxicity than larger particles. This increased toxicity has been associated with elevated malondialdehyde (MDA) content, lipid peroxidation, cellular deformation, and a higher tendency for agglomeration (Lei et al., 2016). However, it has also been reported that nanoparticles with sizes between 30 and 50 nm may show higher internalization efficiency compared to smaller (15–30 nm) or larger (70–240 nm) particles, possibly due to more favorable interactions with membrane receptors (Foroozandeh & Aziz, 2018).

In addition to size, surface interactions between nanoparticles and microalgae can influence their environmental behavior. Microalgal cells generally exhibit a negatively charged surface, which may reduce the bioavailability of nanoparticles through electrostatic repulsion. Nevertheless, these interactions may also lead to direct cellular damage and exacerbate toxic effects in algal cells (Khoshnamvand et al., 2020).

These interactions are largely governed by electrostatic forces between nanoparticles and algal cell surfaces, commonly described through the zeta potential. Microalgal cell surfaces typically display negative zeta potential values due to the presence of functional groups such as hydroxyl, carbonyl, and carboxyl groups in the cell wall matrix (X. Li et al., 2023; Sumathi et al., 2025). The cell wall also acts as a major barrier regulating nanoparticle attachment and transport (Jeon et al., 2023). In contrast, nanoparticles may exhibit either positive or negative surface charges depending on environmental conditions such as pH. For instance, Fe_3O_4 nanoparticles show positive zeta potentials under acidic conditions and negative values under alkaline conditions. These differences strongly influence nanoparticle–cell interactions: positively charged nanoparticles tend to exhibit stronger adsorption and cellular uptake due to electrostatic attraction, whereas negatively charged nanoparticles often show reduced adhesion because of electrostatic repulsion (Al-Awady et al., 2015a; J. Zhang et al., 2020). Moreover, positively charged nanoparticles have been reported to induce stronger aggregation and higher toxicity in freshwater microalgae (McKeel et al., 2024).

3.4.1 Nanotoxicity on primary producers.

Microalgae, as key photosynthetic organisms in surface waters, are particularly vulnerable to the presence of NPs. These particles can anchor to the cell wall, penetrate it, and cause severe damage such as loss of flagella, cell swelling, and alterations in cell function (Pretti et al., 2014).

One of the main toxic effects of nanoparticles in microalgae is the shading effect, which directly affects the chloroplast membrane, where light is captured for photosystem II (PSII), key in the production of ATP and NADPH. TiO_2 nanoparticles, for example, are able to enter cells and aggregate on the chloroplast membrane, inhibiting ATP and NADPH synthesis, which significantly reduces cell proliferation due to a lack of energy for growth (Middepogu et al., 2018).

Studies have shown that exposure to nanoparticles severely affects the photosynthetic activity of microalgae. An 18% drop in PSII activity and a 35% decrease in chlorophyll content induced by nanoparticle exposure have been recorded. Moreover, PSII activity was most affected by nanoparticle dose, while the reduction in chlorophyll content was dependent on both the dose and type of nanoparticle (Chen et al., 2018).

Photosynthesis, the essential mechanism for microalgae to capture solar energy and transform it into ATP and NADPH molecules, is critical not only for their development but also for the production of carbohydrates, organic compounds, and atmospheric oxygen (Lehninger et al., 2002). Therefore, any alteration in this process puts at risk the food chain, which depends on microalgae as primary producers.

It has been reported, for example, that cobalt and copper oxide nanoparticles cause photoinhibition of PSII and reduce photosynthesis in species such as *Platymonas subcordiformis*, *Chaetoceros curvisetus*, and *Skeletonema costatum*, due to decreased light absorbed by shading and photocatalysis effects (Cheloni et al., 2016; Chen et al., 2018; von Moos et al., 2015). In *Chlamydomonas reinhardtii*, exposure to cerium oxide nanoparticles (80 nm, 0.01 g/L) has been shown to cause chlorophyll impairment and photoinhibition of PSII (Saison et al., 2010).

In addition, nanoparticles can modify gene expression in microalgae. For example, a reduction in the transcription of genes associated with photosynthesis has been observed in the presence of TiO_2 and ZnO nanoparticles, as well as alterations in the proteosome, ROS production, and enzymatic activity related to cell hydrolysis (da Costa et al., 2016; Simon et al., 2013). The release of metal ions by nanoparticles, as in the case of copper oxide, interferes with photosynthetic mechanisms and exacerbates oxidative stress (Perreault et al., 2012).

A recent meta-analysis showed that the accumulation of reactive oxygen species in the presence of nanoparticles increases oxidative stress by 90%, with this effect being influenced by dose, type of nanoparticle, and species of microalgae exposed (Nguyen et al., 2020).

On the other hand, diatoms have shown toxic effects similar to those observed in green microalgae when exposed to NPs. In species such as *Phaeodactylum tricornutum*, titanium (TiO_2) and cerium (CeO_2) nanoparticles form aggregates in the culture media, enveloping and entrapping diatom cells. This phenomenon causes a significant inhibition in the quantum yield of photosystem II (PSII), affecting photosynthetic efficiency. Simultaneously, an increase in the following parameters has been observed: Chlorophyll a content, soluble sugars, superoxide dismutase (SOD), and peroxidase (POD) enzyme activities. These increases suggest that diatoms activate antioxidant defense mechanisms in response to nanoparticle-induced oxidative stress (Deng et al., 2017).

Exposure to nanoparticles under visible light generates an overexpression of reactive oxygen species (ROS) in organisms such as cyanobacteria. This accumulation of ROS damages phycobiliproteins (the main photosynthetic pigment of these organisms) and phycobilins (water-soluble pigments). The alteration of these components is the leading cause of the malfunctioning of the photosynthetic system, which inhibits cell growth (Fan et al., 2019).

It is worth mentioning that not all photosynthetic organisms absorb the same wavelengths of the visible spectrum. This difference is due to the variability in the photosynthetic pigments involved in the photochemical mechanisms, which prevents direct competition for ecological niches between different groups of photosynthetic organisms (Geider et al., 2001).

3.4.2 Aquatic plants.

The influence of nanoparticles (NPs) on aquatic plants has been less researched compared to other photosynthetic organisms. However, recent studies have shown that silver nanoparticles (AgNPs) have the ability to accumulate in the roots of species such as duckweed (*Landoltia punctata*) after 24 h of exposure. The formation of silver derivatives in roots is attributed to the activation of plant molecular defenses in response to AgNPs uptake (Stegemeier et al., 2017).

On the other hand, Oukarroum et al. (2013) reported a reduction in cell viability in *Lemna gibba* plants exposed to 1 and 10 mg/L concentrations of AgNPs for 7 days. This phytotoxic effect is associated with uncontrolled production of reactive oxygen species (ROS), leading to severe oxidative stress within the cells. The release of silver ions (Ag^+) inside the cells contributed to the generation of ROS and the observed negative effects (Oukarroum et al., 2013).

When exposed to AgNPs and zinc oxide (ZnO) nanoparticles, tissues of *Spirodela punctata*, another duckweed species, showed a similar pattern of oxidative stress. Stressed plants showed higher total antioxidant capacity and elevated superoxide dismutase (SOD) activity compared to untreated controls, reflecting a cellular defense mechanism to nanoparticle-induced stress (Thwala et al., 2013).

Kim et al. (2011) demonstrated that both AgNPs (at low concentrations of 1 ppm) and TiO_2 nanoparticles (at higher concentrations of 250 ppm) inhibited the growth of aquatic plants *Lemna paucicostata* (Banerjee & Roychoudhury, 2019).

3.5 DIFFICULTIES IN THE RISK ASSESSMENT OF NANOPARTICLES.

Ecological risk assessment is a complex process that involves analyzing the likelihood that the environment (atmosphere, hydrosphere, lithosphere, and biosphere) will be affected due to exposure to stressors. Such ecological effects may involve plants, animals, and ecosystems as a whole, as well as human interactions with these systems (Singh, 2016; Stamatoiu et al., 2012).

Despite these concerns, regulations related to products containing nanoparticles remain limited due to the absence of comprehensive risk assessments that include them. This gap represents a critical challenge in identifying and managing the potential risks of nanoparticles. Grieger et al. (2010) and the OECD (2010) have suggested implementing a prioritized nanoparticle classification, focusing on those with the highest potential risk, in order to encourage immediate research to support the early development of specific regulations (Grieger et al., 2010; Rasmussen et al., 2016).

The classification proposal takes into account:

- Exposure assessments, based on production and use levels.
- Potential risk, determined by toxicity tests (see Table 1).

Based on available information, nanoparticles identified as requiring priority research include:

- AgNPs (silver nanoparticles),
- ZnO (zinc oxide),

- Fe_2O_3 (iron oxide),
- TiO_2 (titanium oxide)

Therefore, current ecotoxicological evaluations **coincide with this trend**, as shown in Table 1, which gathers various results obtained from EC_{50} with different types of nanoparticles.

Table 1. Comparative ecotoxicological assessment of engineered nanoparticles: $\mathrm{EC_{50}}$ values across aquatic organisms. Reemplazar las comas por puntos en los valores decimales.

Nanoparticle	**Species**	**$\mathrm{EC_{50}}$**	**Reference**
$\mathrm{TiO_2}$	*Scenedesmus obliquus*	5.5 mg/L	Iswarya et al., 2017
CdS		21.3 mg/L	
ZnS		94.1 mg/L	
$\mathrm{TiO_2}$	*Heterosigma akashiwo*	141.7 mg/L	(Pikula et al., 2022)
SMB3		252.8 mg/L	
SMB24		3.6 mg/L	
CeO_2 NM-211	*Raphidocelis subcapitata*	8.5 mg/L	(Hund-Rinke et al., 2020)
CeO_2 NM-212		10.9 mg/L	
CeO_2 NM-213		98.7 mg/L	
TiO_2 NM-104		126.9 mg/L	
TiO_2 NM-105		4.7 mg/L	
TiO_2 Eu-doped		0.36 mg/L	
TiO_2 Fe-doped		3.6 mg/L	
TiO_2 No doped		0.06 mg/L	
AgNPs	*S. acuminatus*	0.0385 mg/L	Pham, 2019
	C. gracilis	0.0243 mg/L	
	D. lumholtzi	0.0576 mg/L	
$\mathrm{TiO_2}$	*Dunaliella tertiolecta*	24.10 mg/L	Manzo et al., 2015
$\mathrm{SiO_2}$		187.77 mg/L	
CuO	*R. subcapitata*	12.77 mg/L	Andreani et al., 2020
	L. minor	1.9 mg/L	
ZnO	*R. subcapitata*	4.86 mg/L	
$\mathrm{TiO_2}$	*R. subcapitata*	20 mg/L	
$\mathrm{SiO_2}$	*R. subcapitata*	20 mg/L	
ZnO	*Pseudokirchneriella subcapitata*	0.042 mg/L	Aruoja et al., 2009
TiO_2		5.83 mg/L	
CuO		0.71 mg/L	
AgNPs	*P. tricornutum*	163.5 mg/L	Sendra et al., 2017a
$\mathrm{TiO_2}$	*P. tricornutum*	15.23 mg/L	Broccoli et al., 2021
	T. suecica	11.69 mg/L	
	I. galbana	11.78 mg/L	
	S. obliquus	11.76 mg/L	
	P. subcapitata	29.14 mg/L	
ZnO	*P. tricornutum*	4.41 mg/L	
	T. suecica	4.87 mg/L	
	I. galbana	1.82 mg/L	
	S. obliquus	6.42 mg/L	
	P. subcapitata	3.59 mg/L	
AgNPs	*Chlorella vulgaris*	33.63 mg/L	Khoshnamvand et al., 2020
T-AgNP	*Raphidocelis subcapitata*	0.163 mg/L	Lekamge et al., 2019
C-AgNP		0.155 mg/L	
E-AgNP		0.243 mg/L	
P-AgNPs	*Chlorella vulgaris*	50 mg/L	Zhang et al., 2020
N-AgNPs		70 mg/L	

Nanoparticle	Species	EC$_{50}$	Reference
SMB3 (Mesoporous silicon dioxide without metallic inclusions) *SMB24 (Mesoporous silicon dioxide with metallic inclusions)* *T-AgNP* (tyrosine coating) *C-AgNP* (curcumin coating) *E-AgNP* (epigallocatechin gallate coating) *P-AgNPs* (positively charged, polyethylenimine - PEI - coating) *N-AgNPs* (negatively charged, citrate coated – Cit)			*

If we observe the available data, it is possible to identify the different effective concentrations that have been studied for more than a decade in primary producers. Most reported values fall within the range of milligrams per liter; however, these responses may vary when synthetic nanoparticles undergo modifications in their physicochemical properties. For example, in the case of TiO_2 nanoparticles, undoped TiO_2 has been reported to exhibit an EC$_{50}$ of 0.06 mg/L, indicating a relatively high toxicity compared with other modified forms of TiO_2. In contrast, doping with Fe (Fe-doped TiO_2) and Eu (Eu-doped TiO_2) alters the toxicological response, with EC$_{50}$ values of 3.6 mg/L and 0.36 mg/L, respectively. These differences suggest that chemical modification can significantly influence the biological effects of nanoparticles by altering their physicochemical behavior and interactions with organisms. In particular, structural modifications such as doping, surface functionalization, or compositional changes may regulate nanoparticle reactivity and their interaction with biological systems, thereby influencing their capacity to induce oxidative stress (Bouzidi et al., 2024). Changes in nanoparticle composition and surface chemistry have also been reported to modulate the generation of reactive oxygen species (ROS), which act as key mediators of nanoparticle-induced toxicity. Furthermore, modified nanomaterials may exhibit distinct oxidative responses compared with their unmodified counterparts, leading to differences in cytotoxicity and environmental impact (J. Chen et al., 2023). In this context, ROS are widely recognized as central signaling molecules that initiate downstream toxicity pathways, including oxidative damage, inflammation, and apoptosis (B. Li et al., 2022). Therefore, chemical modification may ultimately alter nanoparticle bioavailability and toxicity mechanisms, possibly through changes in the generation of reactive oxygen species (ROS).

Similarly, a variation in toxicity can be observed with Ag nanoparticles, which show considerably higher toxicity compared to TiO_2. For example, in *S. acuminatus*, *C. gracilis*, and *D. lumholtzi*, the EC$_{50}$ values are extremely low (0.0385 mg/L, 0.0243 mg/L, and 0.0576 mg/L, respectively), indicating high toxicity even at minimal concentrations. This is largely due to the release of silver ions, which can generate oxidative stress and affect essential cellular functions.

In addition, the toxicity of AgNPs varies according to the type of coating. Tyrosine-coated AgNPs (T-AgNP) show an EC$_{50}$ of 0.163 mg/L in *Raphidocelis subcapitata*, while those coated with curcumin (C-AgNP) have an even lower EC$_{50}$ (0.155 mg/L). Those coated with epigallocatechin gallate (E-AgNP) show a slight increase in their EC$_{50}$ (0.243 mg/L), suggesting that certain coatings may modulate toxicity by affecting the stability of nanoparticles and their interaction with the environment.

Another relevant aspect is the difference between the EC_{50} values reported for different species. For example, *Scenedesmus obliquus* presents a relatively high toxicity for TiO_2 (5.5 mg/L), while *Dunaliella tertiolecta* shows an EC_{50} value of 24.10 mg/L. These differences may be due to the physiology of each organism and its ability to tolerate or bioaccumulate nanoparticles.

A clear example is SiO_2 nanoparticles. These exhibit relatively low toxicity compared to TiO_2 and Ag, with EC_{50} values in the range of tens to hundreds of milligrams per liter. However, the inclusion of metals in the SiO_2 structure significantly alters their toxicity.

Mesoporous silicon dioxide without metal inclusions (SMB3) shows an EC_{50} of 252.8 mg/L, indicating low toxicity. However, when metal inclusions (SMB24) are incorporated, the EC_{50} decreases drastically to 3.6 mg/L, significantly increasing toxicity. This suggests that metals present in the structure may modify the solubility of the nanoparticle or facilitate the generation of ROS, which increases cell damage in aquatic organisms.

This difference in toxicity highlights the importance of considering structural and chemical modifications in the risk assessment of nanoparticles, since the simple incorporation of metals can transform a relatively inert nanoparticle into a highly toxic one.

The data analyzed demonstrate that physicochemical modifications in nanoparticles can significantly alter their toxicity, from doping in TiO_2 to coatings on AgNPs or the inclusion of metals in SiO_2. These variations underscore the need for detailed characterization prior to their environmental or biomedical application, as well as the urgency of strategies that reduce their negative impact. In its role, the OECD has proposed a more extensive list of representative nanoparticles, with the aim of better understanding the measurement methods, toxicology, and risk assessment associated with other nanostructured materials (ENMs) (Lai et al., 2018; Rasmussen et al., 2016).

Current environmental challenges include a mismatch between rapid innovation in nanotechnology and current environmental regulations, especially in Latin America. This discrepancy has created regulatory gaps that hinder the effective management of the ecological risk associated with the use and release of synthetic NPs. In addition, nanoparticles experience complex and diverse physicochemical interactions in aquatic ecosystems, which makes it extremely difficult to assess their environmental impacts accurately. This generates uncertainty, complicating the implementation of adequate strategies for their prevention and mitigation. There are no internationally validated standardized methodologies to address this problem, which further limits the ability to assess the risks of the impact of nanoparticles in a consistent manner. In addition, nanomaterial synthesis processes are highly toxic, with persistent residues, sometimes with supernatant products that do not precipitate due to their size and are discharged into water tributaries. This widespread lack of friendly approaches represents a significant threat to the sustainability of aquatic ecosystems, not only because of the synthesis, but also because the massive use of commercial products using nanomaterials is on the rise, as industrial synthesis methods increasingly have greater control over size and shape, at an ever-increasing scale of production incluir referencias.

In this context, green synthesis, or the development of nanoparticles with lower toxicity and greater environmental compatibility, has been widely promoted as an innovative strategy, addressing regulatory challenges and fostering a more sustainable approach to nanotechnology incluir referencias. Nevertheless, this perspective should be viewed with caution, as the mere adoption of greener synthesis strategies does not *per se* guarantee a reduced environmental impact in the final disposal of the material. The sustainability of nanoparticles obtained by green synthesis can only be validated if their degradability, toxicity, and persistence in the environment are evaluated comprehensively, thus avoiding reductionist interpretations that could divert the focus from a holistic assessment of the life cycle of these materials.

3.6 REVIEW OF THE SYNTHESIS OF ENVIRONMENTALLY FRIENDLY NANOPARTICLES.

3.6.1 Principles and fundamentals of green nanoparticle synthesis.

Green synthesis of nanoparticles has emerged as a viable alternative to reduce the environmental impact and toxicity of traditional methods, promoting the production of safer and more environmentally friendly materials (Gupta et al., 2023; Iravani, 2020). This approach seeks to minimize the use of hazardous reagents and decrease waste generation by using reducing agents of biological origin instead, such as plant extracts, microorganisms, and biopolymers (Luque-Jacobo et al., 2023).

Conventional nanoparticle synthesis methods often require extreme temperature and pressure conditions, in addition to the use of potentially toxic precursor chemicals. In contrast, green synthesis is based on sustainable chemistry principles, employing biomolecules such as flavonoids, terpenoids, and polysaccharides to reduce and stabilize nanoparticles (Jayeoye et al., 2024). Several studies have shown that biogenic synthesis of metal nanoparticles and metal oxides yields functional materials applicable in critical areas such as biomedicine, catalysis, environmental remediation, and electronic device development, on the same level as nanoparticles produced through traditional synthesis methods. (Ahmed et al., 2024; Liu et al., 2024). In particular, plant extract-mediated biosynthesis has emerged as a highly efficient strategy, in which secondary metabolites present in plants play a dual role as reducing and stabilizing agents, favoring greater colloidal stability and optimizing reaction kinetics (Piermatti, 2021). These advantages not only consolidate the feasibility of biogenic synthesis but also reinforce its potential as a sustainable approach without compromising the quality or functionality of the resulting nanoparticles. However, the main benefits of this strategy are not that natural extracts are the final product but that the reactive means are the ones that replace traditional chemical methods and minimize contamination by chemical waste from the synthesis process (Girotra et al., 2024).

3.6.2 Benefits and applications of green synthesis in industry.

The implementation of green nanoparticle synthesis in industry has addressed multiple challenges associated with pollution and environmental safety. Among the main successful applications on an industrial scale are:

Antimicrobial coatings in medical devices: Silver nanoparticles synthesized by ecological methods have been implemented in bandages, catheters, and biomedical materials to prevent bacterial infections, reducing the use of antibiotics (Wani & Suresh, 2022).

Ecological cosmetics and sunscreens: Zinc oxide nanoparticles obtained through green synthesis have been incorporated into cosmetic products such as safe UV filters, reducing the environmental impact of conventional sunscreens (Holghoomi et al., 2024).

Ecological fertilizers and pesticides: Biogenic copper nanoparticles have been used in sustainable agriculture to improve crop protection without generating toxic residues (Sadhasivam et al., 2020).

Treatment of contaminated water: Iron nanoparticles synthesized from plant extracts have been applied in environmental remediation processes, removing organic pollutants and heavy metals from water bodies (Huston et al., 2021).

Other biosynthesis applications: Green tea (*Camellia sinensis*) leaf extract (Tran Khac et al., 2023) has been used to synthesize AgNPs by biological reduction of silver ions, taking advantage of its phenolic compounds as natural reducing agents. Neem (*Azadirachta indica*) leaves for the synthesis of Ag, Au, and bimetallic Au@Ag nanoparticles (Shankar et al., 2004) and also aloe vera (Sharma et al., 2022), both have been widely studied due to their antioxidant and ecological metal reduction properties. Fresh leaf extracts of *Ocimum basilicum L*. (basil), *Origanum majorana L*. (marjoram), *Satureja mexicana Briq*. (mint), *Mentha piperita L*. (peppermint) and *Eucalyptus globulus* have also been used to synthesize AgNPs (Hernández-Pinero et al., 2016; Salgado et al., 2023).

The synthesis of nanoparticles has been widely studied using isopolymers or natural biomolecules such as chitosan (Ben Amor et al., 2024), cellulose (Mochochoko et al., 2013), or natural proteins such as albumin or gelatin (Fahim et al., 2024). These are used as stabilizing and reducing agents, significantly reducing toxicity and environmental impact compared to traditional chemical methods. Filamentous fungi such as *Aspergillus niger* and *Penicillium chrysogenum* have also been used in the biosynthesis of silver nanoparticles. In addition, *Penicillium chrysogenum* has been used to synthesize CuNPs and CuO NPs, showing potential antimicrobial and environmental bioremediation applications (Ahmad et al., 2024).

On the other hand, the green synthesis approach not only focuses on the use of biological reduction or oxidation agents, but also on obtaining zinc oxide and titanium dioxide nanoparticles that are synthesized in aqueous routes at low temperature, avoiding the use of organic solvents and high temperatures, decreasing energy consumption and reducing toxic waste (Singh Jassal et al. 2022), in addition to microwave-assisted synthesis systems, because they allow obtaining nanoparticles under mild experimental conditions, in short reaction times with lower energy consumption and significant reduction in the use of toxic solvents (Pechyen et al., 2024).

These practices demonstrate good progress in the concept of green synthesis, emphasizing environmentally responsible, efficient and safer alternative processes in the industry.

3.6.3 Ecotoxicological gaps in sustainable nanomaterial innovation: between green synthesis and real environmental impact.

Despite the above, the accumulation and interaction of nanocontaminants in the environment continues to be one of the main concerns in the scientific field. In this context, it is crucial to redirect the focus towards the final product - the nanoparticles - and not solely towards the synthesis process (Soltys et al., 2021). Adopting an environmentally responsible approach in the design of nanoproducts would allow moving towards extended producer responsibility, thus mitigating the potential ecotoxicological effects *in situ*. For this, it is essential that the green synthesis incorporates ecotoxicological assessments of the final product through representative analyses of the receiving ecosystems, which would contribute to reducing the release of persistent chemical compounds in aquatic, terrestrial, and atmospheric environments.

Thus, as the synthesis of nanoparticles through ecological routes consolidates as an attractive alternative to conventional methods, several studies have reported advances in designing nanomaterials as products that promise natural and biocompatible residues with different environmental matrices. However, a detailed analysis of the literature shows that most of this research lacks ecotoxicological evaluations that support the environmental safety of the materials.

For example, Kulkarni et al. (2023) highlight the advantages of biogenic nanoparticles in biomedical applications, emphasizing their biocompatibility and low toxicity. However, the authors themselves acknowledge the lack of information on their degradation, distribution, and ecotoxicological effects in the receiving environment, which limits their application beyond the laboratory. Similarly, Kucuk et al. (2025) propose nanoparticles obtained from plant extracts for sensors in biological and pharmaceutical environments, but omit to consider their potential impact once released into natural ecosystems.

Even in applications designed for environmental remediation, such as those proposed by Wu et al. (2025) with titanium nanoparticles to remove pesticides in water, no *in situ* studies assessing bioaccumulation or effects on aquatic organisms are reported (Wu et al., 2025). Khatri et al. (2023) present green electrospinning as an innovative strategy to produce biodegradable nanofibers applicable in medicine, remediation, and sustainable packaging, but their analysis focuses on technical aspects of the process, without incorporating environmental toxicity assessments under real conditions (Khatri et al., 2023).

Likewise, Keçili et al. (2025) develop sensors based on materials such as graphene oxides and carbon quantum dots obtained by environmentally friendly methods for detecting antibiotics in food. Although they stand out for their analytical efficiency and sustainability in their synthesis, the adverse effects that could result from their release into the environment are not addressed. In the biomedical field (Keçili et al., 2025), Anwar et al. (2021) highlight the potential of biodegradable nanoparticles for the release of drugs and vaccines. However, they acknowledge that commonly employed polymers, such as PLGA or PEG, can generate acidic by-products for which the environmental toxicity has not been explored outside the clinical context (Anwar et al., 2021).

These examples highlight a critical gap in the development of sustainable nanomaterials. Although significant progress has been made in terms of synthesis and functionality, there is still a lack of comprehensive environmental validation to ensure their ecological safety. Overall, the literature reflects a worrying trend: “green” nanomaterials are promoted as safe and sustainable without standardized studies that assess their toxicity under real environmental conditions. This omission limits the ability to anticipate ecological risks associated with their extensive use and underlines the urgent need to integrate representative ecotoxicological testing from the early stages of nanoproduct design.

3.6.4 Limitations and challenges of green nanoparticle synthesis.

Despite its many advantages, green synthesis faces limitations that hinder its large-scale implementation, such as low reproducibility associated with variability in size and morphology of the obtained nanoparticles, poor industrial scalability due to unresolved technological barriers, and generally longer reaction times compared to conventional chemical methods (Gupta et al., 2023; Holghoomi et al., 2024; Jayeoye et al., 2024).

Even though green synthesis proposes methodologies with lower environmental impact due to the advantages mentioned above, such as mild conditions in the synthesis processes, the use of biological agents or renewable resources, and/or the use of heating and self-reduction systems when microwaves are used, critical challenges remain. Due to their physicochemical nature, the generated nanoparticles can also transform, accumulate, and cause significant ecotoxic effects in aquatic ecosystems. This means that, although the production process may appear environmentally friendly and clean, it does not necessarily guarantee that the final product will be harmless. This paradox implies that the environmental approach should not be limited only to the stages of the synthesis process, but should also consider the entire life cycle of nanoparticles, including their interaction and subsequent transformation in ecosystems. This raises the need to develop synthesis methods to obtain nanoparticles by ecological routes and by containing intrinsic properties that make them less toxic and environmentally compatible.

As authors, we have called the above effect "The Green Synthesis Uncertainty", which refers to the phenomenon in which nanoparticles **synthesized using methods considered ecological or green** can still represent a high ecotoxicological risk once they are released into the environment. In other words, an environmentally friendly production process does not automatically guarantee ecological safety throughout the life cycle of the material. Therefore, it is necessary to comprehensively consider the stability, toxicity, and environmental behavior of nanoparticles and other nanomaterials from the early stages of their design to their final disposal.This paradox uncertainty shows that initial or apparent sustainability does not necessarily guarantee long-term environmental harmlessness, underlining the need for comprehensive and preventive ecological impact assessments throughout the life cycle of any technological innovation. In this sense, it shows how good intentions and 'green' approaches in early stages may not be sufficient to ensure real and lasting sustainability, especially when unexpected negative effects are manifested after implementation or release into the environment.

3.6.7 Recommendations for the nanoparticle industry.

Progress in nanoparticle synthesis has traditionally been focused on process optimization. However, in the current sustainability and environmental regulation context, shifting the focus towards the final nanoproduct is imperative. A key objective is to obtain nanoproducts with higher biodegradability and lower environmental persistence, which will mitigate the risk of bioaccumulation and long-term ecotoxicological effects. To achieve this transition, it is essential for the industry to adopt strategies that not only reduce the environmental impact of synthesis but also guarantee the safety and functionality of the final product throughout its life cycle.

To encourage the adoption of green synthesis in the production of nanoparticles, the following is recommended to the industry:

Optimizing the use of renewable sources: As mentioned in previous sections, integrating plant extracts, microorganisms, and biopolymers as reducing and stabilizing agents in the synthesis of nanoparticles ensures that these components not only minimize the process's toxicity but also positively influence the degradability of the final nanoproduct (Moond et al., 2022).

Establishing strict environmental regulations: Implementing regulations that not only evaluate the toxicity of nanoparticles in their synthesis stage, but also consider their persistence and effects in different environmental compartments before their commercialization (Iravani, 2020). Unlike other pollutants, such as microplastics, which are an environmental waste product of the accumulation and degradation of plastics used for decades, nanoparticles can have an eco-friendly vision from their inception, and that is a problem that can be avoided beforehand, considering all the possible effects exposed, so adopting a preventive vision before environmental regulations is imperative to achieve objectives established by the UNEP.

Promoting the scalability of green synthesis: Investing in technologies that enable large-scale production without compromising the sustainability of the final product, ensuring that the transition to green nanotechnology is technically, economically and environmentally feasible (Ahmed et al., 2024).

3.7 CONCLUSION

Current research on NPs in the environment has demonstrated their increasing presence in aquatic ecosystems. However, important knowledge gaps remain regarding their fate, transformations, and toxicological effects on primary producers.

This review critically evaluates the most recent findings on the interaction of NPs with aquatic systems, demonstrating that current ecotoxicological assessments may underestimate actual environmental risks due to simplified experimental conditions and inadequate regulatory frameworks. The influence of surface chemistry and aggregation dynamics further complicates toxicity predictions, making a paradigm shift in risk assessment methodologies imperative.

Despite growing awareness of these risks, significant knowledge gaps remain regarding chronic exposure effects, mixture toxicity, and specific responses among species at different trophic levels. Furthermore, although the use of green synthesis methods shows promise, it raises new concerns due to the absence of standardized protocols and effective regulation, which could introduce novel bioactive compounds with unknown ecotoxicological profiles.

In the face of these challenges, it is recommended that future research adopt integrative approaches that combine high-resolution physicochemical characterization, realistic exposure scenarios, and multi-biomarker assessments in environmentally relevant species. Similarly, it is urgent to develop a regulatory framework that considers the complexity of the behavior of nanoparticles as products, including their transformations, trophic transfer, and potential long-term impacts, to ensure the safe design, use, and disposal of nanomaterials in aquatic systems.

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AUTHORS’ CONTRIBUTIONS.

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CAPÍTULO 4

First evidence of anthropogenic TiO_2 nanoparticles occurrence in Chilean rivers

Primera publicación científica

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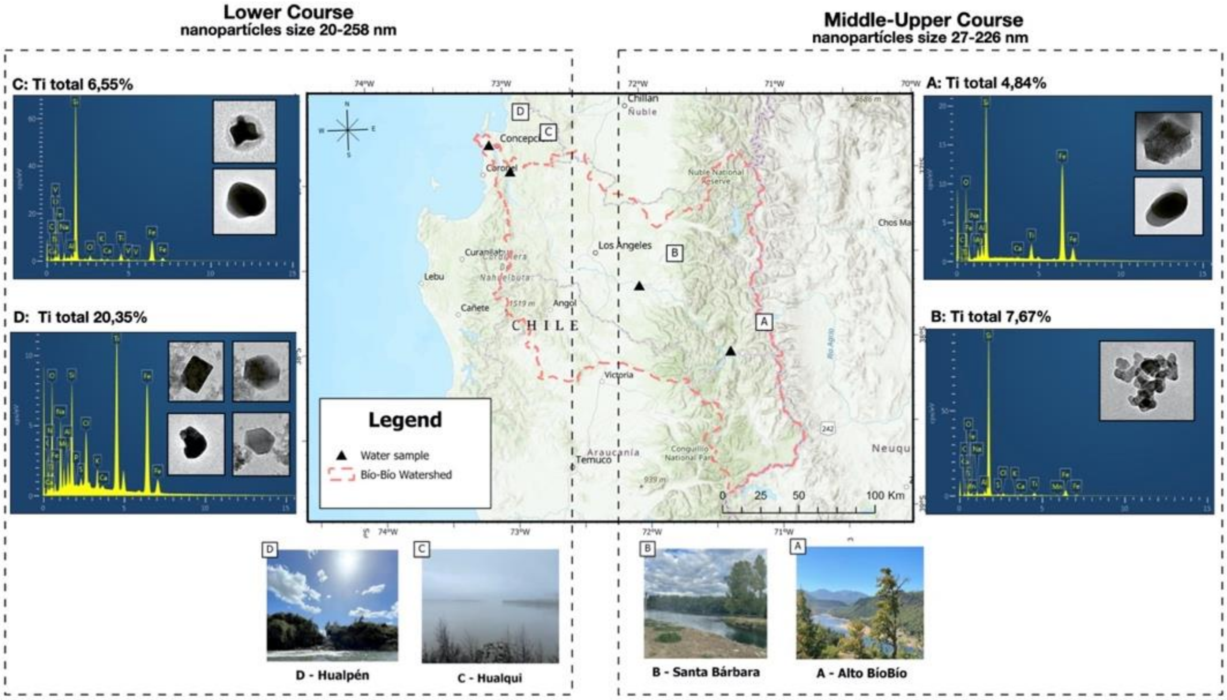
Graphical abstract



ABSTRACT

This study marks the first recorded case of TiO_2 nanoparticle pollution in Chilean rivers, indicating significant progress in understanding the distribution of nanowaste and its effects on a global scale. By investigating four different locations, including the outlet of a wastewater treatment plant during summer, winter and spring, the research revealed varied concentrations of TiO_2 nanoparticles, with a notable range between 17.6 μg L^{-1} during the summer and 22.9 μg L^{-1} in spring in downstream river sections. The study used transmission electron microscopy to characterize nanoparticles, observing sizes between 10 and 206 nm, and an EDS detector confirmed titanium proportions of 4.84 % to 20.35 % by dry weight. These TiO_2 nanoparticles, predominantly in Rutile and Anatase forms, denote a significant environmental presence, especially considering the low population densities of the sampling areas. The findings highlight the urgent need for international awareness and routine monitoring of nanowaste, advocating for preventive actions in the production of nanomaterials and adaptive management strategies in tune with the dynamic nature of water systems and environmental changes, both for places densely and sparsely populated.

Keywords: TiO_2 nanoparticle, Surface water, Nanowaste, Pollution, Field study

4.1. INTRODUCTION

Over time the nanotechnology industry has become among the most versatile, improving various chemical substances with unique properties according to their use, function, and fate (Yadav & Ahmaruzzaman, 2022). The characteristics according to which these substances are classified as nanoparticles are size below 100 nm and defined structures with specific functions that form molecular agglomerates or aggregates with weak or strong bonds that allow them to react with other components of the medium (Ale et al., 2019; Cascio et al., 2015; Zhang et al., 2022). However, the efficacy and long-term stability of these nanoparticles remain poorly understood, particularly for those engineered with a specific application in mind without adequately considering their eventual environmental disposal. This gap in understanding represents a potential environmental hazard, as the oversight of post-use disposal practices can lead to ecological disturbances. Such disruptions may contribute to imbalances in natural processes, affecting ecosystem homeostasis (Banerjee & Roychoudhury, 2019; Deng et al., 2017; Fan et al., 2019; Kim et al., 2011; Oukarroum et al., 2013; Stegemeier et al., 2017).

In recent years, TiO_2 nanoparticles have been incorporated into various everyday and domestic products. They can be found in cosmetic additives (Morais et al., 2022), medications (Farshbaf et al., 2022), and foods (dos Santos et al., 2020). TiO_2 has chemical stability properties as an amphoteric oxide, and is an n-type semiconductor, which makes it sensitive to light, absorbing mainly UV radiation, making it the most used photocatalyst to degrade organic molecules. It is also used as a white pigment, anticorrosive coating, gas sensor, UV-ray absorber in cosmetic products, and generally in the ceramic industry (Khan et al., 2020, 2023; Rai et al., 2019).

Synthetic nanoparticles of TiO_2 lack trace elements within their structure and typically exhibit a size below 100 nm, white pigmentation, and an isoelectric point less than 3. Their composition primarily consists of anatase and/or pure rutile, sometimes coated with small amounts of aluminum and/or silica to enhance technological properties. The affinity of these nanoparticles with organic matter is contingent upon the type of surfactant they are associated with. Consequently, one can anticipate the presence of nanoparticulate structures associated with various forms of organic matter, including lipids or in their free form (Campos et al., 2022; Liao & Liao, 2007). TiO_2 nanoparticle production accounts for around 37 % of total nanoparticle production, and it is one of the most used nanomaterials worldwide (Yadav & Ahmaruzzaman, 2022); this statistic implies a high probability that TiO_2 will be found as waste in natural water bodies due to increases in the use of these products (Keller et al., 2010).

There are studies that show the existence of these nanoparticles downstream of urban settlements (Bäuerlein et al., 2017). Complementarily, anthropogenic input of TiO_2 nanoparticles through wastewater treatment plants inside and outside the facilities has been reported in different parts of the world (Westerhoff et al., 2011), as well as their input through water runoff of rain to different river systems (Boenisch, 2020; Nabi et al., 2021). The quantification and identification of nanoparticles allow evaluating the long-term effects on ecosystems exposed to TiO_2 nanoparticles; This information can help define possible scenarios after the release of nanoparticles to the environment, considering bioavailability and transport in aqueous solutions. In Chile it has been shown that existing wastewater treatment systems do not have adequate technology to eliminate emerging contaminants (Rozas et al., 2016). Therefore, it does not guarantee that nanowaste is removed within the system. Furthermore, there is an information gap due to the lack of studies on nanopollutants, so there is not enough evidence to expand current regulations and prevent the degradation of the water available in Chile for consumption, this being an important issue to address, especially in through new scenarios due to the effect of climate change. The Biobío River is one of the main rivers in Chile, it has a network of rivers that contribute to the drainage basin and has a high socioeconomic value for the region and the country, providing various ecosystem services, including water extraction for irrigation in the agricultural and forestry industry, drinking water supply and hydroelectricity. energy production, among others (Díaz et al., 2018). Currently, the Biobío basin supplies drinking water to 1.2 million people, who in turn discharge domestic water through the main wastewater treatment plants, closing the cycle of use and reuse of this resource. This is why it is important to quantify the life cycle of TiO_2 nanoparticles, considering that there is currently no monitoring of the consumption of the products that contain them, so it is necessary to know the quantities and ways in which they enter the aquatic systems after its application.

The objective of this study is to explore the presence of TiO_2 nanoparticles in one of the main rivers of Chile, determining the concentrations, morphologies and sizes of these nanoparticles.

Furthermore, the study seeks to evaluate the environmental influence on the origin of TiO_2, differentiating anthropogenic and natural sources.

4.2. MATERIAL AND METHODS

4.2.1. Study area and samples collection

Situated in south-central Chile, the Biobío River spans between latitudes 36°42′ S and 38°49′ S, and longitudes 71° W to 73°20′ W, covering a catchment area of 24,264 km². At 380 km in length, it stands as one of Chile's largest basins. Its hydrological profile is complex, featuring nival origins in the Alto BíoBío region and pluvial contributions in its middle course, which often result in seasonal flooding, predominantly in the winter and spring months (Caro, 2004). Human activities variably impact the river along its course, exploiting its resources for a range of purposes. This is set against a backdrop of significant biological processes and hydrodynamic shifts that are seasonally driven.

This study examines the influence of human activities on nanoparticle dissemination in the Biobío River, integrating both anthropogenic and natural seasonal fluctuations. Sampling was strategically conducted in line with the distinct climatic seasons of the southern hemisphere—specifically during the summer, winter, and spring of 2022. The research was carried out at four key locations along the river: Alto Bíobío, Santa Bárbara, Hualqui, and Hualpén, chosen to elucidate the gradient of contamination (Figure 4.1).

Figure 4.1: Study area location in 1 and 2, and points at which sampling was performed during 2022 in A, B, C, and D.

At each sampling site, 3 surface water samples were collected no more than 30 cm from the surface in 1 L amber glass bottles in triplicate and in 10 L HDPE-quality plastic jugs. Before their use, the bottles and jugs were washed with phosphate-free neutral detergent and then with 10 % hydrochloric acid (Sigma Aldrich, EMSURE® ACS, ISO, Reag. Ph Eur) for at least 24 h. They were subsequently rinsed three times with running water and then submerged in ultrapure water (Easy pure, Centro-EULA, Chile) for 24 h. To dry the glass bottles, they were kept in a stove at 60 °C for another 24 h. The jugs were dried in open air face down, avoiding contact with any type of trace contaminants, for 24 h. During the sampling, before each sample was taken the bottles and jugs were primed three times with surface water from each sampling point before the definitive water samples were taken. The bottles with samples were handled with nitrile gloves to avoid any contamination from outside the sampling location. The samples were stored in the dark at 4 °C until further analysis.

4.2.2. ICP and ICP-MS analysis

The methodology for sample preparation and elemental analysis via ICP-MS and ICP was carried out with precision. Initially, the samples were subjected to digestion: each was homogenized and placed in Teflon containers, each with a capacity of 50 mL. To this, 3 mL of Suprapur® concentrated nitric acid was added. The mixture was then heated until the volume was reduced to 10 mL. Subsequently, the concentrates were transferred to 50 mL plastic volumetric flasks, supplemented with an additional 5 mL of nitric acid, and then diluted to the marked volume with ultrapure water.

Elemental concentrations of titanium (Ti), with a limit of detection (LOD) of 0.02 µg L^{-1}, niobium (Nb, LD 0.001 µg L^{-1}) and vanadium (V, LD 0.085 µg L^{-1}) were quantified. These analyzes aimed to distinguish natural titanium dioxide (TiO_2) nanoparticles from those of anthropogenic origin, following the approach suggested by Nabi (2021). The determination was carried out using an ICP-MS (Thermo Scientific iCAP RQ). Additionally, iron (Fe, LD 1 μg L^{-1}), manganese (Mn, LD 1 μg L^{-1}) and aluminum (Al, LD 5 μg L^{-1}) were incorporated into the study and evaluated by ICP using an optical emission spectrophotometer (Perkin Elmer Optima 8000).

Calibration of the analytical instruments was performed using Certipur $TiCl_4$ in HCl (1000 ± 2.00 g L^{-1} Ti), a vanadium (V) ICP standard (1000 mg L^{-1}) and a niobium (Nb) standard (1000 ± 5 μg mL^{-1}). In a mixture of nitric and hydrofluoric acid). Multielement standards for Fe, Mn, and Al were used for ICP analysis (Baird & Bridgewater, 2017).

4.2.3. Nanoparticle detection using TEM and VP-SEM with EDS detector

The methodology for nanoparticle observation and analysis used a Talos F200 G2 transmission electron microscope (Thermo Scientific) equipped with a high-resolution CETA 16M CMOS camera. This configuration, including an in 3D.

Sample preparation involved triplicate transfers to 50 mL Falcon tubes, followed by a 10 min sonication period. This was followed by a tiered centrifugation protocol: 500 rpm for 4 min, 3500 rpm for 5 min and finally 5000 rpm for 30 min, with meticulous pipetting of the sample after each step, as recommended by Phillipe et al. (2022). Samples were then mounted on Formvar/carbon coated TEM 200 mesh copper grids and dried at 60 °C, 10 μL drops were concentrated onto the grid as many times as necessary to ensure a smooth and homogeneous grid surface. low magnification.

The comparative analysis used a titanium (IV) oxide standard (mixture of rutile and anatase, particle size < 100 nm) prepared at 10 μg L−1 for TEM observation, following the same procedures applied to the water samples.

Additional observations were made using a Zeiss EVO MA10 variable pressure scanning electron microscope (VP-SEM) with an Oxford x-act EDS detector, where AZtec 6.0 SP1 software facilitated data analysis. Samples were coated with carbon to a thickness of 10 nm and spot spectra were acquired using automated settings over a period of 6 min.

For nanoparticle dimension analysis, ImageJ version 1.54c, a Java-based open source imaging software, was used.

4.2.4. Statistical analyses

The Shapiro–Wilk test was applied to assess the normality of the data distribution of each recorded variable. In addition, Kruskal–Wallis was used to determine the existence of statistically significant differences in the recorded variables according to the “Location” and “Season” factors. Finally, an analysis of variance (ANOVA) was carried out to determine the existence of statistically significant differences in the recorded variables according to the “Location” and “Season” factors. The program used was Statistical analysis was conducted using R Studio version 4.3.1.

4.3. RESULTS AND DISCUSSION

In the four sampling points studied, a clear similarity was evident according to their physicochemical characteristics and seasonal variations (summer, winter and spring) in the Alto Biobio and Santa Bárbara sites located in the upper middle zone of the river, according to the recorded field data. The same similarity is observed in the Hualqui and Hualpén sampling points located in the lower part of the river. However, these last two points differ from the two points mentioned above. These differences stand out even more when the factor of the gradient of urban activity is considered, since as one advances along the course of the river, urban settlements increase and with it the discharges from the treatment plant into the river (Vera et al., 2013). Therefore, the Hualqui and Hualpén sampling sites are the ones with the greatest urban influence because the main wastewater treatment plants are located, and according to the information provided by the Superintendence of the Environment (2019), they treat the domestic waters of more than 559,908 inhabitants (Table 4.1)

In Figure 3 (dendrogram), the previously mentioned observations are visually depicted. A sizable cluster encompasses all the assessed localities along the Biobío River, emphasizing the notable similarity between the localities of Alto Biobío and Santa Bárbara across multiple stations. Within the second group, comprising the towns of Hualqui and Hualpén, a substantial divergence is evident during winter compared to the rest of the localities.

The dendrogram clearly illustrates the subdivision of the four sampling points based on their chemical and physical characteristics into two distinct groups. The upper-middle course is characterized by the grouping of Alto Biobío and Santa Bárbara, while the lower course of the Biobío River accommodates the points of Hualqui and Hualpén.

Table 4.1. Locations and descriptions of the main activities at the sampling points.

Sampling station on the Biobío River	No. of inhabitants	Coordinates	Altitude (meters above sea level)	Area (Km^2)	Main economic activities in the location[1]
Alto Biobío	6775	38º05'03"S 71º20'39"W	1312	2124.6	Rural: commerce, tourism, agriculture, livestock, and fishing.
Santa Bárbara	14592	37º40'20"S 71º02'34"W	214	1254.9	Urbanized: Agriculture, forestry, fishing, commerce, transport, tourism, and water supply and wastewater management.
Hualqui	26201	36º58'06"S 72º57'52W	30	530.5	Urbanized: Commerce, transport, service activities, tourism, agriculture, forestry, fishing, manufacturing, and water supply and wastewater management.

Sampling station on the Biobío River	No. of inhabitants	Coordinates	Altitude (meters above sea level)	Area (Km^2)	Main economic activities in the location[1]
Hualpén[2]	97273	36º48'24''S 73º06'26''W	6	53.5	Urbanized: manufacturing, water supply and wastewater management, commerce, transport, service activities, lodging services, and food services.

[1] data provided by the Library of the National Congress of Chile, with a projection to 2020; [2] The Hualpén point is at the discharge of the main wastewater treatment plant of Concepción, with tertiary treatment.

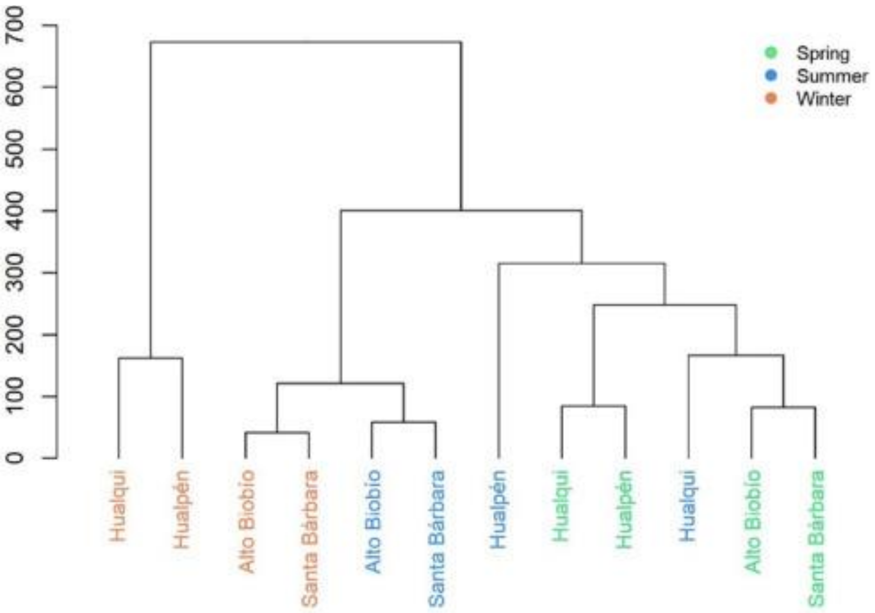


Figure 4.2. Similarity analysis (dendrogram) based on untransformed data matrix of variables recorded in surface water at different locations along the course of the Biobío River in different seasons.

4.3.1. ICP-MS analyses

The ICP-MS analyzes of the water samples showed the presence of total Ti in all sites in concentrations of the order of µg L^{-1}, with higher concentrations found in Alto Biobío in summer 24.6 µg L^{-1}, Hualqui in winter 39.5 µg L^{-1} and Hualpén in spring 22.9 µg L^{-1} (Fig. 4.3). The analysis carried out for total Ti, Al, Fe, V, Nb and Mn, in surface waters, shows the existing profile in the sampling areas, with the predominant presence of Fe and Al and, to a lesser extent, Mn, while for Nb and V values were below the detection limit. It is important to describe the concentration of the elements mentioned above to contrast with Ti and thus be able to establish the possible origins of TiO_2 present in the surface water (Vidmar et al., 2022).

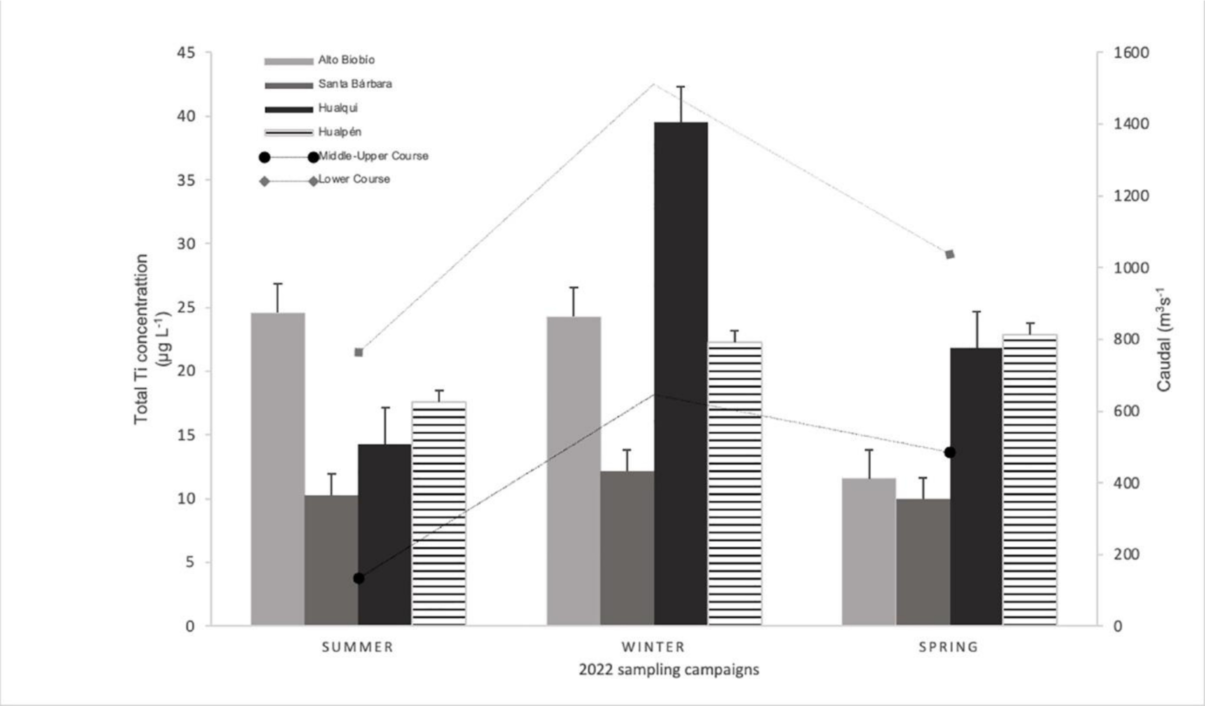


Figure 4.3. Total Ti results obtained via ICP-MS by campaign in the Biobío River and flows at the different sampling points; from left to right: Alto Biobío, Santa Bárbara, Hualqui, Hualpén. The standard error bar represents the analysis of three samples taken at the same sampling point in each season.

Figure 4.3 illustrates the fluctuation in total Ti concentrations (left Y-axis) along the river in correlation with measured flow (right Y-axis), during summer, winter, and spring. The summer data reveal a gradual increase in Ti concentrations from Santa Bárbara to Hualpén, this coincides with the decrease in flow in this period of the year, evidencing the concentrations of solutes in the river. However, surprisingly, in Alto Biobío, characterized by minimal human interference, in the same season the highest concentration was recorded with 24.6 µg L^{-1}.

This unexpected result may be attributed to the influence of Ti concentration resulting from ice melting on the mountain. With a river flow of 135 $m^3\ s^{-1}$, the lowest among all points measured, the Ti peak could be linked to atmospheric transport of nanoparticles from urban areas. These particles may condense and encapsulate in the coldest mountain points during winter, subsequently releasing between spring and summer.

This hypothesis finds support in the research of Azimzada et al. (2020), which highlights climatological influences, particularly in cold climates, on the transport of TiO_2 nanoparticles (Azimzada et al., 2020). On the other hand, it should be noted that in Andean areas at the sampled points, the type of soil through which the river passes contains Ti minerals in nature, highlighting magnetite-ulvöspinel (Fe_2TiO_4) and ilmenite-Hematite ($FeTiO_3$), According to a report from the National Geological Service of Chile in 2003, the soil formations present in the basin influence the composition of the water that flows over the territory. The upper part, where the river originates, is composed of sedimentary volcanic rocks, sandstones, paraconglomerates, andesitic and dacitic lavas and intercalations of ignimbrites, limonites and limestones. The soils are mainly composed of OM2c, KT2 and PPI3 and according to these geological codes (Figure 4.4) these have high Fe contents and formation of Fe oxides, explaining the high concentrations of Fe found in surface waters, these soils being the main soils that naturally present Fe-Ti interactions (Henriquez, 1978). Furthermore, according to reports from the General Directorate of Water of Chile (DGA, 2022), in this area of the upper middle course the flow is much lower than at the mouth of the river (Fig. 4.3); Therefore, there is an effect of the concentration of Ti in the area. According to this effect and considering the effects of evaporation in summer, it would be interesting in a future study to sample Andean lakes to analyze the possibility of nanoparticles arriving through atmospheric transport. In the midstream Santa Bárbara region, analysis of variance of Ti, Al, and Fe concentrations (Fig. 4.5) demonstrated minimal seasonal fluctuation, with Ti levels remarkably consistent, ranging only between 10 and 12 $\mu g\ L^{-1}$ throughout the year.

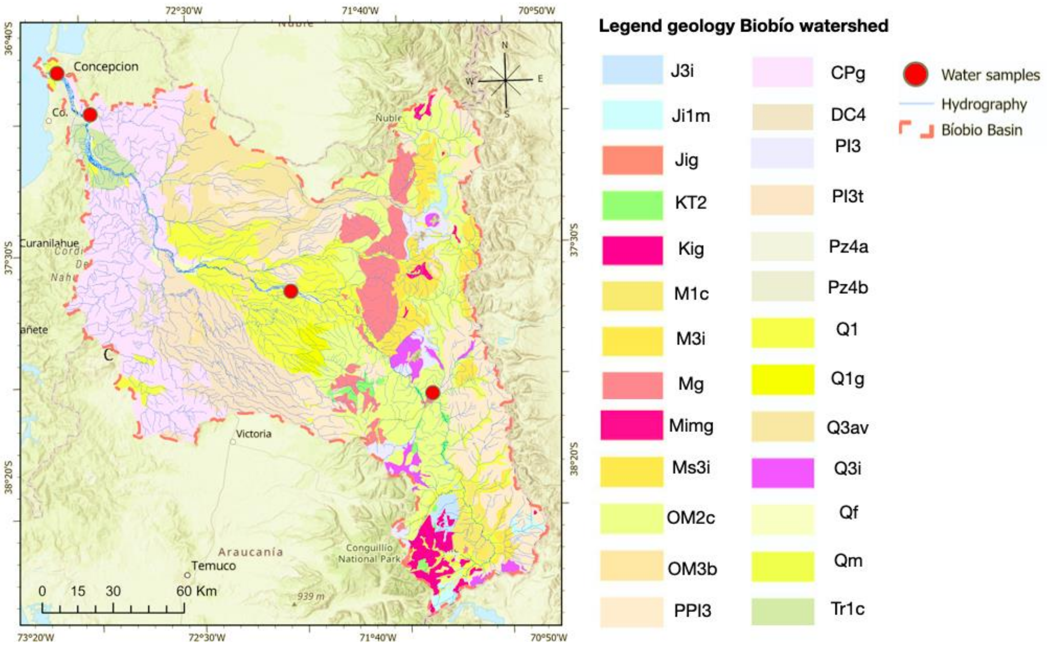


Figure 4.4. Geological formations and drainage network of the Biobío River basin. Esta información fue extraída de donde?

This stability likely reflects river flow dynamics and local soil composition, suggesting that the Santa Bárbara site can serve as a reference point for establishing natural background levels against which contamination can be measured. Therefore, future studies should consider incorporating an upstream sampling location to differentiate between baseline Ti contributions from the Alto Biobío region and potential nanoparticle contaminants arising from urban activities in Santa Bárbara. The third sampling site is located in Hualqui, located in the lower part of the river basin. At this sampling point, the highest Ti concentration is found in winter (39.5 $\mu g\ L^{-1}$) compared to the other sampling points sampling. This is not unexpected, since upstream of Hualqui is the confluence of another important river in the hydrographic basin that contributes to the main course of the Biobío River. This has a spring with soil formation characteristics similar to the Biobío River, it rises in a lake located in the middle of volcanic sequences where other studies reported the existence of TiO_2 concentrations (Vera et al., 2013).

Another important factor to consider is that this tributary river also receives interactions from large cities located in the middle course of the region and that could enhance the contributions of Ti (Albornoz Tapia, 2019). Therefore, the Ti peak observed at the Hualqui sampling point is not surprising, especially in winter due to precipitation that contributed to an increase in the flow of the Laja River of around 700 mm year^{-1} in 2022 (DGA, 2022), which could drag these particles towards the Hualqui area. At the last sampling point, few of the Ti contributions observed could be of natural origin, since the soil in the area is of type CPg and Q1, formed by conglomerates, breccias, sandstones, shales and limestones that interact with contributions of Ca and Si, not related to basaltic soils linked to oxide minerals that affect natural Ti contributions. Therefore, the Ti concentrations observed in Hualpén in summer (17.6 µg L^{-1}), winter (22.3 µg L^{-1}) and spring (22.3 µg L^{-1}) are mostly of anthropogenic origin.

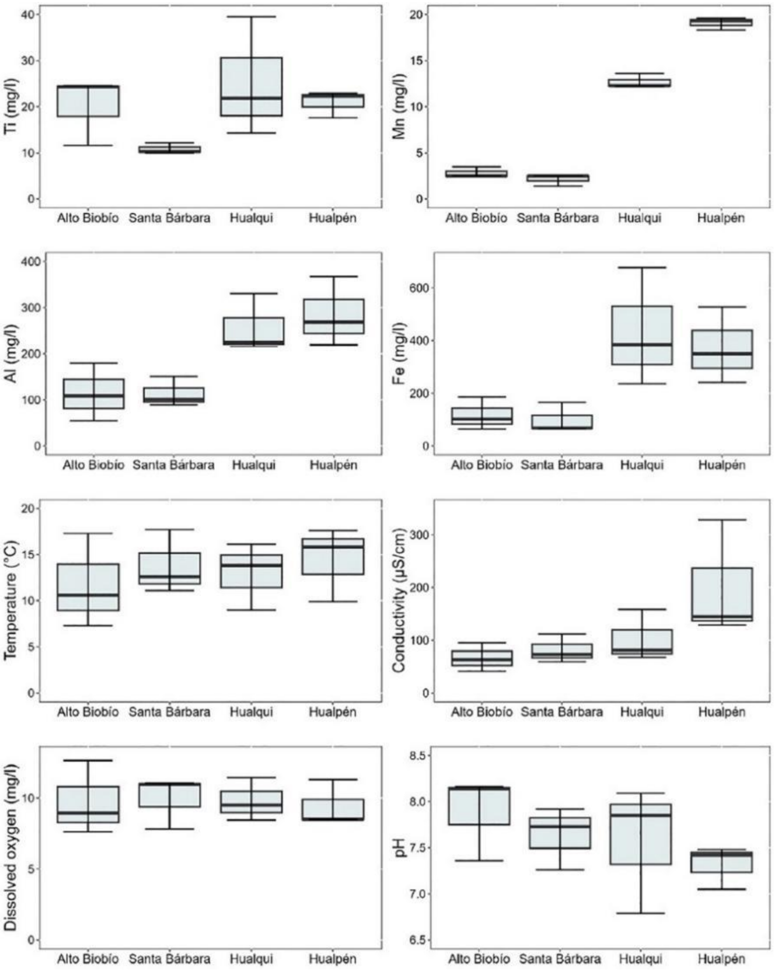


Figure 4.5. Spatial variation of the most representative variables recorded in surface water along the course of the Biobío River. The locations are presented by decreasing elevation from left to right. Center lines in the boxes represent mean values.

The mixed behavior of the sampled river, with increases in flow in spring and winter, makes it difficult to establish a relationship between anthropogenic and natural contributions of Ti. The use of Fe and Al for the normalization of Ti allows us to date the natural and anthropogenic contributions of Ti (Fig. 4.6) in summer, winter and spring. When contrasting this information with spatial analysis (Fig. 4.4) of the concentrations of Ti, Al, Fe and Mn, a marked trend of lower variability is observed in the upper course of the Biobío River compared to the lower course, where the Hualqui points are located and Hualpén. Additionally, an increase in the concentration of Al and Fe was observed along the course of the river to the lower part. On the other hand, for Ti, in Hualpén an increase in concentration is observed as time passes during 2022. This information reveals and confirms a greater influence of anthropogenic contributions that are enhanced by temperature gradients in different areas sampled. The temperature contrast in the lower part of the river is between 0.3 and 5 °C greater than in the upper course, which causes greater evaporation of water. Furthermore, increasing drought conditions have been recorded in the area in recent years, decreasing the amount of water available and at the same time concentrating solutes in the river (Yevenes et al., 2018). Under these conditions, pollutants and micropollutants can accumulate more easily in the aquatic system, highlighting the need to regulate and control emissions entering rivers with characteristics similar to those of the Biobío River.

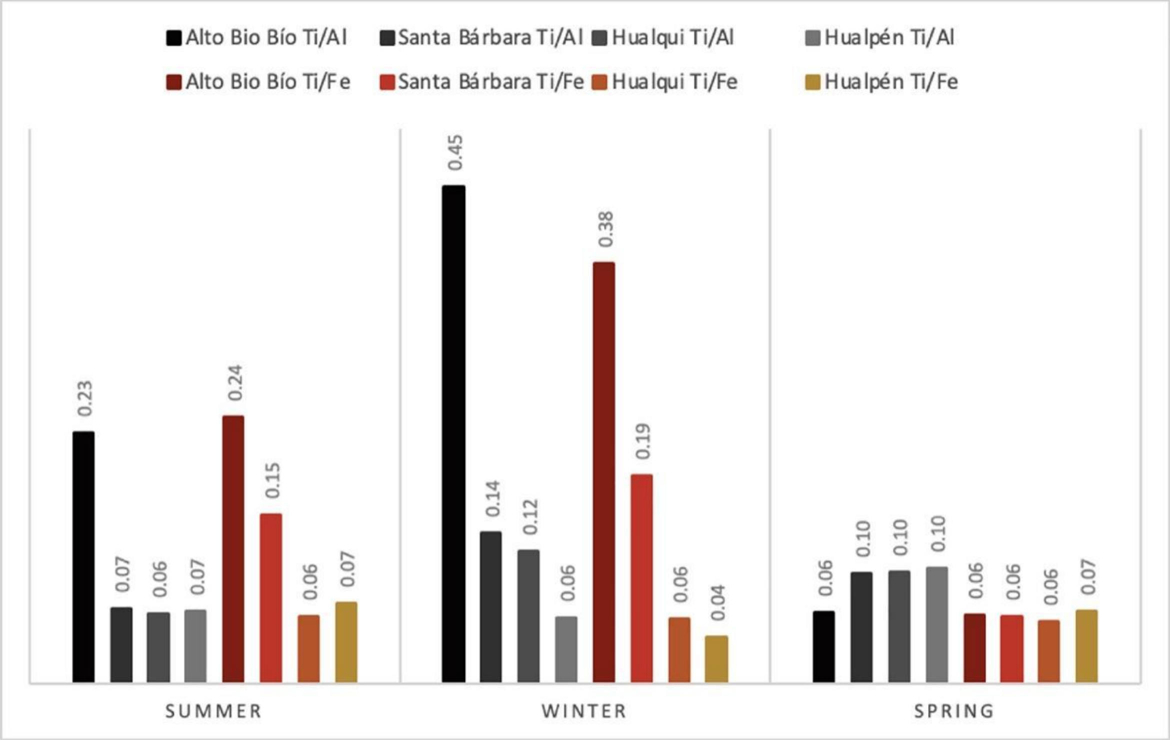


Figure 4.6. Ti/Al element ratio in grayscale and Ti/Fe in red scale; variations by season and sampling point are shown.

In a future study it would be important to complement this research with isotopic analyzes of the elements Ti, Al and Fe to more precisely trace their origins. For their part, these results coincide with studies on contributions from soils and rivers carried out in other parts of the world, in which it has been estimated that the proportion of TiO_2 in minerals is approximately 0.25-0.95 % of the total, a very low percentage (Greber et al., 2021). Despite the difficulty in interpreting the origin of the TiO_2 nanoparticles, these data allow inferences to be made about the sampling points that are important to consider to construct an adequate mineralogical baseline and to be able to trace the origins of the dual-origin nanoparticles that enter the rivers. with characteristics similar to those of the Biobío River.

Another tracer of the potential anthropogenic origin of TiO_2 nanoparticles is Mn. This element is associated with TiO_2 nanoparticles because it is commonly used as a dopant in synthetic anatase and rutile structures (Sukhdev et al., 2020), which are employed in energy harvesting and storage technologies (Latif et al., 2022), dye production, and industrial processes in the paper industry (Mohamed et al., 2007).

ICP-MS results show that at the Alto Biobío and Santa Bárbara sites, where Ti is strongly associated with natural sources, Mn concentrations were below the detection limit during both summer and winter sampling campaigns. This suggests that the nanoparticles present at these sites are likely not related to anthropogenic inputs.

In contrast, at the lower course of the river, Mn concentrations ranging between 12.3 and 19.6 $\mu g\ L^{-1}$ were detected during the same sampling seasons. Mn concentrations showed statistically significant differences among sampling stations, according to the Kruskal–Wallis test (Table 4.2). Furthermore, Mn concentrations exhibited significant positive correlations with conductivity, suggesting a potential influence of anthropogenic inputs in the downstream sections of the basin.

According to field observations, between the Santa Bárbara and Hualqui points, the activity in areas near the river is industrial, with pulp plants and the active construction (2023) of a series of wind farms in areas that directly impact the Biobío River and its tributary rivers. These industrial activities could explain the increase of Ti in water samples.

In the principal components graph (Fig. 4.7), PC1 axis reflects 80.22 % of the total variance, explained by the variability of the concentrations of Fe and Al, which according to the ordination analysis appear to be different between localities in the upper middle zone and the lower zone of the Biobío River. Furthermore, the variance captured by the PC2 axis (16.52 %) reflects the variability in conductivity values in Hualqui and Hualpén between different stations. This could be a consequence of the concentrations of solutes in the lower area of the river, where there is also estuarine influence, since the Hualpén sampling point is located almost at the mouth of the Biobío River.

Table 4.2. Summary of statistical parameters

Shapiro-Wilk test

parameter	W	p-value	
Ti	0.88068	0.0894*	High values of the W statistic (>0.8) together with low significance values (p>0.005) dictate that the hypothesis that the variable data present a normal distribution not be rejected, except for the conductivity values.
Mn	0.81541	0.0141*	
Al	0.95803	0.7554*	
Fe	0.88509	0.1019*	
Temperature	0.9219	0.302*	
Conductivity	0.76213	0.00359	
O2	0.90994	0.213*	
pH	0.94471	0.5614*	

Test Kruskal Wallis

BY LOCATION		
parameter	**p-value**	
Mn	0.01842*	P-values <0.005 indicate the existence of significant differences; thus, only Mn concentrations presented statistically significant differences between locations.
Conductivity	0.1207	
BY SEASON		
parameter	**p-value**	
Mn	0.942	
Conductivity	0.116	

Analysis of Variance

BY LOCATION		
parameter	**p-value**	
Ti	0.2093	
Al	0.01376*	P-values<0.005 indicate the existence of significant differences; thus, Al and Fe concentrations presented statistically significant differences between locations, while the values of the temperature and dissolved oxygen variables presented statistically significant differences between seasons.
Fe	0.03951*	
Temperature	0.8642	
O2	0.9903	
pH	0.5437	
BY SEASON		
parameter	**p-value**	
Ti	0.3432	
Al	0.9136	
Fe	0.4984	
Temperature	0.0002655*	
O2	0.0004612*	
pH	0.1776	

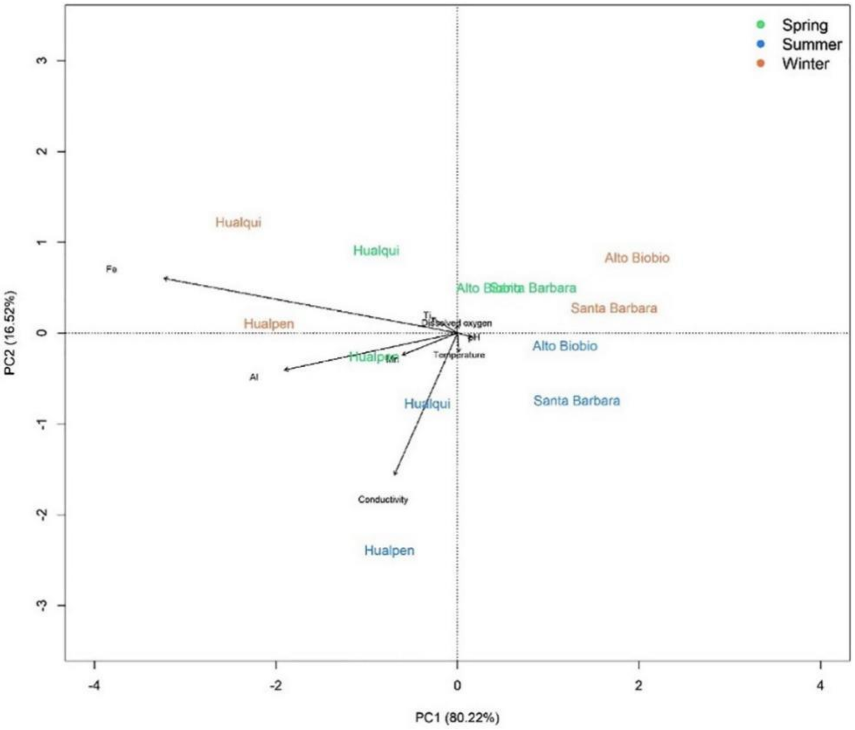


Figure 4.7. Principal component analysis (PCA) based on variables recorded in surface water at different locations along the course of the Biobío River in different seasons, represented by different colors.

In this sense, soils can represent a source of nanoparticles for the surface water of the Biobío River. For example, in agriculture, Fe nanoparticles are used to increase nutrient absorption and germination (Santás-Miguel et al., 2023). Consequently, the application of nanoagrochemicals could increase their content in the soil and crops (Rajput et al., 2021). According to the CONAF Territorial Information System, Hualqui covers an area of 2,694 hectares of agricultural land (Corporación Nacional Forestal, 2020). This site could represent a seasonal source of nanoparticles transported by runoff with winter precipitation, which could end up into river watersin Hualpén.

4.3.2. TEM analysis

To analyze the TiO_2 nanoparticles through TEM in the surface water samples, the shapes and sizes of the standard of Titannium (IV) oxide nanoparticles, mixture of rutile and anatase at 10 µg L^{-1} in MilliQ water were compared (Fig. 4.9 A and B). The first observation in the prepared standards is the agglomerates of different defined shapes found in the standards, ranging from sizes < 100 nm as individual nanoparticles to > 100 nm in agglomerates. Despite these observations, it is possible to identify the forms of anatase (Fig. 4.9-A) and it is more difficult to identify Rutile (Fig. 4.9-B) according to the Bravais lattices (Iadonisi et al., 2014; Sengupta & Sarkar, 2015).

Regarding surface water samples, nanoparticle structures were found less frequently in the Alto Biobío. However, those that were observed were cubic (Fig. 4.9-C) and oval (Fig. 4.9-D) in shape, with sizes in the range between approx. 120–230 nm. These structures are associated with the organic matter of the samples, which is expected since TiO_2 nanoparticles of natural origin tend to be hydrophobic and have a greater affinity for this matter, unlike manufactured TiO_2 nanoparticles that have a wide coating variability, which can be hydrophilic or hydrophobic (Campos et al., 2022).

At the Santa Bárbara sampling point it was more common to find defined structures of TiO_2 nanoparticles in nanometric sizes < 100 nm. The observed nanoparticles were in individual states with tetragonal shapes characteristic of Anatase with one side with a size of 46 nm (Fig. 4.9-E) and tetragonal agglomerates characteristic of Rutile (Fig. 4.9-F). These shapes are consistent with TiO_2 nanoparticle structures synthesized using Pechini methods (Hajizadeh-Oghaz, 2019; Vargas Urbano et al., 2011). According to field observations, the sampling site was influenced by camping activities, so the presence of the observed nanoparticles is a consequence of this activity, identifying Santa Bárbara as the first point in this study where the entry of TiO_2 nanoparticles from recreational activities on the river.

At the Hualqui sampling point (Fig. 4.9-G, H, I and J) nanoparticle structures were observed more frequently than in Alto Biobío and Santa Bárbara, observing different types of structures that coincide with some observed at the previous points. Fig. 4.9-D and J show shapes and sizes that agree with those observed in the Alto Biobío, with a size of approximately 229 nm (Fig. 4.9-D) and 259 nm (Fig. 4.9-J), while Fig. 4.9 G and H, and show similarity to the homoagglomerate observed at the Santa Bárbara sampling point (Fig. 4.9-F), with a size of < 100 nm each. However, in Hualqui heteroagglomerated forms are observed, as seen in Fig. 4.9 G and I, where the nanoparticles are superimposed in different ways, which can be explained because at this point there is an increase in conductivity due to the concentration of solutes in the area, along with an increase in pH that promotes heteroagglomeration of nanoparticles (Labille et al., 2015). It is important to describe this, since these conditions promote the interaction of nanoparticles with other components in the environment, such as organic matter (Fig. 4.9-H), heavy metals, emerging contaminants and any other compounds present in surface water, increasing the probability of ecological risks. as the negative effects of settleable colloidal nanoparticles increase (Morelli et al., 2018), causing interaction with substrate-removing species that forage or bury themselves in the sediment (Bhagat et al., 2020).

This mixture of structures found in Hualqui is consistent with the increase in total Ti concentrations found at this sampling point, showing the coexistence of TiO_2 nanoparticles of natural and anthropogenic origin. Although TEM and ICP-MS analyzes are not sufficient to distinguish between the two origins (Philippe et al., 2018), it is possible to demonstrate their existence at sizes < 100 nm and larger.

Regarding the last stretch of the river, at the Hualpén sampling point, nanoparticle structures were observed more frequently, especially in summer and spring. The observed nanoparticle structures are well defined and have sizes between 50–200 nm. In this area there is a direct discharge from a wastewater treatment plant, which provides tangible evidence that the synthetic nanoparticles contained in everyday products are not retained during domestic water purification processes, and enter aquatic ecosystems without any filter type. Fig. 4.9-K, L, M and N show nanoparticles with very defined tetragonal and octahedral shapes, with a length range of approximately 85–120 nm. These structures can be clearly seen in the anatase and rutile phases. Rhombohedral shapes characteristic of the brookite TiO_2 phase are also seen (Fig. 4.9-K and N). The anatase octahedrite structures are slightly larger than those of rutile and share structural similarities, as stated by Akakuro (2020). If we compare the frequency of nanoparticle sightings at this sampling point with the total Ti concentration determined (Fig. 4.4), it can be concluded that the concentration of TiO_2 nanoparticles at this point is approximately 17.6-22.9 $\mu g\ L^{-1}$ between summer and spring. Furthermore, fewer agglomerates of nanoparticles and more defined structures are observed than the colloids observed in the Alto Biobío (Fig. 4.9-D).

To finish verifying the existence of the TiO_2 nanoparticles observed, SEM-DLS analysis was carried out on the surface water samples. These analyzes confirmed the Ti content in them, in quantities between 4.84 % and 20.35 % of the dry weight. The brightest crystals were analyzed first and then the bottom of the sample, always finding the element present. Element mapping revealed a uniform presence of Ti in all samples indicating crystalline nature.

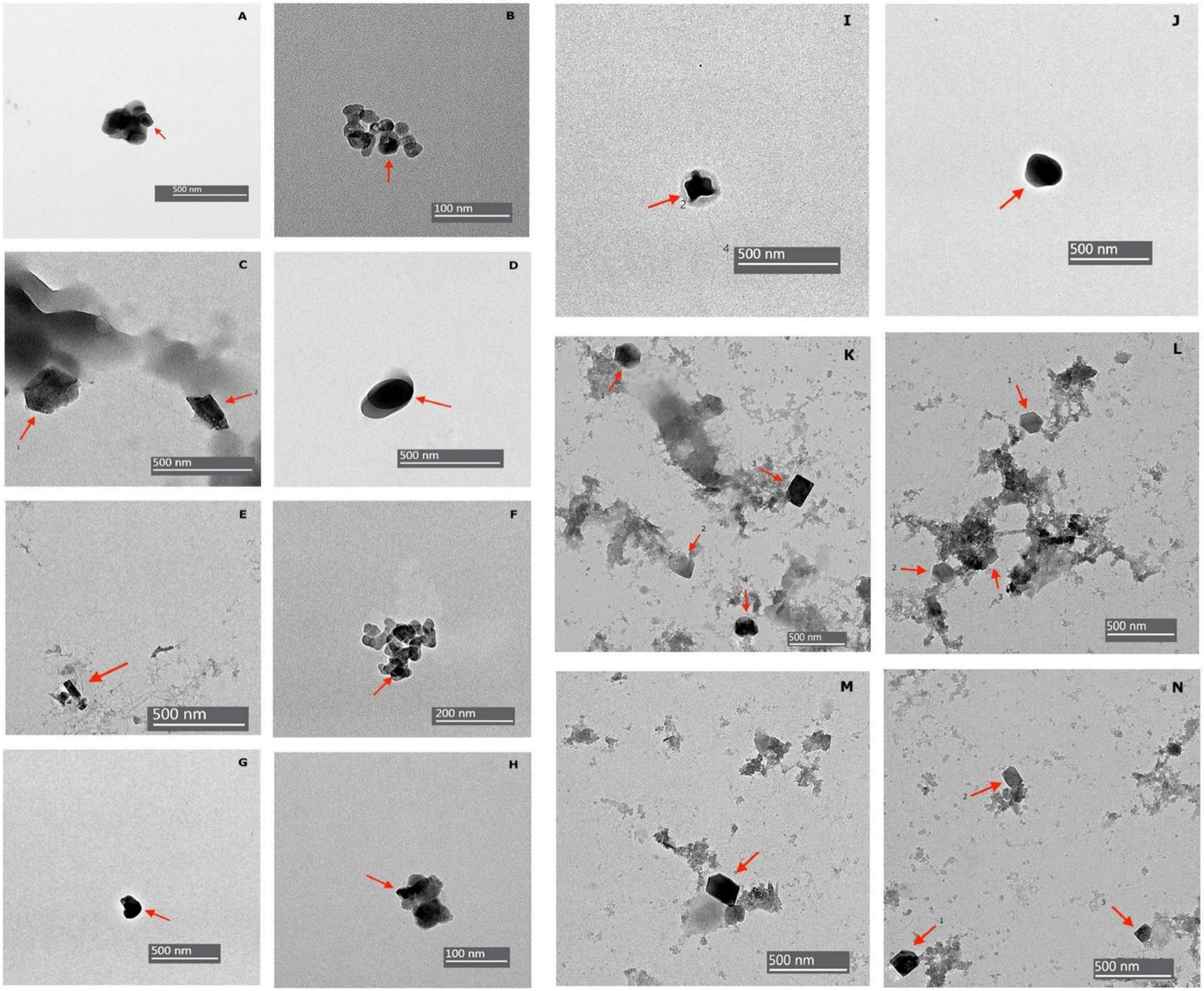
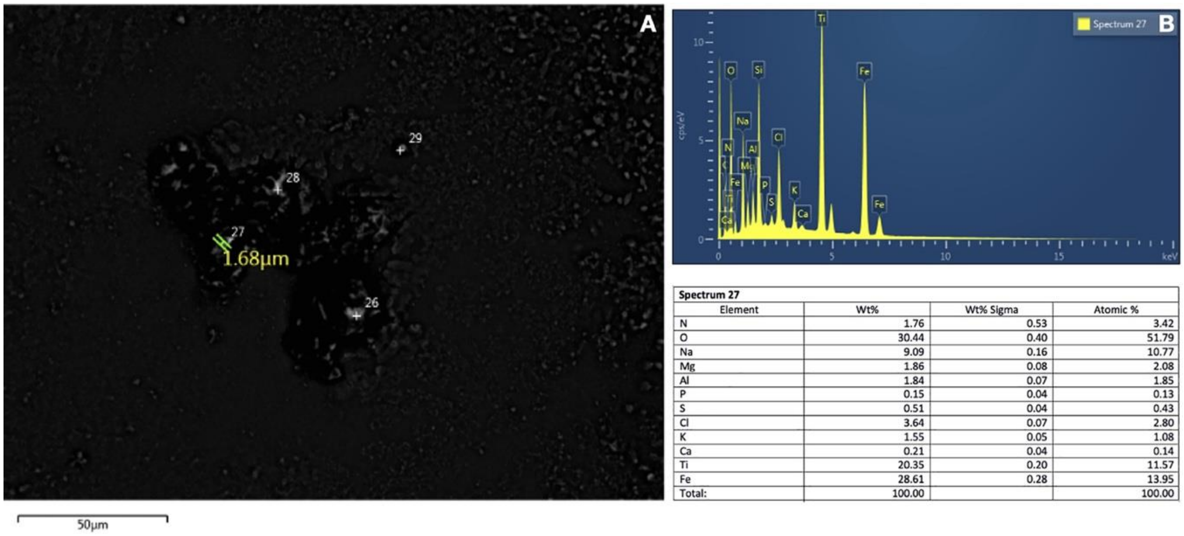


Figure 4.9. Transmission electron microscope (TEM) analysis of standard of Titannium (IV) oxide nanoparticles, mixture of rutile and anatase (MKCR1332) and of surface water samples at each sampling point: A and B: anatase and rutile standards at concentrations of 10 μg L−1 and size < 100 nm; C and D: Alto Biobío samples; E and F: Santa Bárbara samples; G, H, I, and J: Hualqui samples; K, L, M, and N: Hualpén samples.

For the Hualpén sample, in Fig. 4.10-A, a cloud with bright spots is observed that indicates the presence of metals, similar to those observed in TEM when zooming in on the samples to reach nanometric scales, the spectrum scanned over the particles indicates the high Ti content present in the sample, as well as the presence of Fe and Al but to a lesser extent in the scanned point, these results agree with the ICP-MS analyzes where these same elements were observed predominantly, however at SEM-DLS it is possible to observe the nanoparticles present in the sample in a more localized way, so it can be discriminated when Ti predominates and confirm that the nanoparticles observed in TEM are Ti nanoparticles of anthropogenic origin.



Spectrum 27			
Element	Wt%	Wt% Sigma	Atomic %
N	1.76	0.53	3.42
O	30.44	0.40	51.79
Na	9.09	0.16	10.77
Mg	1.86	0.08	2.08
Al	1.84	0.07	1.85
P	0.15	0.04	0.13
S	0.51	0.04	0.43
Cl	3.64	0.07	2.80
K	1.55	0.05	1.08
Ca	0.21	0.04	0.14
Ti	20.35	0.20	11.57
Fe	28.61	0.28	13.95
Total:	100.00		100.00

Figure 4.10. Scanning electron microscope analysis with DLS element detector of Hualpén surface water. A: nanoparticle images obtained via SEM; B, spectrum that confirms the existence of elemental Ti.

According to the different sizes of TiO_2 nanoparticles observed in the water samples, it is important to note that we are in the presence of a potential environmental risk, a situation that can be extrapolated to countries demographically and geomorphologically similar to those seen in this research. Authors have described that size is crucial to determine whether synthetic nanoparticles have ecotoxicological effects; establishing that the size of 100 nm is considered a threshold to establish different levels of toxicity (Banerjee & Roychoudhury, 2019), because lengths less than 100 nm correspond to highly dangerous nanoparticles, since they can enter cells without any difficulty (Middepogu et al., 2018). On the other hand, nanoparticles between 100 and 200 nm have surface interactions with cells and can affect or limit cellular functions, thus causing malfunctions in their biochemical processes (Perreault et al., 2012). Finally, nanoparticles with a size > 200 nm can aggregate or agglomerate, interacting with the same particle. According to what was mentioned above, in this study it was possible to identify all the size ranges that are potential environmental risk (Table 4.3), taking a sense of urgency to establish baselines to establish the contributions of elements to surface water through of erosion or other natural processes to determine the concentration of possible xenobiotics that violate the stability of ecosystems through the ecotoxicological impacts of nanoparticles of anthropogenic origin.

Table 4.3. Comparison of nanoparticle sizes between commercial standards and nanoparticles found in the Biobío River.

Sample	**Image**	**Length (nm)**			
		1	2	3	4
St. TiO_2 10 µg L^{-1}	A	74.043 ▲	-	-	-
	B	24.865 ▲	-	-	-
Alto Biobío spring	C	172.296 ✷	120.535 ✷		-
	D	229.053 ◇		-	-
Santa Bárbara summer	E	27.855 ▲	-	-	-
Santa Bárbara winter	F	46.314 ▲	-	-	-
Hualqui summer	G	157.135 ✷	-	-	-
	H	20.587 ▲		-	-
	I	95.957 ▲		-	-
	J	258.941 ◇	-	-	-
Hualpén summer	K	115.281 ✷	170.21 ✷	182.275 ✷	206.221 ◇
	L	79.782 ▲	54.241 ▲	75.219 ▲	-
	M	83.218 ▲	-	-	-
	N	126.535 ✷	87.121 ▲	85.678 ▲	
▲: very dangerous; ✷: dangerous; ◇: moderately dangerous.					

4.4. CONCLUSIONS

Research on the presence and distribution of TiO_2 nanoparticles within the Biobío River has yielded definitive results. In the four strategically chosen sampling locations, our study has determined that the concentrations of TiO_2 nanoparticles varied significantly with seasonality, recording concentrations that ranged between 17.6 µg L^{-1} and 39.5 µg L^{-1}. These findings indicate a complex interaction between anthropogenic influences and natural river dynamics.

Using advanced TEM and VP-SEM analyses, we have observed a variety of nanoparticle shapes and sizes, demonstrating the diverse nature of TiO_2 nanoparticles in this river system. Our results have shown that nanoparticles found in river surface waters span sizes from 10 to 206 nm, and a significant proportion exist in the form of rutile and anatase.

Through precise ICP-MS and ICP measurements, we have quantified not only Ti but also associated trace elements such as Fe, Mn, and Al, further elucidating the physicochemical profile of the water samples. This comprehensive analytical approach has revealed a marked trend in the distribution and concentration of TiO_2 nanoparticles, suggesting possible sources and transport mechanisms.

The evidence suggests that TiO_2 nanoparticles are present along the river, regardless of proximity to urban activities, however an increase in concentrations is observed downstream of urban settlements, particularly defined structures corresponding to synthetic nanoparticles during the seasons. winter and spring. These findings highlight the river's role as a sink for TiO_2 nanoparticles, with possible implications for the ecology and human health of the basin.

This study provides a significant contribution to our understanding of nanodebris in aquatic environments, demonstrating the widespread distribution of TiO_2 nanoparticles in the Biobío River and establishing a clear seasonal pattern in their appearance. This research lays the foundation for future studies aimed at unraveling the complex fate and transport mechanisms of nanoparticles in aquatic ecosystems.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Gester G. Gutiérrez: Writing – original draft, Writing – review & editing, Validation, Visualization, Resources, Software, Methodology, Project administration, Funding acquisition, Investigation, Conceptualization, Data curation, Formal analysis. Alessandra Perfetti-Bolaño: Data curation, Writing – review & editing. Manuel Meléndrez: Writing – review & editing, Methodology. Karla Pozo: Funding acquisition. Ilaria Corsi: Conceptualization, Methodology, Writing – review & editing. Ricardo O. Barra: Writing – review & editing, Conceptualization, Supervision. Roberto Urrutia: Writing – review & editing, Conceptualization, Investigation.

DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Gester reports financial support was provided by National Agency for Research and Development. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CAPÍTULO 5

Toxicological Responses of Photosynthetic Genes in *Chlorella vulgaris* Exposed to Environmentally Relevant Concentrations of TiO_2 Nanoparticles

Segunda publicación científica

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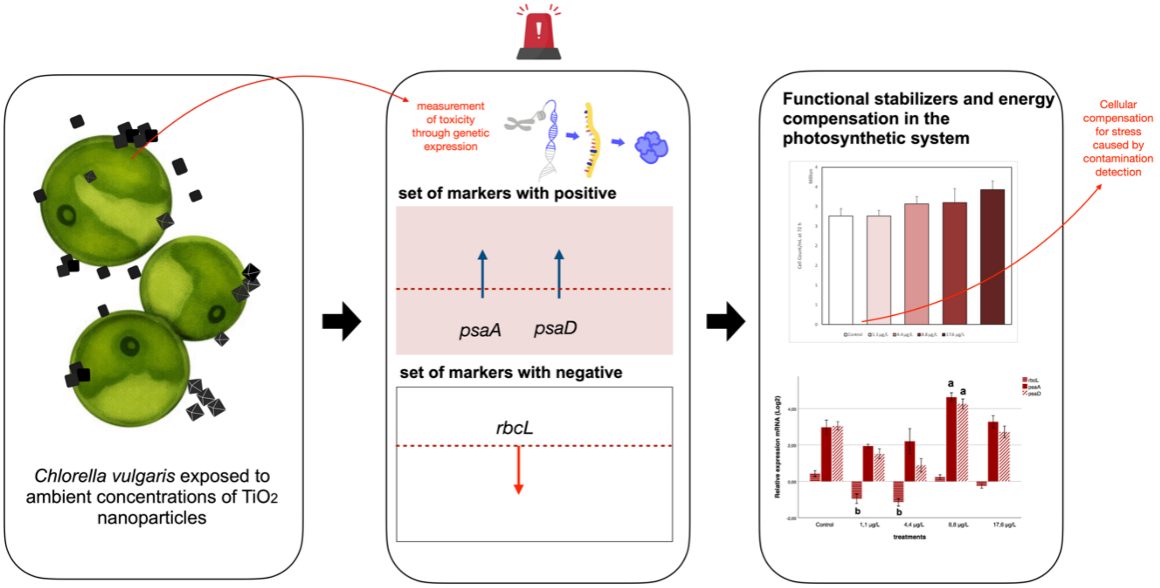
Graphical Abstract



ABSTRACT

Engineered nanoparticles are increasingly released into aquatic environments, raising concerns about their effects on primary producers. Titanium dioxide nanoparticles (TiO_2 NPs), one of the most widely used nanomaterials, are frequently detected at low concentrations in surface waters. Here, we investigated the impact of environmentally relevant TiO_2 NP concentrations (1.1–17.6 µg/L) on the freshwater microalga *Chlorella vulgaris* by combining standardized growth inhibition bioassays with transcriptional analysis of photosynthesis-related genes. Cultures were exposed for 72 h following OECD TG 201, and cell density, growth factor (GF), and specific growth rate (µ) were determined to validate bioassay reliability. Gene expression of six photosynthetic genes (*atpB*, *psaA*, *psaB*, *psaD*, *psbA*, and *rbcL*) was quantified by RT-qPCR and normalized against 18S rRNA. Statistical analyses included Shapiro–Wilk and Levene's tests, followed by one-way ANOVA with Bonferroni or Dunnett's T3 post hoc tests. The results showed a hormetic growth response, with stimulation at intermediate NP concentrations and no inhibition at the highest dose. At the molecular level, *rbcL* was significantly repressed at 1.1–4.4 µg/L, while *psaA* and *psaD* were upregulated at 8.8–17.6 µg/L, indicating compensatory reinforcement of photosystem I. These divergent transcriptional trajectories demonstrate that molecular endpoints reveal sublethal effects not evident from cell counts alone. Overall, this study highlights the potential of photosynthesis-related genes as early biomarkers for detecting nanoparticle-induced stress in aquatic primary producers.

Keywords: titanium dioxide nanoparticles; *Chlorella vulgaris*; photosynthesis; gene expression; biomarkers; hormesis; aquatic ecotoxicology

5.1. INTRODUCTION

The increasing production and use of engineered nanoparticles (NPs) has led to their continuous release into aquatic environments, raising concerns about long-term ecological risks. Among them, titanium dioxide nanoparticles (TiO_2 NPs) are among the most widely used nanomaterials and are frequently detected in natural waters due to their extensive application in industrial, cosmetic, and consumer products [1–4]. Their nanoscale size (<100 nm) enhances reactivity and photochemical activity [5–13], but also promotes direct interactions with cellular components, potentially disrupting essential processes such as photosynthesis and energy metabolism in aquatic organisms [14–22].

Despite the growing attention on NP pollution, important knowledge gaps remain regarding their effects at environmentally realistic concentrations. Most ecotoxicological studies have focused on the milligram-per-liter range, where acute toxicity or mortality is observed, but these exposure levels do not reflect the subtle and sublethal effects that occur at microgram-per-liter concentrations commonly found in aquatic systems [23–27]. Such effects in primary producers may alter productivity, food web dynamics, and eco-system stability.

To explore these sublethal responses, we selected *Chlorella vulgaris*, a model fresh-water microalga widely used in standardized algal growth inhibition tests (OECD TG 201). This species plays a key role as a primary producer, is sensitive to pollutants, and is ecologically relevant due to its central role in aquatic trophic chains. Unlike *Raphi-docelis subcapitata* or *Desmodesmus subcapitata*, *C. vulgaris* possesses a robust cell wall and chloroplast structure [28,29], making it particularly suitable for evaluating nanoparticle interactions, including agglomeration and photosystem disruption.

Previous studies have shown that TiO_2 NPs can alter chloroplast membranes, inhibit ATP and NADPH synthesis, and disrupt photosystem II (PSII), ultimately reducing photosynthetic efficiency [19,30]. However, these effects remain poorly characterized at environmentally relevant concentrations, particularly at the molecular level. Gene expression analysis has emerged as a sensitive tool to detect early responses to contaminants before visible toxicity occurs. Focusing on photosynthesis-related genes such as *psaA, psaB, psaD, psbA, rbcL*, and *atpB* can provide valuable insights into how nanoparticles affect energy capture, electron transport, and carbon fixation pathways [31–34]. Importantly, this approach is consistent with nanotoxicology work in green microalgae where sublethal endpoints—including PSII performance and transcriptional changes—have been used to uncover early effects of metal-oxide nanoparticles [35–40].

Most ecotoxicological studies on nanoparticles in microalgae, however, have relied on single, independent endpoints such as growth inhibition, pigment content, or chlorophyll fluorescence. While informative, these approaches often overlook the complexity of cellular stress responses and fail to identify sublethal mechanisms. Recent studies demonstrate that integrative approaches provide a more realistic perspective: genome-wide molecular profiling has shown that prolonged exposure to nanomaterials can trigger adaptive responses undetectable by conventional growth assays [41]. Similarly, combined physiological and molecular analyses in *Chlorella vulgaris* exposed to silver nanoparticles revealed distinct modes of toxicity between ionic and nanoparticulate forms, highlighting the limitations of evaluating endpoints in isolation [42]. These findings underscore the importance of linking standardized bioassays with mechanistic bi-omarkers to improve the ecological relevance of nanoparticle risk assessment. In addition, this integrative approach is highly relevant for environmental considerations when nanoparticles are proposed as solutions in agriculture or pollutant remediation at low doses. By combining classical and molecular endpoints, such analyses provide a broader perspective on both the short- and long-term consequences of nanoparticle doping, ensuring a more balanced assessment of their potential benefits and risks [43].

Finally, hormesis—a biphasic response characterized by low-dose stimulation and high-dose inhibition—is well documented in algae exposed to chemical stressors, including nanomaterials [44]. Understanding how gene-expression patterns align with hormetic growth responses is crucial to predict potential changes in biomass production and ecosystem balance.

The present study aimed to evaluate the effects of TiO_2 NPs at environmentally relevant concentrations (1.1–17.6 µg/L) on the growth and photosynthetic gene expression of *C. vulgaris*. Specifically, we sought to: (i) determine whether TiO_2 NPs induce hormetic responses at the cellular level; (ii) assess the dose-dependent regulation of photosynthesis-related genes; and (iii) explore the potential of these genes as early molecular biomarkers of nanoparticle stress in aquatic primary producers.

5.2 METHODOLOGY.

5.2.1 Analysis of TiO_2 nanoparticle standard

For toxicity testing, a stock suspension was prepared using certified commercial TiO_2 nanoparticles (rutile/anatase mixture, particle size < 100 nm, ≥ 99.5% trace metals basis) obtained from Aldrich (Product No. 637262, Batch No. BCCF1510), manufactured in China and certified by Quality Assurance, Buchs, Switzerland. The nanoparticles were supplied as a dry powder and were dispersed in algal growth medium to prepare the stock suspension used for the exposure experiments [59]. The physicochemical specifications, including primary particle size, crystalline phase composition, and purity, were provided by the manufacturer. Therefore, additional characterization techniques such as dynamic light scattering (DLS), zeta potential, or energy-dispersive X-ray spectroscopy (EDS) were not performed.

To confirm particle identity and morphology, Raman spectroscopy coupled with atomic force microscopy (AFM) was performed using a Horiba LabRAM Nano 800 system. This combined technique enabled verification of the crystalline phase through Raman analysis and provided high-resolution AFM images of nanoparticle surface topography and agglomeration behavior. AFM revealed the formation of larger agglomerates at higher concentrations, offering three-dimensional insight into clustering patterns. In addition, high-resolution transmission electron microscopy (TEM; Talos F200 G2, Thermo Scientific) equipped with a CMOS CETA 16M camera was used to determine particle size, morphology, and fine agglomeration structures at the nanoscale. TEM images confirmed the concentration-dependent increase in agglomerate size, complementing AFM observations and strengthening the characterization of the nanoparticle standard.

TiO_2 NPs were selected because they are among the most widely produced nanomaterials worldwide, with extensive applications in cosmetics, paints, coatings, and food additives [60]. Due to these widespread applications, TiO_2 NPs are frequently detected in wastewater effluents and surface waters at environmentally relevant concentrations [61,62]. Their high production volume and recurrent environmental occurrence make them a representative model contaminant for aquatic ecotoxicology.

5.2.2 Biological analysis.

The test microalga *Chlorella vulgaris* (strain CCM-UDEC 051) was obtained from the FICOLAB culture collection (Faculty of Natural and Oceanographic Sciences, Universidad de Concepción, Chile) and maintained under standard OECD TG 201 (2011) culture conditions [59]. *C. vulgaris* was chosen because it is widely distributed in freshwater, estuarine, and even wastewater environments, reflecting its ecological persistence and tolerance to variable conditions [63,64]. This broad occurrence highlights its ecological relevance, as it plays a central role as a primary producer in aquatic food webs. Moreover, *C. vulgaris* has been successfully used in ecotoxicological assessments under different stressors, including nutrient-rich effluents, confirming its robustness as a test organism [65]. Importantly, given its relatively resilient physiology, adverse responses in *C. vulgaris* may suggest that more sensitive phytoplankton species could be even more affected, reinforcing its protective value as an indicator in nanoparticle risk assessment.

The growth inhibition assay was performed by exposing *C. vulgaris* cultures to a series of environmentally relevant concentrations of TiO_2 nanoparticles (mix of rutile and anatase phases). A stock suspension of 1000 µg/L was prepared, from which serial dilutions were made to obtain final exposure concentrations of 1.1, 4.4, 8.8, and 17.6 µg/L. These levels were selected based on previous environmental monitoring of the Biobío River [66], where TiO_2 nanoparticles were detected at comparable concentrations. All treatments, including the control, were performed in triplicate (three independent biological replicates), with a culture volume of 10 mL per replicate in sterile glass vessels, ensuring adequate nutrient availability and light penetration throughout the experiment.

The exposure period was set to 72 h, following OECD TG 201 recommendations, to maintain microalgae in the exponential growth phase during the assay. Extending the exposure to longer periods (e.g., 96 h) could result in nutrient depletion and entry into stationary phase, potentially confounding growth inhibition and gene expression measurements.

Cell density and size were recorded every 24 h using an Olympus optical microscope. To verify the validity of the bioassay, the specific growth rate (µ), growth factor (GF), and coefficient of variation (CV%) were then calculated as follows:

$$\mu = \frac{\ln N2 - \ln N1}{t2 - t1} \tag{1}$$

$$GF = \frac{Nt}{N0} \tag{2}$$

$$CV\% \frac{SD}{Mean} \times 100 \tag{3}$$

where N1 and N2 represent the cell densities at times t1 and t2, respectively, used for the calculation of the specific growth rate (µ). N0 and Nt represent the initial and final cell densities used to calculate the growth factor (GF), and t1 and t2 correspond to the start and end times of the measurement interval.

Once the bioassay was completed at 72 h, cell counting for cell inhibition and measurement of cell size was performed under an Olympus optical microscope. Then, the extraction of genetic material was started.

5.2.3 Obtaining RNA from *Chlorella vulgaris*

Chlorella vulgaris pellets were obtained by centrifugation at 14,000 rpm and 4 °C using a CR 22G III-HITACHI ultracentrifuge (Hitachi Koki Co., Ltd., Life-Science In-struments Division, Tokyo, Japan), completely removing the supernatant. The cell lysis process was carried out by adding 90 µL of Lysis Binding Solution Concentrate Mag-MAX™-96 Total RNA Isolation Kit (Qiagen, Hilden, Germany) and 10 µL of Plant RNA Isolation Aid (cat. #AM9690) to each sample, resulting in a total lysate volume of 100 µL. After pre-centrifugation at 14,000 rpm to remove cell debris, the clarified superna-tant was recovered, and a 50 µL aliquot was used to continue the RNA extraction proto-col according to the manufacturer's instructions (MagMAX™-96 Total RNA Isolation Kit, Thermo Fisher Scientific).

5.2.4 Primer design

Table 5.1 presents the list of primers designed for the selected genes, together with their functions in cell metabolism. The primers were generated using the gene sequences available at NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 9 September 2024). For *18S* and *psaA*, primers were designed de novo in this study using consensus sequences obtained from Geneious Prime® 2023.1.1, as no specific GenBank accession was available. The remaining primers were designed based on sequences retrieved from GenBank. The *18S* rRNA gene was selected as a reference gene and used as an internal standard for normalization of gene expression data.

Table 5.1. List of reference genes designed for the study of gene expression in *Chlorella v.*

Gene Name	GenBank Accession or Source	Forward Prime (5′3′)	Reverse Prime (5′3′)	Tm	%GC	PCR Product
18S	♦ Designed in this study	AACGGCTACCACATCCAAGG	GTCCCACCCGAAATCCAACT	55	55	250 pb
atpB	EF113499.1	CCAATTCACCGTTCAGCACC	TTTCCCTACACCTGCACCAC	55	55	144 pb
psaA	♦ Designed in this study	AAATGCAGACGTTGGTGGTG	CGCTAAACTGCCGAGACCTA	55	50	256 pb
psaB	GQ423926.1	GCCCAATGGATTCAAGCAGC	AGAACCACGAGCGTCAAGAG	55	55	255 pb
psaD	MG596028.1	AGCGTGTATAACCCAGCTCG	AGTGTCCTCCTCATACGCCT	55	55	267 pb
psbA	KX066373.1	CGTTGCCGGTGTATTTGGTG	ACAACTGGCCAAGCAGCTAA	55	55	240 pb
rbcL	MK295221.1	GCACGCTGTAATTGACCGTC	TCAACAAGAGCTGGCATGTG	55	55	285 pb

♦ Designed in this study (based on consensus sequences in Geneious Prime® 1 January 2023).

Amplification efficiency and linearity were assessed by constructing standard curves for each target gene, including *atpB*, *psaA*, *psaB*, *psaD*, *psbA*, and *rbcL*, and the 18S reference gene. Analyses were performed on a QuantStudio 3 thermal cycler (Applied Biosystems) using Design & Analysis Software v2.7.0. The threshold cycle (TC) quantification method was employed, and the standard curve was generated using the Primary Analysis v1.8.0, Standard Curve v1.7.0 module. The parameters evaluated included the slope of the calibration curve (Slope), the coefficient of determination (R^2), the amplification efficiency (Eff%), and the standard error. The sheath temperature was set at 105 °C, and a three-step melting stage analysis was applied.

5.2.5 RT-qPCR

The mRNA concentration of each sample was determined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with 1 µL of extract. A total of 500 ng of RNA was reverse transcribed into cDNA following the manufactur-er's instructions for the M-MLV Reverse Transcriptase kit (Promega). Quantitative PCR (qPCR) was performed to determine the relative expression levels of six target genes (atpB, psaA, psaB, psaD, psbA, and rbcL) using KAPA SYBR® FAST qPCR Master Mix (2X) (KAPA BIOSYSTEMS, Cape Town, South Africa) in a QuantStudio 3 thermal cycler (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA).

Cycling conditions were an initial denaturation at 95 °C for 8 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s, and a final melting curve stage. Each reaction was performed in triplicate (three technical replicates) for each of the three biological replicates per treatment.

5.2.6 Statistical analysis.

All statistical analyses were performed using IBM SPSS Statistics 25 (IBM Corp., Armonk, NY, USA). For bioassay data (cell density and growth inhibition), the normality of residuals was verified using the Shapiro–Wilk test, and the homogeneity of variances was assessed with Levene's test. When assumptions were met, a one-way analysis of variance (ANOVA) was performed to determine significant differences among treatments, followed by Tukey's post hoc test for pairwise comparisons. The significance level was set at 0.05.

For the determination of the effective concentration (EC_{50}), lowest observed effect concentration (LOEC), and no observed effect concentration (NOEC), Probit analysis and ANOVA were applied. Growth factor (GF) and specific growth rate (µ) were calculated according to OECD TG 201 (2011), and the coefficient of variation (CV%) was computed across replicates to assess variability.

Relative quantification of photosynthetic gene expression was performed using a modified standard curve method as described by Steinberg et al. (2012) [67]. For each target gene (*atpB, psaA, psaB, psaD, psbA*, and *rbcL*) and the reference gene 18S rRNA, standard curves were generated from serial dilutions of cDNA to determine slope, coefficient of determination (R^2), and amplification efficiency (Eff%). When amplification efficiencies deviated from the ideal 90–110% range, efficiency values were explicitly incorporated into the expression calculations. In each experimental condition, expression of the target genes was normalized to 18S rRNA, used as a stable endogenous reference in this model. Relative expression was calculated according to the following equation:

$$\textit{Relative expression} = \frac{mRNA\ of\ target\ gene}{mRNA\ of\ 18S} \tag{4}$$

All data are presented as mean ± standard deviation (SD) from three independent biological replicates, each measured in technical triplicate.

Statistical significance was indicated in figures and tables by asterisks ($p < 0.05$; one-way ANOVA, Dunnett's post hoc test), and this convention was applied consistently across all graphs and tables.

5.3 RESULTS

5.3.1. Standard analysis of TiO_2 nanoparticles

The effects of TiO_2 nanoparticles on *Chlorella vulgaris* cultures at environmentally relevant concentrations were evaluated after confirming nanoparticle identity and purity through physicochemical characterization. Raman spectroscopy (Figure 5.1A) showed spectral peaks matching those of the anatase crystalline phase, with a high-quality index (HQI = 99.39) confirming a strong spectral match. Complementary AFM imaging (Figure 5.1B–D) revealed the surface morphology of dispersed nanoparticles, demonstrating concentration-dependent agglomeration patterns at 10, 50, and 100 µg/L. Transmission electron microscopy (TEM) (Figure 5.2A–D) further confirmed that primary particles were smaller than 100 nm, while agglomerates exceeded 100 nm, with aggregate size increasing proportionally to nanoparticle concentration. The predominance of anatase was clear, whereas rutile detection was less consistent due to differences in Bravais lattice structure [9,45]. Together, these results confirm the physicochemical identity of the TiO_2 nanoparticle standard and highlight the direct implications of agglomeration dynamics for subsequent bioassay behavior.

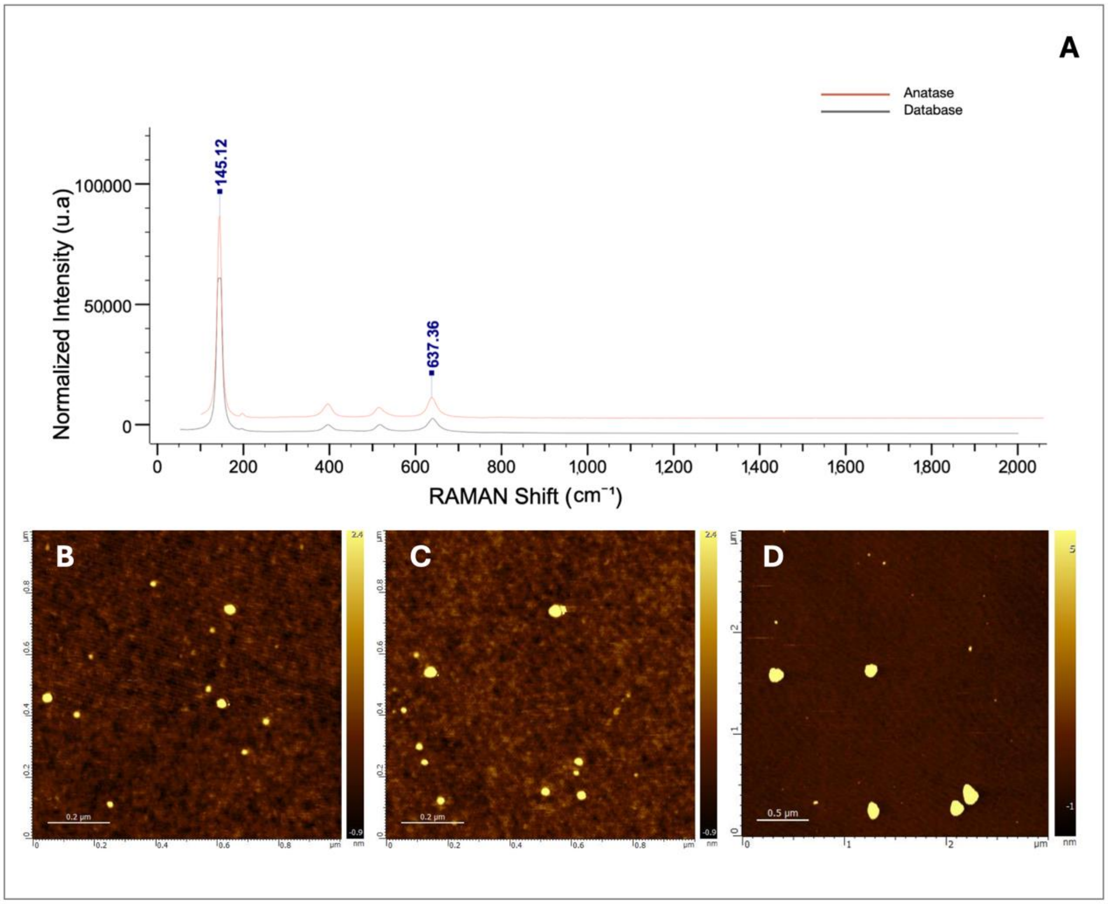


Figure 5.1. (A) Raman spectrum of TiO_2 nanoparticles showing characteristic anatase peaks, confirming the crystalline phase of the material. (B–D) Atomic force microscopy (AFM) images of TiO_2 nanoparticles dispersed in algal growth medium at stock concentrations of 10 µg/L (B), 50 µg/L (C), and 100 µg/L (D). AFM analysis revealed concentration-dependent agglomeration patterns, with larger aggregates observed at higher nanoparticle concentrations.

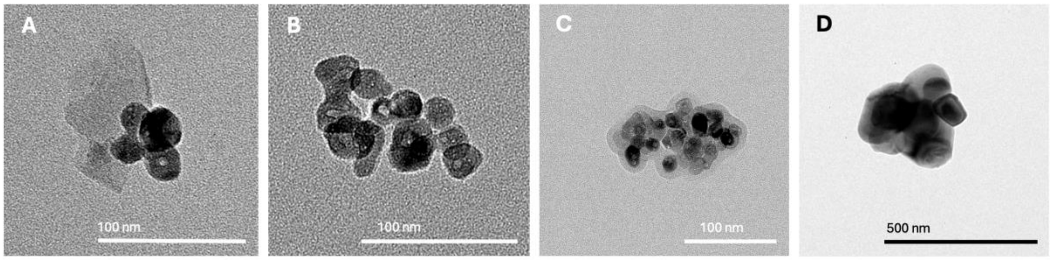


Figure 5.2. Transmission electron microscopy (TEM) images of commercial TiO_2 nanoparticles dispersed in algal growth medium at environmentally relevant concentrations. Panels show agglomeration patterns at 1.1 µg/L (A), 4.4 µg/L (B), 8.8 µg/L (C), and 17.6 µg/L (D).

5.3.2. Cell inhibition assay

The validity of the bioassay was confirmed following the criteria established in OECD Guideline 201 (2011), using growth data from *C. vulgaris* cultures maintained under controlled laboratory conditions. Key parameters—including mean (σ), standard deviation (SD), growth factor (GF), specific growth rate (µ), and coefficient of variation (CV%)—were calculated based on triplicate measurements from three independent biological replicates per treatment.

The control met all OECD 201 validity criteria, achieving a growth factor (GF) of 19.90 (threshold ≥16), indicating sufficient cell proliferation during the 72 h exposure period. The specific growth rate (µ) ranged from 0.89 to 1.00 d^{-1}, consistent with the expected range for optimal *C. vulgaris* growth.
Assay reproducibility was evaluated through the coefficient of variation (CV%), which was 6.66% in the control (<20%), indicating low variability and high precision. All treatment groups also exhibited CV% values below 20%, confirming the reliability of the experimental setup for subsequent ecotoxicological interpretation (Figure 5.3).

These findings confirm that the bioassay met the validation criteria established by OECD 201 (2011), supporting the reliability of subsequent gene expression analyses conducted within this ecotoxicological framework. For the *C. vulgaris* cell inhibition assay, a Dunnett's T3 post hoc test was applied to compare the control group with the different TiO_2 concentrations (1.1 µg/L, 4.4 µg/L, 8.8 µg/L, and 17.6 µg/L). Figure 3 shows the cell density recorded at the end of the 72 h bioassay.

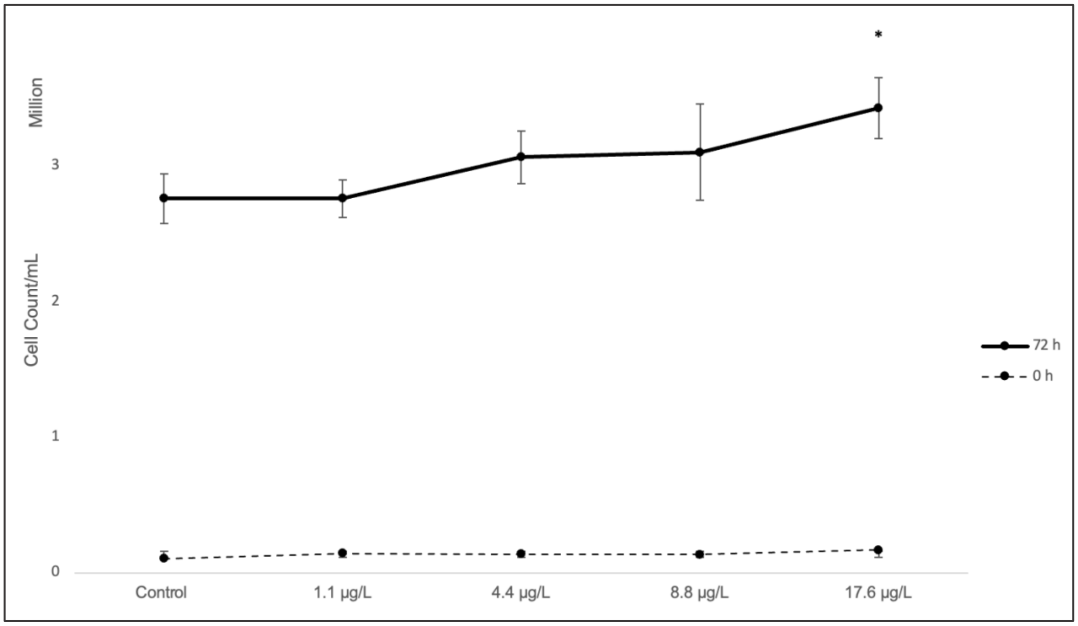


Figure 5.3. Cell density of *Chlorella vulgaris* after 72 h exposure to TiO_2 nanoparticles (1.1–17.6 µg/L). Data are expressed as mean ± SD (n = 3 biological replicates). The dashed line indicates the initial density at 0 h, while the solid line represents the final mean density after 72 h. An asterisk indicates a significant difference compared with the control (* $p < 0.05$; one-way ANOVA, Dunnett's post hoc test).

The control group achieved a growth factor (GF) of 26.2 (threshold ≥ 8) and a coefficient of variation (CV%) of 6.7%, both within the acceptable range defined by OECD 201 (CV% < 20%). All treatment groups also met these criteria, confirming the validity of the bioassay for assessing ecotoxicological effects on microalgae.

The statistical analysis using Dunnett's T3 post hoc test revealed evidence of growth stimulation in *C. vulgaris* cultures. At 72 h, comparisons between the control group and the treatments with 1.1 µg/L and 4.4 µg/L of TiO_2 showed no statistically significant differences.

However, the comparison with 8.8 µg/L revealed a mean difference of −28.33 ($p = 0.090$), indicating a trend toward statistical significance, although not reaching the conventional threshold ($p < 0.05$). In contrast, the highest concentration (17.6 µg/L) showed a highly significant difference from the control (mean difference = −61.00; * $p < 0.001$), marking the point at which the stimulatory effect on cell proliferation becomes evident in *C. vulgaris* cultures (Table 5.2).

Table 5.2. Results of Dunnett's T3 post hoc test comparing cell densities between *Chlorella vulgaris* exposed to different TiO_2 nanoparticle concentrations and the control group after 72 h of exposure.

(I) Group	(J) Treatment	Mean Difference (I–J)	Standard Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	1.1 µg/L	5.6667	2.9059	0.633	−15.3685	26.7018
	4.4 µg/L	−24.6667	8.2731	0.372	−98.8309	49.4976
	8.8 µg/L	−28.3333	4.5216	0.09	−65.8700	9.2033
	17.6 µg/L	−61.000 *	2.0000	<0.001	−72.9276	−49.0724

* The difference in means is statistically significant at the 0.05 level

This response may be partially attributed to the formation of larger nanoparticle agglomerates at higher concentrations (Figure 1B), which reduces their effective surface area and bioavailability, thereby limiting nanoparticle–cell interactions and mitigating potential toxic effects [46].

In natural aquatic environments, where dissolved solute concentrations are typically higher than under controlled laboratory conditions, the agglomeration behavior of nanomaterials may become even more unpredictable, potentially exacerbating ecological risks. This contrasts with the risks reported in studies where smaller nanoparticles exhibit higher reactivity and greater potential for internalization into photosynthetic organisms [17,47].

However, the increase in both the concentration and size of TiO_2 nanoparticle agglomerates may also intensify ecological risk by promoting excessive microalgal biomass accumulation. This process can contribute to eutrophication, which in turn leads to trophic imbalances and deteriorated water quality, ultimately affecting biodiversity and ecosystem health.

These results indicate that TiO_2 nanoparticles, from an environmentally relevant concentration of 17.6 µg/L, trigger a hormetic response in *C. vulgaris* cell density. This biphasic effect reinforces the selection of this concentration range for evaluating early molecular responses through gene expression analysis. Detecting such sublethal biochemical signals is essential for strengthening ecotoxicological assessments of nanomaterials, as they may not cause acute toxicity but still disrupt cellular metabolism.

5.3.3. Standardization of primers

For the gene expression analyses, first, the set of primers synthesised for the *18S*, *atpB*, *psaA*, *psaB*, *psaD*, *psbA*, and *rbcL* genes was standardised. The corresponding standard curves were then performed to verify the efficiency and linearity of the primers, as shown in Table 5.3.

Table 5.3. Efficiency and linearity parameters of primer quantification by qPCR for *Chlorella vulgaris.*

Gen	Slope	R^2	Y-Inter	Eff%	Error
18S	−3.448	0.997	18.29	95.004	0.051
atpB	−3.2	0.981	23.149	105.341	0.113
rbcL	−3.294	0.983	20.448	101.194	0.107
psaA	−2.951	0.944	25.448	118.227	0.179
psaB	−3.375	0.985	26.552	97.832	0.106
psaD	−2.912	0.758	29.714	120.531	0.412
psbA	−3.458	0.983	20.29	94.637	0.114

Efficiency values were within the acceptable range of 90–110%, supporting reproducible amplification for the *psaB* and *psbA* genes. These targets demonstrated optimal efficiency and consistent amplification, validating their suitability for relative quantification. In contrast, the *psaA* gene showed an efficiency slightly above 110%, suggesting possible overamplification, while *atpB* also exceeded the ideal threshold. Therefore, future analyses should consider optimizing the reaction conditions for *psaA* and *atpB* to improve assay performance.

5.3.4. Gene expression

Prior to the ANOVA, Levene's test was applied to evaluate the assumption of homogeneity of variances across treatments for each target gene. The test revealed that variances were homogeneous for *atpB, rbcL*, and *psbA* ($p > 0.05$), whereas significant heterogeneity was detected for *psaA, psaB,* and *psaD* ($p < 0.05$). In all cases, the subsequent ANOVA remained valid because corrections were applied through Bonferroni and Dunnett T3 post hoc procedures when necessary (Table 3).

The one-way ANOVA showed significant treatment effects for five out of six genes examined ($p < 0.05$). Only psbA failed to show statistically significant variation among treatments ($p = 0.748$) and was therefore excluded from further analysis and discussion.

For atpB, the one-way ANOVA indicated a significant overall effect ($p = 0.003$). However, subsequent post hoc tests did not reveal significant differences between individual treatments and the control (Table 5.4; Appendix A Table A1). This suggests that, although a global trend was detected, the variability among replicates limited the identification of specific concentration-dependent effects.

For *rbcL*, a consistent downregulation was observed at 1.1 and 4.4 µg/L compared with the control ($p < 0.05$), indicating inhibition of carbon fixation pathways at lower nanoparticle concentrations. In contrast, *psaA* exhibited a significant upregulation at 8.8 µg/L ($p = 0.018$), while *psaD* was strongly induced at both 8.8 and 17.6 µg/L ($p < 0.05$), suggesting activation of photosystem I components under elevated nanoparticle exposure.

psaB also displayed significant differences at 8.8 µg/L ($p < 0.05$); however, its expression profile showed greater variability across treatments, making the overall trend less consistent compared to other genes.

In summary, significant and consistent concentration-dependent alterations were detected for *rbcL, psaA, psaD*, and *psaB*, whereas *atpB* showed an overall ANOVA effect without treatment-specific significance, and *psbA* remained unaltered across all conditions.

These transcriptional responses across all six genes are summarized in Figure 5.4, which illustrates the relative expression profiles after 72 h exposure to TiO_2 nanoparticles.

Table 5.4. Levene's test for homogeneity of variances and one-way ANOVA results for photosynthesis-related genes in *C. vulgaris* exposed to TiO_2 nanoparticles. Significant values are indicated with an asterisk ($p < 0.05$).

Test of Homogeneity of Variances					
Dependent Variable	Levene Statistic	df1	df2	Sig.	
atpB	2.433	4	10	0.116	
rbcL	0.912	4	10	0.493	
psaA	3.625 *	4	10	0.045	
psaB	7.192 *	4	10	0.005	
psaD	6.332 *	4	10	0.008	
psbA	2.238	4	10	0.138	
ANOVA					
Dependent Variable	Sum of Squares	df1	Mean Square	F	Sig.
atpB	15.913	4	3.978	8.300	0.003 *
rbcL	1.853	4	0.463	14.568	0.000 *
psaA	882.128	4	220.532	8.992	0.002 *
psaB	10.981	4	2.745	7.092	0.006 *
psaD	615.215	4	153.804	13.031	0.001 *
psbA	1.901	4	0.077	0.484	0.748

Taken together, these results indicate that TiO_2 nanoparticles can elicit both repression and stimulation of photosynthesis-related genes in *C. vulgaris*, in a concentration-dependent and gene-specific manner, supporting the occurrence of hormetic-like responses at the molecular level.

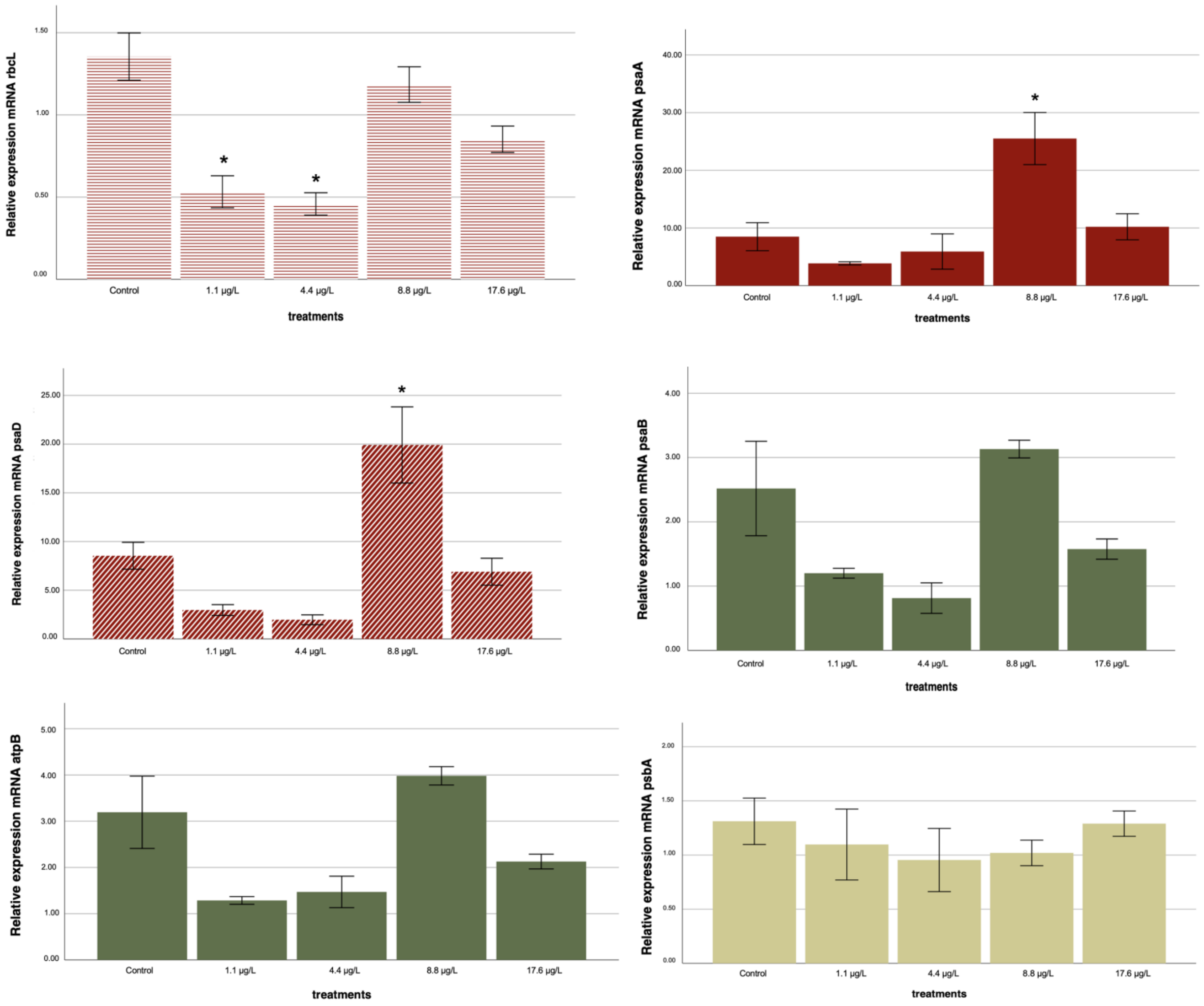


Figure 5.4. Relative mRNA expression of six photosynthesis-related genes in *C. vulgaris* after 72 h exposure to TiO_2 nanoparticles (1.1–17.6 µg/L). Expression values were calculated using the standard curve method, normalized to 18S rRNA, and are presented as relative expression ratios. Data are shown as mean ± 1 standard deviation (n = 3 biological replicates). Asterisks indicate significant differences compared with the control (* $p < 0.05$; one-way ANOVA, Dunnett's post hoc test). Bar colors are used only for visual grouping: red, genes showing at least one significant change; green, genes showing changes without statistical significance; and yellow, genes without detectable alterations under any treatment.

The transcriptional responses of *C. vulgaris* to TiO_2 nanoparticles were not monotonic but instead followed dose-dependent trajectories that paralleled the hormetic pattern observed in cell density. At the lowest concentrations tested (1.1 and 4.4 µg/L), *rbcL* expression was significantly repressed, indicating an early inhibition of carbon fixation. However, this repression was progressively alleviated at higher concentrations, with a partial recovery at 8.8 µg/L and a return to near-control levels at 17.6 µg/L, suggesting an acclimatory adjustment of the photosynthetic machinery. In contrast, *psaA* exhibited a pronounced induction at 8.8 µg/L, reflecting a transient upregulation of PSI reaction-center components, but this effect diminished at 17.6 µg/L, where expression remained slightly above the control but no longer statistically significant. *psaD* followed a distinct trajectory, showing strong upregulation at 8.8 µg/L and maintaining elevated expression at 17.6 µg/L, pointing to a sustained reinforcement of PSI electron-transfer capacity under stronger stress.

Together, these gene-specific patterns reveal a coordinated but divergent regulatory strategy: the repression of *rbcL* at low doses coincides with a limitation in carbon assimilation, while the induction of PSI components at intermediate and high doses likely serves to sustain electron transport and balance energy metabolism under nanoparticle stress. This molecular behavior mirrors the hormetic stimulation of cell growth observed in the bioassay, where intermediate doses promoted cell proliferation before the effect attenuated at the highest concentration. Thus, the integrated response suggests that *C. vulgaris* initially restricts carbon fixation but subsequently engages compensatory mechanisms through PSI activation, providing a mechanistic basis for the hormetic phenotype detected at the cellular level.

This divergent transcriptional behavior, characterized by low-dose repression of *rbcL* and the induction of *psaA* and *psaD* at intermediate to high exposures, provides molecular evidence consistent with the hormetic growth response observed in the bioassay. These patterns are illustrated in Figure 5.5.

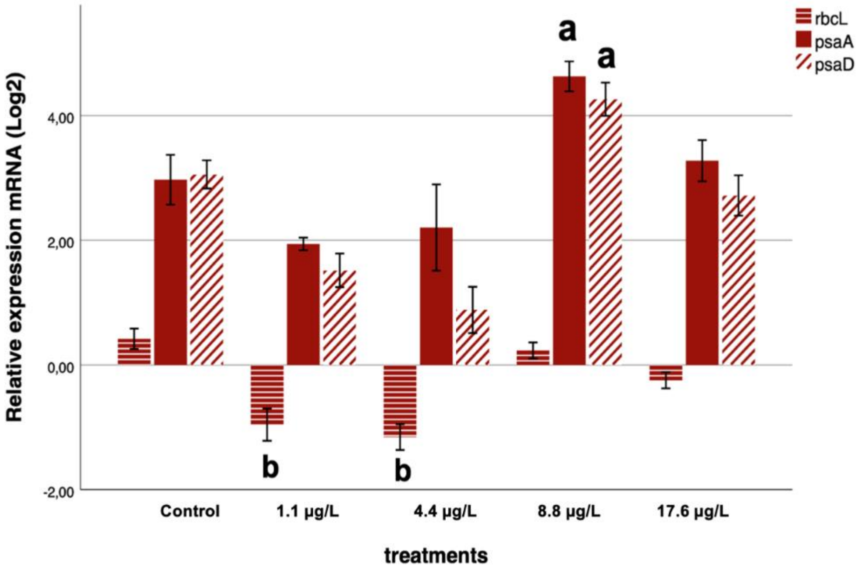


Figure 5.5. Relative mRNA expression (log$_2$) of *rbcL*, *psaA* and *psaD* genes in *Chlorella vulgaris* after 72 h exposure to TiO$_2$ nanoparticles (1.1–17.6 µg/L) compared to the control. Data were normalized against the 18S reference gene and are expressed as mean ± 1 standard deviation (n = 3 biological replicates). Letter a denotes significant overexpression, whereas letter b denotes significant repression of genes (*p* < 0.05; one-way ANOVA followed by Bonferroni post hoc test).

5.4. Discussion

5.4.1. Adaptive Response: Positive Regulation

The induction of *psaA* and *psaD* at intermediate and high nanoparticle concentrations illustrates an adaptive molecular strategy of *C. vulgaris*. Rather than a linear dose-dependent effect, the upregulation of these photosystem I subunits reflects the organism's capacity to reconfigure electron transport pathways under stress. PSI functions as the terminal electron acceptor in the photosynthetic chain, and its reinforcement may ensure continuity of ATP and NADPH production when other parts of the system, such as carbon fixation via *rbcL*, are compromised. This type of adjustment has been associated with enhanced cyclic electron flow around PSI, a mechanism known to contribute to photoprotection by dissipating excess energy and stabilizing the redox state of chloroplasts [34].

Notably, *psaD* maintained elevated expression even at the highest exposure, whereas *psaA* induction was transient. This divergence suggests a fine-tuned regulation in which certain PSI components are preferentially sustained to guarantee electron transfer stability. Such molecular plasticity is consistent with previous reports of algae under oxidative or nanoparticle stress, where targeted reinforcement of PSI allowed acclimation without requiring generalized upregulation across the entire photosynthetic machinery. These findings highlight that the adaptive response of *C. vulgaris* is not a uniform overexpression, but a selective reinforcement of key nodes in the electron transport network [33,48].

5.4.2. Toxic effect: Negative regulation

The repression of *rbcL* at 1.1 and 4.4 µg/L reveals an early toxicological impact of TiO_2 nanoparticles on the photosynthetic machinery of *C. vulgaris*. *rbcL* encodes the large subunit of RuBisCO, the key enzyme responsible for CO_2 fixation. Its transcriptional downregulation at low doses suggests that even environmentally relevant concentrations of nanoparticles can initially compromise carbon assimilation, limiting primary productivity. Such inhibition may result from direct oxidative stress or from indirect effects related to nanoparticle agglomeration, which alters bioavailability and cellular uptake. Although speculative in this study, previous reports indicate that smaller aggregates at lower concentrations can remain more bioavailable and interact more efficiently with cells, thereby producing stronger inhibitory effects than at higher doses, where larger agglomerates tend to form and reduce effective toxicity [49].

At higher concentrations (8.8–17.6 µg/L), *rbcL* expression returned to near-control levels, indicating an apparent acclimatory adjustment. However, whether this recovery reflects a truly sustained adaptation or a transient compensation remains uncertain. It is possible that enhanced PSI activity (*psaA* and *psaD* induction) provides a temporary stabilization of ATP and NADPH pools, allowing RuBisCO activity to be restored despite ongoing nanoparticle stress. Over longer exposures, this compensatory balance may collapse if energy demands exceed the buffering capacity of PSI. Therefore, the normalization of *rbcL* at elevated concentrations should be interpreted cautiously: it may represent either genuine resilience of algal metabolism or a short-lived adjustment preceding eventual decline [30].

5.4.3. Hormetic observations: Cell density and gene regulation

The transcriptional dynamics observed in this study provide a mechanistic basis for the hormetic growth pattern detected in *C. vulgaris* cultures exposed to TiO_2 nanoparticles. At low concentrations (1.1–4.4 µg/L), repression of *rbcL* reflects an early limitation of carbon fixation, consistent with the absence of growth stimulation in the cell-count bioassay. This initial inhibition may be linked to the higher bioavailability of small nanoparticle aggregates at low concentrations, which could penetrate cells more effectively and disrupt photosynthetic metabolism.

At 8.8 µg/L, however, the strong induction of *psaA* and *psaD* coincided with the hormetic stimulation of cell density, suggesting that reinforcement of PSI electron transport provided the energetic compensation required to overcome the temporary bottleneck in carbon assimilation. This transcriptional reconfiguration allowed cells to sustain ATP/NADPH production and redirect energy flow, supporting enhanced proliferation at intermediate exposure levels.

At 17.6 µg/L, the response diverged: *psaD* remained strongly induced while *psaA* declined, and *rbcL* normalized. This pattern corresponds to the attenuation of hormesis observed at the cellular level, indicating that while compensatory mechanisms persist, they may represent a precarious balance rather than a stable adaptation. The persistence of elevated *psaD* expression at the highest concentration suggests a prioritization of PSI stability under stress, but whether this reflects long-term acclimation or merely a transient adjustment remains uncertain.

Altogether, these results align with the conceptual framework of hormesis, where low-dose inhibition is followed by compensatory stimulation at intermediate levels and attenuation under stronger stress. The molecular data presented here provide direct evidence that the hormetic phenotype in microalgal growth emerges from the interplay between early repression of carbon fixation and compensatory induction of PSI genes [44,50].

It should be noted that the mechanistic links proposed between transcriptional changes (*psaA, psaD, rbcL*) and the hormetic growth response remain correlative in nature. While our data reveal consistent expression patterns that align with growth dynamics, no direct measurements of photosynthetic performance (e.g., Fv/Fm, electron transport rate), enzyme activity (RuBisCO), or ATP/NADPH levels were performed. Therefore, the interpretation presented here should be regarded as a proposed model integrating gene expression signals with cellular outcomes. Similar observations have been reported in the recent literature, where hormetic responses to nanomaterials were linked to differential gene expression but emphasized as correlative rather than causal, highlighting the need for integrative studies that combine molecular and physiological endpoints [51]. Future work in this direction will be essential to validate and refine the mechanistic framework proposed here.

5.4.4. Ecological implications: A balance between adaptation and risk

The molecular and cellular responses described here also have implications for environmental risk assessment (ERA) of nanoparticles in aquatic systems. Traditional ERA endpoints, such as growth inhibition measured by cell counts, may underestimate risk when hormesis occurs. In the present study, cell density indicated stimulation at intermediate TiO_2 concentrations, which could be misinterpreted as a neutral or even beneficial effect if evaluated in isolation. However, the repression of *rbcL* at low doses demonstrated that key photosynthetic functions were already compromised at concentrations where no visible growth inhibition occurred. This discrepancy underscores the limitations of relying exclusively on growth-based endpoints.

By integrating gene expression biomarkers into ERA frameworks, it becomes possible to detect sublethal alterations that precede or accompany hormetic stimulation. Such early molecular signals provide a more sensitive and mechanistic basis for predicting ecological consequences, particularly for primary producers that underpin aquatic food webs. Without these molecular insights, risk assessments could overlook subtle disruptions in carbon assimilation pathways, leading to contradictory conclusions: apparent growth stimulation on the one hand, but impaired photosynthetic capacity on the other.

Although cultures were continuously agitated to minimize nanoparticle sedimentation, it should be noted that agglomeration was not directly measured during the bioassays. Instead, TEM and AFM analyses performed on TiO_2 suspensions in algal growth medium demonstrated concentration-dependent agglomeration, providing indirect evidence to contextualize the observed biological responses. Based on these findings, we infer that at lower concentrations, nanoparticles remain more dispersed, increasing their surface area and interaction potential with algal cells, which may account for the stronger inhibitory effects detected under these conditions. In contrast, at higher concentrations, the formation of larger aggregates likely reduces effective bioavailability by decreasing surface area and favoring sedimentation of clusters, thereby attenuating cellular responses. This interpretation is consistent with previous studies reporting similar dynamics in algal systems [49,52–54]. Such considerations highlight the importance of combining particle characterization with biological endpoints to strengthen mechanistic interpretations in nanoparticle risk assessment.

Ultimately, the combined use of classical bioassays and molecular biomarkers offers a more comprehensive evaluation of nanoparticle impacts. In the case of TiO_2, this integrative approach reveals that the hormetic growth response is not simply benign, but reflects an underlying physiological stress that could compromise ecosystem functioning under long-term or community-level exposure scenarios [55,56].

To integrate these findings, a conceptual scheme was developed to summarize the molecular and cellular responses of *Chlorella vulgaris* to TiO_2 nanoparticles (Figure 5.6). The diagram highlights the downregulation of *rbcL* at low concentrations and the induction of *psaA* and *psaD* at intermediate and high exposures, linking these transcriptional adjustments with the hormetic growth pattern observed. This visual synthesis reinforces the interpretation that molecular regulation underlies the balance between stimulation and attenuation across exposure levels.

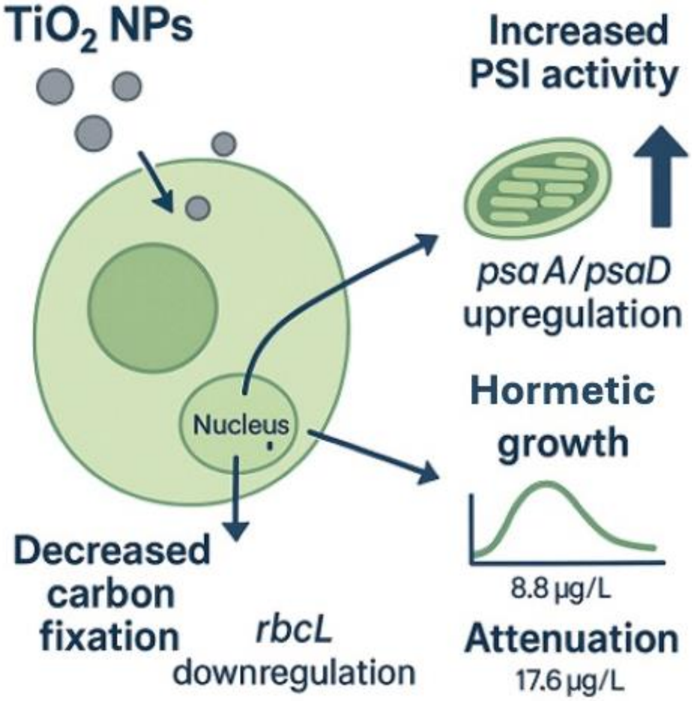


Figure 5.6. Conceptual scheme summarizing the molecular and cellular responses of *Chlorella vulgaris* exposed to TiO_2 nanoparticles. The downregulation of *rbcL* at low concentrations indicates an early impairment of carbon fixation, whereas the upregulation of *psaA* and *psaD* reflects the reinforcement of PSI electron transport under stress. These transcriptional adjustments explain the hormetic growth pattern observed at 8.8 µg/L and its attenuation at 17.6 µg/L, linking gene regulation with physiological and ecological outcomes.

5.4.5. Study limitations

One limitation of the present study is that direct quantification of TiO_2 nanoparticle uptake by *Chlorella vulgaris* was not performed. Nevertheless, complementary TEM and AFM analyses conducted in algal growth medium at environmentally relevant concentrations provided valuable evidence of concentration-dependent agglomeration patterns, supporting the interpretation of exposure conditions. Another limitation relates to the bioassay design, since hydrodynamic parameters such as zeta potential, PDI, or DLS were not measured in the actual exposure system. These parameters could have provided additional insights into nanoparticle stability and effective exposure during the bioassay. Despite these constraints, the combined Raman, TEM, and AFM analyses ensured a robust confirmation of nanoparticle identity, morphology, and aggregation behavior under experimental conditions.

Another limitation of this study is that the amplification efficiencies of *psaA* (118%) and *psaD* (120%) exceeded the recommended range (90–110%). Although melt curve analysis and gel electrophoresis confirmed the specificity of the amplicons, such values may introduce uncertainty in relative quantification. Therefore, the results for *psaA* and *psaD* should be interpreted with caution. However, their consistency with the expression trends of other photosynthetic genes and the observed physiological responses reinforces the reliability of the conclusions.

5.4.6. Perspectives on metabolomics and integrated omics approaches

In addition to transcriptional responses, growing evidence indicates that nanoparticles can also induce significant metabolomic alterations in microalgae. TiO_2 NPs have been reported to disrupt amino acid, lipid, and carbohydrate metabolism, leading to shifts in cellular energy balance and biochemical composition [57,58]. Such metabolomic changes often occur at sublethal concentrations and may precede visible growth effects, making them valuable early biomarkers of nanoparticle stress. Recent reviews further emphasize that integrating metabolomics with transcriptomics is essential to link gene regulation with functional metabolic outcomes and to achieve a systems-level understanding of nanoparticle impacts [58]. Although metabolomic profiling was not included in the present study, future work combining transcriptomic and metabolomic approaches will provide a more comprehensive perspective on the mechanisms of nanoparticle toxicity in primary producers.

5.5. Conclusions

This study demonstrates that environmentally relevant concentrations of TiO_2 nanoparticles elicit complex, gene-specific responses in *C. vulgaris*, which cannot be fully captured by conventional growth assays alone. While cell counts revealed a hormetic pattern with growth stimulation at intermediate exposures, molecular data showed that key photosynthetic genes were differentially regulated: *rbcL* was repressed at low doses, while *psaA* and *psaD* were induced at intermediate to high concentrations. These divergent trajectories highlight the dual nature of nanoparticle effects, where apparent stimulation at the cellular level coexists with underlying transcriptional stress.

From a risk assessment perspective, these findings emphasize the need to integrate classical endpoints with molecular biomarkers. Reliance on growth-based measurements alone could lead to contradictory or misleading interpretations, underestimating the potential for subtle disruptions in primary productivity. By incorporating gene expression profiles into environmental risk frameworks, a more sensitive and mechanistic evaluation of nanoparticle impacts can be achieved, improving our ability to predict ecological consequences.

Overall, the results indicate that TiO_2 nanoparticles can induce hormetic responses in microalgae through an interplay between early repression of carbon fixation and compensatory induction of PSI components. Such molecular plasticity provides short-term resilience, but its sustainability under prolonged or community-level exposure remains uncertain. Future studies should therefore combine long-term bioassays with multi-level biomarkers to refine the ecological risk assessment of engineered nanoparticles in aquatic ecosystems.

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Appendix A

Table A1. Analysis of one-way ANOVA for the genes *atpB*, *rbcL*, *psaA*, *psaB*, *psaD and psbA.*

Post Hoc Tests—Bonferroni							
Dependent Variable	**(I) Treatments**	**(J) Treatments (µg/L)**	**Mean Difference (I–J)**	**Std. Error**	**Sig**	**Lower Bound**	**Upper Bound**
atpB	Control	1.1	1.906	0.144	0.071	−0.118	3.9308
		4.4	1.722	0.589	0.123	−0.302	3.7464
		8.8	−0.788	0.344	1.000	−2.812	1.2364
		17.6	1.065	0.276	0.889	−0.959	3.0894
rbcL	Control	1.1	0.823 *	0.169	0.002	0.301	1.3441
		4.4	0.897 *	0.118	0.001	0.375	1.4181
		8.8	0.170	0.188	1.000	−0.351	0.6918
		17.6	0.503	0.139	0.062	−0.019	10.244
psaA	Control	1.1	4.627	0.479	1.000	−9.854	19.1084
		4.4	2.575	5.293	1.000	−11.907	17.0561
		8.8	−17.020 *	7.830	0.018	−31.501	−2.5383
		17.6	1.718	3.920	1.000	−16.199	12.7634
psaB	Control	1.1	1.317	0.133	0.268	−0.502	3.1367
		4.4	1.705	0.411	0.073	−0.115	3.5240
		8.8	−0.614	0.238	1.000	−2.433	1.2057
		17.6	0.942	0.273	0.934	−0.877	2.7613
psaD	Control	1.1	5.570	0.959	0.752	−4.476	15.6157
		4.4	6.567	0.872	0.413	−3.479	16.6127
		8.8	−11.375 *	6.772	0.023	−21.421	−1.3293
		17.6	1.638	2.399	1.000	−8.408	11.6837
psbA	Control	1.1	0.215	0.567	1.000	−0.952	1.3817
		4.4	0.358	0.505	1.000	−0.809	1.5247
		8.8	0.292	0.204	1.000	−0.875	1.4594
		17.6	0.022	0.202	1.000	−1.145	1.1890

* The difference in means is statistically significant at the 0.05 level.

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CAPITULO 6

Highlights

- Evaluation of TiO_2 nanoparticles toxicity in aquatic ecosystems
- Bioadsorption confirmed via SEM-EDS
- Feeding assay reveals increased toxicity via trophic transfer
- Ecological implications for nanoparticle pollution

ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) have emerged as emerging contaminants with a high potential impact on aquatic ecosystems. This study evaluates the ecotoxicological effects of environmentally relevant concentrations of TiO_2 NPs on *Daphnia magna*, a microcrustacean widely used as a bioindicator in toxicity assessments. Acute and chronic bioassays were conducted to determine the effects of direct exposure and ingestion through *Chlorella vulgaris* contaminated with TiO_2 NPs.

The results indicated that TiO_2 NPs did not induce significant acute toxicity in *D. magna*, as the 50% immobilization concentration (CI_{50}) was not reached. However, in chronic bioassays, prolonged exposure resulted in a reduced reproductive rate in exposed organisms. Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) analyses confirmed the bioadsorption and potential bioaccumulation of TiO_2 NPs in key anatomical structures of *D. magna*, suggesting alterations in its biological functionality.

The findings emphasize the need for more sensitive bioassays to evaluate the toxicity of nanomaterials, particularly those that assess effects along the trophic chain. The implementation of feeding assays proved to be an effective strategy for evaluating the transfer of toxicity from primary producers to secondary consumers. This study contributes to the understanding of the ecological impact of nanocontaminants and highlights the importance of their monitoring in aquatic ecosystems.

Keywords: Titanium dioxide nanoparticles (TiO_2 NPs), *Daphnia magna*, Feeding assay, Ecotoxicology, Trophic transfer impact.

6.1 INTRODUCCIÓN

Siendo el agua un recurso hídrico importante para el desarrollo de la vida, su demanda ha ido en aumento junto con el crecimiento de la población y de las economías de muchos países por lo que puede ser considerada actualmente como un recurso estratégico (Leonard et al., 2025; Murgatroyd et al., 2022; Santos Coelho et al., 2022). Como consecuencia de este crecimiento poblacional y amplio desarrollo de las actividades industriales, el ser humano libera diariamente diversos contaminantes a los cursos de agua natural, por lo que la producción de aguas contaminadas es cada vez mayor (Kim et al., 2024; Wang et al., 2024). La aparición de enfermedades y el desarrollo de la química analítica, han permitido que la comunidad científica centre sus estudios en la identificación y cuantificación de los diferentes contaminantes que pueden estar presentes en diferentes reservorios de agua, ya sean contaminantes orgánicos, inorgánicos, y agentes patógenos (Kumar & Ram, 2024).

El desarrollo de la industria ha impulsado la aparición de nuevas sustancias químicas, como plaguicidas, fragancias y preservantes de alimentos. Actualmente, la preocupación se centra en la escala de tamaño de estos contaminantes emergentes, incluyendo microplásticos y nanopartículas. Estos compuestos no solo plantean riesgos debido a su composición química y las concentraciones detectadas en diversas matrices ambientales, sino también por sus propiedades fisicoquímicas, las cuales influyen directamente en su interacción con los ecosistemas en los que se encuentran presentes (Osman et al., 2023; Strawn, 2021).

Desde hace más de una década, su impacto ha sido objeto de debate, especialmente en lo que respecta a su disposición final y los potenciales efectos sobre el medioambiente, afectando directamente a los productores primarios y alterando las dinámicas ecológicas de los sistemas naturales (ver Capítulos I y IV). Como ocurre con la identificación de cualquier nuevo grupo de contaminantes emergentes, el desarrollo y aplicación de técnicas analíticas resulta fundamental para evaluar su presencia en el medioambiente. Además, la creación de modelos experimentales, permite simular en el laboratorio los posibles efectos y dinámicas de estas nuevas fuentes de contaminación, proporcionando una mejor comprensión de su impacto ambiental y facilitando el diseño de estrategias de mitigación.

Los reportes que se han hecho sobre el modo de acción de las nanopartículas cuando llegan al medio ambiente van desde la adsorción de estas en la materia orgánica, la interacción en las células de los productores primarios hasta sus multiples efectos por la alteración de propiedades fisicoquímicas con otras sustancias del medio ambiente acuático (Mahana et al., 2021; Wu et al., 2022; Yu et al., 2025; Zhang et al., 2022). En cuanto a sus propiedades como tiempos de vida media, solubilidad, biodegradabilidad, entre otros, dependen de la propiedad intrínseca de las nanopartículas ya sea por su origen orgánico o inorgánico, como también de los factores ambientales que existan en el medio en que se encuentren siendo determinantes para los efectos tóxicos que estos desarrollen (Canuel et al., 2021; Poletaev et al., 2024).

Los bioensayos agudos y crónicos son ampliamente utilizados para evaluar efectos tóxicos, debido a sus múltiples ventajas, entre las que destacan la obtención de resultados de calidad en períodos de tiempo relativamente cortos, su alta eficiencia, costos reducidos y beneficios significativos. Estas características han favorecido el desarrollo y aplicación de una amplia variedad de bioensayos empleando distintos organismos (Gallo et al., 2023; Grigg et al., 2024; Pease et al., 2021; Raymundo et al., 2024; Xiong et al., 2022), Sin embargo, a momento de escoger, se deben tener encuentra los siguientes parámetros: organismos con una alta disponibilidad y que representen el medio a evaluar, estabilidad genética y uniformidad de la población, alta y constante sensibilidad (Mkandawire et al., 2014; Oliva et al., 2021; Rivetti, 2017).

Los bioensayos con microalgas verdes han sido utilizados en estudios ecotoxicológicos durante más de cuatro décadas y han sido estandarizados a nivel internacional mediante normativas (ISO, 2012; OECD, 2011) En particular, la prueba de inhibición del crecimiento algal se ha establecido como un bioindicador clave para evaluar la contaminación ambiental. Este ensayo, de aplicación sencilla, consiste en exponer células de microalgas a diferentes concentraciones de un contaminante dentro de un volumen definido, permitiendo así determinar sus efectos tóxicos o deletéreos sobre el crecimiento y la viabilidad celular. La sensibilidad de las microalgas a una amplia gama de compuestos químicos las convierte en herramientas fundamentales para la evaluación del impacto de sustancias potencialmente peligrosas en los ecosistemas acuáticos.

Sin embargo, en el capítulo anterior en este estudio, ya logramos observar los nuevos desafíos impuestos por los nanocontaminantes y que no pueden ser resueltos del todo con los modelos de bioensayos establecidos por la OECD (2011) e ISO (2012). Por lo que es sumamente importante considerar otras formas de evaluar toxicidad de forma indirecta por nanocontaminantes a través de la cadena trófica, como por ejemplo el feeding con la finalidad de establecer una línea de efectos a través de las tramas tróficas acuáticas, se recomienda iniciar con organismos unicelulares representativos del medio ambiente estudiado. En el caso de aguas continentales, las microalgas verdes constituyen un punto de partida adecuado, seguidas por el siguiente eslabón trófico, los microcrustáceos.

Como un modelo de consumidor primario en los microcrustaceos, podemos encontrar los grupos de cladóceros, con las especies del género *Daphnia* sp ampliamente utilizadas como organismos de referencia en pruebas de toxicidad (Nederstigt et al., 2022; Schmid et al., 2023; Tai et al., 2024; Tongur & Yıldız, 2021; Zheng et al., 2024). La amplia distribución geográfica, el importante papel que cumplen al interior de la comunidad zooplanctónica, la facilidad de cultivo en el laboratorio, la reproducción partenogenética, el corto ciclo de vida y una alta frecuencia reproductiva, han hecho de este grupo un modelo para la evaluación de toxicidad, a nivel mundial. Los ensayos de toxicidad con *D. magna*, permiten determinar la letalidad potencial de sustancias puras, aguas residuales domésticas e industriales, lixiviados, aguas superficiales o subterráneas y agua potable, entre otros (Instituto Nacional de Normalización (INN), 1999; United States Environmental Protection Agency (EPA), 1996). Además por su características filtradoras es posible determinar una toxicidad crónica, a través del ingreso de contaminantes a su sistema digestivo, permitiéndonos observar posibles cambios en los organismos a través del tiempo (ISO, 2001), sobre todo si se trata de una escala nanométrica y pruebas de concentraciones ambientales.

En este contexto, el modelo conformado por *Chlorella vulgaris* y *Daphnia magna* resulta ideal para la evaluación de toxicidad mediante ensayos de alimentación (feeding assays) (Manríquez-Guzmán et al., 2023; Tao et al., 2024; Vilar et al., 2022; Yin et al., 2022).

El presente estudio tiene como objetivo evaluar la transferencia de los efectos tóxicos derivados de la interacción nanopartículas-organismos en sistemas acuáticos. Para ello, se empleó *Chlorella vulgaris* y *Daphnia magna* como modelos tróficos representativos en ensayos de alimentación (feeding assays), considerando concentraciones ambientalmente relevantes.

Específicamente, se busca analizar los efectos tóxicos de las nanopartículas en *Daphnia magna*, con énfasis en su reproducción. Comparar los efectos observados en bioensayos tradicionales de reproducción con *Daphnia magna* frente a los obtenidos mediante feeding assays, en los cuales *Chlorella vulgaris* se utilizará como fuente de alimento. Esto permitirá evaluar la eficacia y aplicabilidad del enfoque basado en feeding assays.

Los resultados de este estudio contribuirán a una mejor comprensión del impacto de los nanocontaminantes en las cadenas tróficas acuáticas y servirán de base para el desarrollo de estrategias de monitoreo y mitigación de sus efectos en el ambiente.

6.2. METODOLOGÍA

6.2.1 Preparación de solución stock de nanopartículas de Mix TiO_2.

El estándar utilizado para cada medio preparado fue de nanopartículas de óxido de Titanio (IV), es una mezcla de rutilo y anatasa (MKCR1332) adquirido a través de Merck-Sigma Aldrch.

Para la realización de los concentrados con microalgas y nanopartículas para alimento, se utilizó la especie *Chlorella vulgaris* y se preparó una solución stock de 1000 μgL^{-1} en medio de cultivo para microalgas según OECD, 2011.

Para los ensayos de toxicidad aguda y crónica con microcrustáceo se utilizó la especie *Daphnia magna* y se preparó una solución stock de 1000 μgL^{-1} en medio de cultivo según ISO/10706.

Para todos los caso en la preparación de la solución stock y diluciones posteriores antes de ser aforada cada solución, estas fueron sometidas a sonicación por 2 h, para dispersar bien las nanopartículas, Finalmente se trasvasijó a un matraz aforado de 1 L y se continuó con el aforo hasta su medida final. Una vez obtenidas las soluciones stock de cada compuesto, se procedió a preparar las diferentes diluciones para cada caso de ensayo.

6.2.2 Concentrados de microalgas verdes *Chlorella vulgaris* para feeding.

El crecimiento de microalgas para alimento se realizaron en tubos esterilizados de 50 mL de vidrio de boro silicato con tapa de algodón estériles. Las concentraciones a las que se hicieron crecer los cultivos fueron: 3,9 $\mu g\ L^{-1}$, 7,8 $\mu g\ L^{-1}$ y 15,6 $\mu g\ L^{-1}$. Los cultivos se iniciaron con un inóculo de microalgas de 100.000 células por mL y se dejaron crecer hasta una densidad de 5.000.000 de células por mL como mínimo para comenzar a alimentar los bioensayos crónicos con *Daphnia magna* durante 21 días. Cada tratamiento fue realizado con 10 replicas.

6.2.3 Bioensayo con *Daphnia magna*.

Se utilizó *Daphnia magna* (Figura 6.1), aclimatadas para bioensayos agudos y crónicos de acuerdo con ISO/10706:2000, se obtuvieron del laboratorio de Bioensayos del Centro EULA de la Universidad Concepción, Chile. Se midieron los parámetros fisicoquímicos de acuerdo con las exigencias de las normativas internacionales para asegurar la calidad de los bioensayos. Para el bioensayo agudo se utilizaron neonatos de *D. magna*, menores a 24 h de vida. Cinco de estos organismos fueron depositados en envases de 50 mL, que contenían 10 mL de las distintas concentraciones preparadas de nanopartículas, esto se hizo por cuadruplicado en cada tratamiento y se mantuvieron sin renovación ni alimentación durante 48 h. Las concentraciones a ensayar correspondieron a 62,5 µg L^{-1}, 125 µg L^{-1}, 250 µg L^{-1}, 500 µg L^{-1} y 1000 µg L^{-1}. Los resultados a registrar son inmovilidad de los organismos cada 24 horas y el parámetro calculando es CI_{50} (concentración de Inmovilidad del 50% de la población expuesta). Los datos obtenidos fueron analizados mediante análisis Probit a través del programa IBM SPSS.

Para el caso de los bioensayos crónicos semi-estático con renovación cada 48 horas de medio de cultivo y alimento, el régimen de alimentación tanto para los bioensayos de exposición directa como para los de feeding, se estandarizaron con 300 µL de microalgas y 300 µL de solución proteica preparada en el laboratorio.

Las concentraciones empleadas para los ensayos crónicos se obtuvieron de los ensayos agudos en el cual al determinar LC_{50} se obtiene los limites inferiores obtenidas en el ensayo agudo, en donde las concentraciones no produjeron mortalidad en los dáfnidos. Se realizó recambio de agua y alimento cada 48 h la solución proteica preparada contenía: 3,15 g harina de pescado, 1,3 g de levadura y 0,25 g de alfalfa, disuelto en 500 mL de agua mili Q.

El ensayo se realizó a temperatura y fotoperiodo controlados (20 ± 1°C y 16 horas oscuridad y 8 horas de luz), durante los 21 días. Los resultados registrados fueron el número de crías obtenidas durante el periodo de duración del bioensayo, con los cuales se pudieron evaluar los parámetros de NOEC (Concentración más alta donde se puede observar un efecto) y LOEC (Concentración más baja donde se observan efectos), Los datos obtenidos fueron analizados a través de ANOVA de una vía con el test de Dunnet utilizando el programa estadístico IBM SPSS.

Para cada caso de ensayo mencionado anteriormente se realizó un control negativo con agua reconstituida y un control positivo con $K_2Cr_2O_7$.

6.2.4 Obtención y caracterización de imágenes de nanopartículas en *Daphnia magna*

Para la caracterización morfológica y evaluación de la interacción de nanopartículas de dióxido de titanio (NP-TiO_2) con *Daphnia magna,* se empleó un microscopio electrónico de barrido de emisión de campo (FE-SEM) ZEISS, modelo Gemini SEM 360. Este equipo cuenta con un voltaje de aceleración ajustable en un rango de 20 V a 30 kV, optimizado a 10V para minimizar el daño en las muestras biológicas. Se utilizó un emisor de efecto de campo tipo Schottky, que proporciona una alta estabilidad y resolución en la adquisición de imágenes.

Las muestras fueron preparadas siguiendo un procedimiento de fijación y deshidratación para evitar la colapsabilidad de los tejidos biológicos. Posteriormente, se recubrieron con una fina capa de oro-paladio mediante un sistema de *sputtering* para mejorar la conductividad y reducir la acumulación de carga. Se empleó un porta muestras de 12 posiciones, donde tres de ellas fueron utilizadas para aplicaciones analíticas con ángulo variable, permitiendo la observación de diferentes planos estructurales de los organismos.

La adquisición de imágenes se realizó en modo de presión variable utilizando el detector de corriente de cascada (C2D), lo que permitió obtener imágenes a muy bajo voltaje sin dañar las muestras. Además, el sistema cuenta con un detector de electrones secundarios y retrodispersados, lo que facilitó la observación de la morfología superficial y la composición elemental de las estructuras expuestas a NP-TiO_2.

Fig. 6.1: Imagen obtenida a través de microscopio electrónico de barrido de emisión de campo, marca ZEISS, modelo Gemini SEM 360. La imagen corresponde a la especie *Daphnia magna* con vista lateral en estado de neonato menor a 24 horas de eclosionado.

Para confirmar la presencia y distribución de las nanopartículas en las estructuras de *Daphnia magna*, se utilizó un detector de espectroscopía de dispersión de energía (EDS) de 80 mm, incorporado en dos puertos coplanares, lo que permitió mejorar la resolución mediante la adquisición de un mayor número de cuentas. Esto facilitó la identificación de la distribución del titanio en la superficie y en las estructuras internas del organismo.

6.3. RESULTADOS Y DISCUSIÓN

6.3.1 Ensayo de toxicidad aguda y crónica sobre *Daphnia magna* con nanopartículas de TiO_2.

Tabla 6.1: Números de Individuos inmóviles después de 48 h de exposición.

Réplicas	**Tipo de tratamiento (µg L^{-1})**					
	Control	62,5	125	250	500	1000
1	0	0	0	3	1	1
2	0	1	1	0	1	2
3	0	1	1	1	3	4
4	0	0	1	1	2	2
Total *D. magna* Inmóviles	**0**	**2**	**3**	**5**	**7**	**9**

La Tabla 6.1, presenta los datos de inmovilidad obtenidos de los neonatos de *D. magna* expuestos a la nanopartícula de TiO_2 durante 48 h de exposición. Se observa inmovilidad en *D. magna*, a medida que aumenta la concentración de las nanopartículas, sin embargo, esta respuesta no es suficientemente significativa para evidenciar toxicidad aguda, por lo que no se obtiene un CI_{50}.

Debido a lo anterior, estos resultados a pesar de ver que si es posible ver una pequeña repuesta sobre los individuos bajo las normativas establecidas para estos ensayos (NCh 2083;1999, ISO/10701, EPA 712-C-96-120:1996, Standard Methods For The Examination Of Water And Wastewater 23th: 2017), los resultados a informar son que no se detectó toxicidad aguda.

Esto respalda la necesidad de abordar nuevos métodos más sensibles frente a los nuevos contaminantes, A pesar de que se observa una leve respuesta adversa en los organismos expuestos a concentraciones más altas de nanopartículas, la falta de una reducción marcada en la movilidad sugiere que *D. magna* presenta cierta tolerancia o que los efectos de las nanopartículas de TiO_2 pueden no manifestarse inmediatamente a través de la inmovilidad. Este hallazgo es consistente con estudios previos (ver capítulo 3, 4 y 5) que han indicado que los efectos tóxicos de las nanopartículas pueden depender de múltiples factores, como la agregación en el medio, la biodisponibilidad y la interacción con materia orgánica disuelta, lo que puede reducir la fracción bioaccesible del contaminante.

6.3.2 Comparación de feeding assay y exposición directa sobre *Daphnia magna*.

Los resultados del ensayo comparativo entre exposición directa y feeding assay muestran diferencias claras en la respuesta crónicas de *Daphnia magna* frente a las nanopartículas de TiO_2 (Tabla 6.2).

Tabla 6.2. Exposición de *Daphnia magna* a nanopartículas de TiO_2

Tratamiento **Directo**	Promedio	SD	Varianza	Valor t (Dunnett)	Límite Inferior	Límite Superior
Control	54,2	3,1	9,7	-	-	-
2,2 µg/L	51,7	2,4	5,6	2,3	-1,4	6,4
4,4 µg/L	49,6	3,5	12,3	4,6	-0,1	9,3
8,8 µg/L	49,9	3,1	9,4	4,3	-0,1	8,7
17,6 µg/L	49,5	3,8	14,3	4,7	-0,2	9,6
Tratamiento por **ingesta**	Promedio	SD	Varianza	Valor t (Dunnett)	Límite Inferior	Límite Superior
Control	61,0	3,1	9,8	-	-	-
2,2 µg/L	51,8	22,7	517,1	9,2	-16,4	34,8
4,4 µg/L	51,3	26,7	711,1	9,7	-20,3	39,7
8,8 µg/L	49,7	25,8	667,8	11,3	-17,7	40,3
17,6 µg/L	37,1	19,2	367,4	23,9*	2,4	45,5

*. La diferencia de medias es significativa en el nivel 0.05.

En el tratamiento de exposición directa, los promedios de reproducción variaron entre 49,5 y 54,2 neonatos por hembra, sin registrarse diferencias significativas en relación con el control ($p > 0,05$). Los valores t de Dunnett se mantuvieron entre 2,3 y 4,7, todos por debajo del umbral de significancia, lo que indica que la exposición directa a las concentraciones ensayadas (2,2–17,6 µg/L) no generó un efecto adverso evidente sobre la tasa reproductiva. Estos resultados sugieren que, bajo condiciones de exposición directa, las nanopartículas de TiO_2 tienden a permanecer aglomeradas, reduciendo su biodisponibilidad y, por ende, su toxicidad efectiva hacia *D. magna.*

Por el contrario, en el ensayo por ingesta (feeding assay), los efectos fueron marcadamente distintos. Se observó una disminución progresiva en el número promedio de neonatos a medida que aumentó la concentración de nanopartículas, desde 61,0 en el control hasta 37,1 neonatos/hembra a 17,6 µg/L. El análisis estadístico mediante la prueba de Dunnett mostró un incremento sostenido en los valores t, alcanzando significancia estadística a la concentración más alta ($t = 23,9$; $p < 0,05$), lo que nos indica un LOAEC de 17,6 µg/L y un NOAEC de 8,8 µg/L. Esta respuesta confirma que la exposición indirecta, mediada por la ingesta de microalgas contaminadas con NP-TiO_2, produce un impacto negativo significativo en la reproducción de *D. magna*.

Estos resultados coinciden con la hipótesis de que las nanopartículas pueden experimentar una bioacumulación trófica a través de los productores primarios, aumentando su biodisponibilidad y toxicidad en niveles tróficos superiores. La reducción significativa en la reproducción observada en el feeding assay podría deberse a la internalización de nanopartículas a través del alimento, lo que incrementa el contacto directo con tejidos intestinales y células germinales, afectando procesos metabólicos esenciales y la producción de energía.

En conjunto, la comparación entre ambos tratamientos evidencia que la vía de exposición modula fuertemente la respuesta biológica, siendo la ingesta una ruta más crítica que la exposición directa. Este patrón concuerda con lo descrito por estudios previos (Baun et al., 2008; Luo et al., 2025), donde la exposición alimentaria a nanopartículas resulta en una mayor retención y toxicidad, incluso a concentraciones ambientalmente relevantes.

Por tanto, los resultados obtenidos refuerzan la importancia de considerar la transferencia trófica y los efectos subletales en la evaluación del riesgo ecológico asociado a nanopartículas en sistemas acuáticos, ya que la toxicidad observada no depende únicamente de la concentración, sino de la vía de ingreso y la transformación de las partículas a lo largo de la red trófica.

6.3.3 Estimación de EC_{50} en la inhibición de reproducción por feeding assay

El análisis Probit aplicado a los datos de reproducción de *D. magna* expuestas durante 21 días a nanopartículas de TiO_2 mediante la vía trófica (feeding assay) permitió estimar un valor de EC_{50} de 17,8 µg/L (IC_{95}%: 11,9–61,4 µg/L), con un ajuste lineal significativo ($R^2 = 0,916$) (Tabla 6.3, Figura 6.2). La tendencia observada evidencia una disminución progresiva en la tasa de reproducción a medida que aumenta la concentración de nanopartículas ingeridas, alcanzando un 49% de inhibición en el tratamiento más alto.

Tabla 6.3. Análisis Probit para EC_{50}, Inhibición de reproducción de *D. magna* por feeding assay en 21 días de exposición indirecta con microalgas contaminadas con TiO_2

Tratamiento por ingesta	Número de sujetos	Respuesta Observada	Respuesta esperada	Residuos	Probabilidad	Estimación EC_{50}	LI	LS
Control	100	0	6,2	-6,2	0,1	**17,8**	**11,9**	**61,4**
2,2 µg/L	100	15	8,9	6,2	0,1			
4,4 µg/L	100	16	12,3	3,6	0,1			
8,8 µg/L	100	19	21,8	-3,3	0,2			
17,6 µg/L	100	49	49,2	-0,2	0,5			

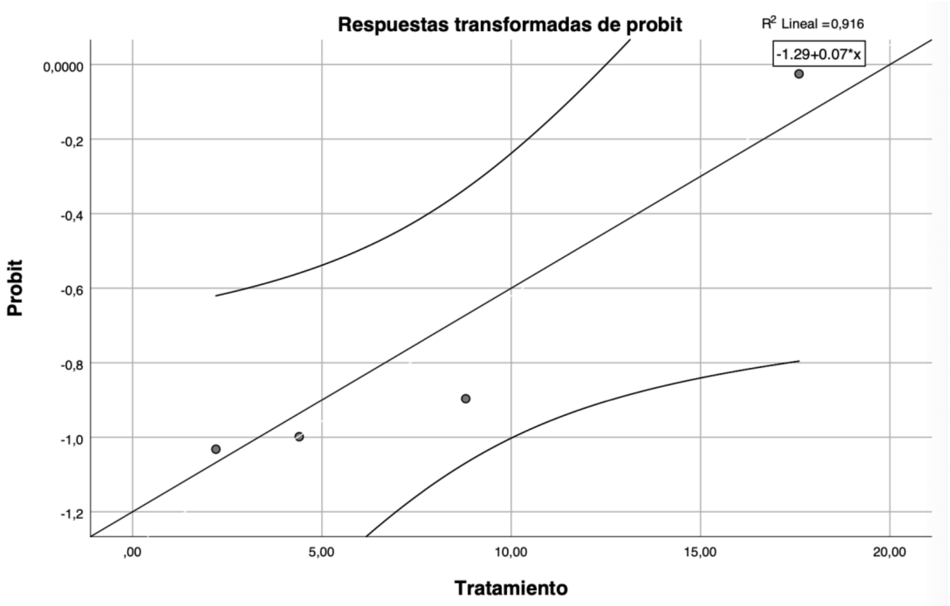

Figura 6.2: Efectos de reproducción en *D. magna* por aplicación por ingesta de microalgas contaminadas con NP-TiO_2

Un hallazgo destacable es que el EC_{50} coincide con el LOAEC (17,6 µg/L) obtenido en la prueba de Dunnett (Tabla 6.2). Esta concordancia sugiere que la concentración a partir de la cual se observaron diferencias significativas respecto al control es también aquella en la que se produce una reducción del 50% en la capacidad reproductiva. Tal superposición refuerza la consistencia estadística y biológica del resultado, indicando que a partir de ese punto se produce un cambio fisiológico crítico en el organismo.

Desde una perspectiva ecotoxicológica, este punto de inflexión puede interpretarse como una transición fisiológica crítica en *Daphnia magna*, donde los mecanismos de compensación metabólica —como la detoxificación intestinal o la redistribución energética hacia funciones vitales— dejan de ser suficientes para mantener la reproducción. En concentraciones inferiores, el organismo podría sostener su homeostasis, redistribuyendo energía hacia la reproducción o el mantenimiento celular;

sin embargo, una vez alcanzado el umbral de ~17–18 µg/L, la carga interna de nanopartículas ingeridas excede su capacidad de regulación, provocando una disminución abrupta en la fecundidad.

La pendiente del modelo Probit (0,07) respalda esta interpretación, al reflejar una relación dosis-respuesta pronunciada: pequeñas variaciones en la concentración producen cambios notables en la proporción de organismos afectados. Este comportamiento sugiere un fenómeno de acumulación fisiológica y umbral más que un efecto lineal progresivo, coherente con los mecanismos de toxicidad por ingestión de nanopartículas descritos previamente (Bhagat et al., 2020; S. Li et al., 2014). En este tipo de respuesta, la toxicidad se manifiesta de manera abrupta una vez saturadas las rutas de excreción o neutralización de partículas, generando daños sostenidos en la función intestinal y en el metabolismo energético de los ovarios.

Asimismo, la elevada varianza observada en los tratamientos intermedios (4,4–8,8 µg/L) podría asociarse a diferencias individuales en la capacidad de ingestión o al grado de aglomeración de las nanopartículas adheridas a las microalgas, lo que condiciona la dosis efectiva interna. Este comportamiento también es consistente con la naturaleza no homogénea de la exposición trófica y con la heterogeneidad fisiológica propia de los ensayos crónicos con *Daphnia magna*.

En conjunto, los resultados sugieren que el efecto reproductivo observado no responde únicamente a la concentración externa, sino a una acumulación trófica efectiva que desencadena una respuesta fisiológica límite. Este patrón se ha documentado en otros estudios de transferencia trófica, donde la exposición alimentaria resulta más tóxica que la exposición directa por provocar la internalización de nanopartículas en tejidos reproductivos y hepáticos (Manríquez-Guzmán et al., 2023)

Desde la perspectiva del riesgo ecológico, la coincidencia entre EC_{50} y LOAEC adquiere especial relevancia, pues implica que el umbral de toxicidad crónica para *D. magna* se encuentra dentro del rango de concentraciones ambientales detectadas en el río Biobío (17–22 µg/L). Esto refuerza la hipótesis de que la transferencia trófica de nanopartículas puede generar efectos subletales ecológicamente significativos bajo condiciones ambientales realistas, comprometiendo la estabilidad poblacional de especies clave en la red trófica pelágica.

Los resultados obtenidos en el feeding assay revelan que la exposición a nanopartículas de TiO_2 mediante el consumo de *C. vulgaris* genera efectos adversos mayores a los observados en la exposición directa. Pudiéndose observar que en algunas concentraciones, los efectos por vía trófica fueron incluso más pronunciados, lo que sugiere una posible bioacumulación de nanopartículas en las microalgas antes de ser consumidas por *D. magna*. Este hallazgo concuerda con los resultados de Yin *el al*. (2022), quienes encontraron que el uso de *Daphnia* como organismo modelo en estudios ecotoxicológicos permite evaluar la acumulación de contaminantes emergentes en los niveles tróficos inferiores y sus efectos en consumidores secundarios (Yin et al., 2022).

La ausencia de toxicidad aguda en concentraciones ambientales pero la presencia de efectos crónicos en la reproducción de *D. magna* sugiere que la exposición prolongada a nanopartículas de TiO_2 puede tener impactos ecológicos a largo plazo. Tal como lo reportaron Liu *el al*. (2022), en su estudio sobre la bioacumulación de nanopartículas en sistemas acuáticos, la persistencia de estas partículas en el medio puede generar efectos subletales que afecten la dinámica de las poblaciones de zooplancton y, por consiguiente, el equilibrio de los ecosistemas acuáticos (Liu et al., 2022).

6.3.4 Caracterización morfológica mediante SEM y EDS

En el plano ventral observada en la figura 6.3, se pueden observar estructuras como las patas filtradoras y la boca de *Daphnia*, que están orientadas hacia el interior del caparazón en donde además se pueden observarse parcialmente sus órganos internos.

En la Figura 6.4, Se pueden observar con mayor detalle el caparazón con vista superior y los apéndices internos del individuo.

El análisis de microscopia electrónica de barrido (SEM) reveló la presencia de nanopartículas de TiO_2 adheridas a la superficie del caparazón y en las estructuras filtradoras de los neonatos de *Daphnia magna* expuestos a concentraciones ambientalmente relevantes de NP-TiO_2 (Figura 6.4). La espectroscopía de dispersión de energía (EDS) confirmó la presencia de Ti en las muestras analizadas, lo que sugiere una posible bioadsorción y bioacumulación en estructuras anatómicas clave, esto comprometen la movilidad y eficiencia alimentaria en especies planctónicas, estos resultados muestran que la impregnación de NP-TiO_2 en la estructura cálcica del exoesqueleto de *D. magna* podría afectar su integridad estructural y funcionalidad.

Figura. 6.3: Imagen obtenida a través de SEM. Vista ventral de un neonato de *Daphnia magna* expuesta a nanopartícula de TiO_2.

Se ha reportado que la acumulación de nanopartículas en sistemas biológicos puede inducir alteraciones mecánicas que comprometen la flexibilidad y resistencia del exoesqueleto, dificultando la locomoción y la eficiencia alimentaria (Wang et al., 2023). Por otra parte, se ha reportado que las nanopartículas de TiO_2 pueden modificar la integridad del exoesqueleto y afectar la fisiología de filtración de *D. magna*, lo que podría alterar su capacidad de ingestión de alimento y, en consecuencia, su desempeño ecológico en ambientes contaminados (Trinh et al., 2022).

La acumulación de NP-TiO_2 en las estructuras filtradoras sugiere una posible interferencia con la capacidad de alimentación de *D. magna*, reduciendo la tasa de ingestión y afectando su balance energético. Esto concuerda con hallazgos previos de Di Nica et al. (2022), quienes observaron que la exposición a contaminantes emergentes en *D. magna* puede alterar su comportamiento de alimentación y locomoción, comprometiendo su supervivencia a largo plazo (Di Nica et al., 2022).

Dado que las NP-TiO_2 están ampliamente presentes en sistemas acuáticos debido a su uso industrial y cosmético, es fundamental realizar estudios de largo plazo que evalúen su persistencia en el ambiente y su potencial de transferencia trófica. Asimismo, se recomienda investigar la interacción de las NP-TiO_2 con otros contaminantes ambientales para comprender mejor su toxicidad en condiciones de exposición múltiple (Wang et al., 2022).

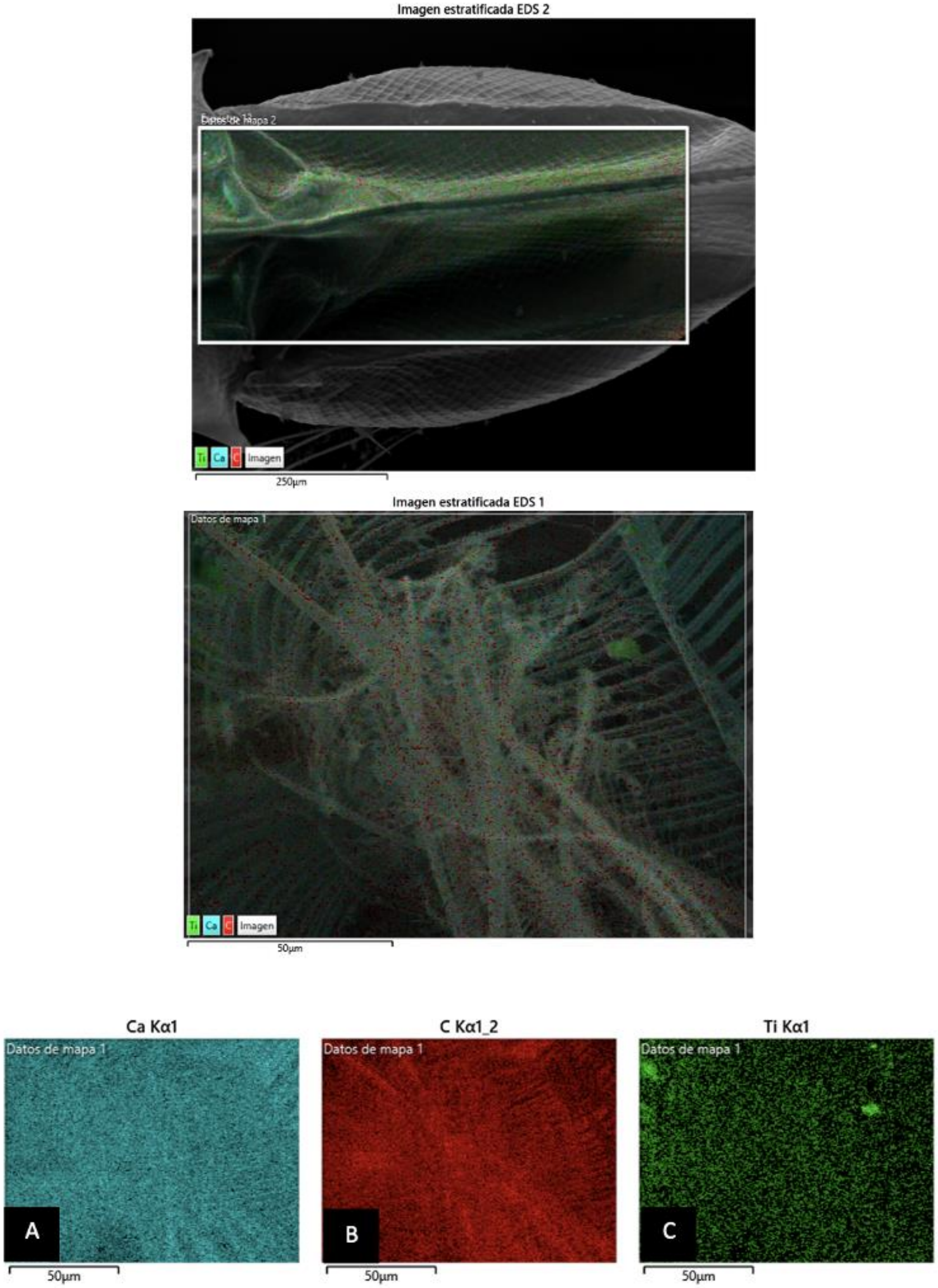

Fig. 6.4: Apéndices internos de neonato de *Daphnia magna* expuesta a nanopartículas de TiO_2. A: muestra las estructuras cálcicas propias del exoesqueleto de *Daphnia magna*; B- muestra la presencia de carbono orgánico propia del alimento proteico el cual complementa la dieta de los individuos y C-corresponde a las nanopartículas de TiO_2 adheridas a las estructuras internas de los individuos.

6.4 CONCLUSIONES

Los resultados obtenidos en este capítulo evidencian que los efectos de las nanopartículas de TiO_2 sobre *D. magna* dependen fuertemente de la vía de exposición, mostrando diferencias sustanciales entre la exposición directa y la exposición trófica mediante el feeding assay.

En los ensayos de exposición directa, las concentraciones evaluadas (2,2–17,6 µg/L) no produjeron efectos significativos en la tasa de reproducción, lo que sugiere una baja biodisponibilidad de las nanopartículas en suspensión. Este comportamiento puede atribuirse a procesos de aglomeración y sedimentación que limitan el contacto efectivo con el organismo, reduciendo la fracción realmente activa en el medio.

Por el contrario, la exposición por ingesta reveló una inhibición progresiva y significativa de la reproducción, con diferencias estadísticamente detectables a 17,6 µg/L (LOAEC) y una relación dosis-dependiente bien definida (t de Dunnett = 23,9; $p < 0,05$). El análisis Probit confirmó este patrón, determinando un EC_{50} de 17,8 µg/L (IC_{95}%: 11,9–61,4 µg/L), valor coincidente con el LOAEC, lo que indica un punto crítico de transición fisiológica en el que los mecanismos homeostáticos del organismo se ven superados.

Esta coincidencia entre el EC_{50} y el LOAEC refuerza la consistencia estadística y biológica de los resultados, sugiriendo que la concentración crítica a partir de la cual se produce una reducción significativa de la reproducción también representa el umbral de afectación poblacional. La pendiente positiva del modelo Probit (0,07) y el alto coeficiente de determinación ($R^2 = 0,916$) reflejan una respuesta dosis-respuesta abrupta, característica de procesos fisiológicos saturables donde la capacidad de detoxificación y redistribución energética resulta insuficiente.

En términos ecológicos, los resultados confirman que la vía alimentaria es la principal ruta de exposición y bioacumulación de nanopartículas en organismos filtradores, aumentando su toxicidad crónica y el riesgo de transferencia trófica. La disminución significativa en la capacidad reproductiva de *D. magna* a concentraciones ambientalmente detectadas (17–22 µg/L) sugiere un impacto potencial sobre la dinámica poblacional de esta especie clave en la red trófica acuática.

En conjunto, los resultados de este capítulo demuestran que las nanopartículas de TiO_2 pueden ejercer efectos subletales relevantes a concentraciones ambientales, especialmente mediante mecanismos de exposición indirecta, y que su evaluación ecotoxicológica requiere considerar las vías tróficas y los procesos de bioacumulación para una interpretación realista del riesgo ecológico. Estos hallazgos establecen una base sólida para el análisis posterior de riesgo ambiental y la interpretación integrada de los efectos observados en los niveles tróficos superiores

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La presente investigación constituye la primera evidencia documentada de la presencia de nanopartículas de dióxido de titanio (TiO_2) en el río Biobío, un sistema fluvial de alta relevancia ecológica y socioeconómica para el centro-sur de Chile. Las concentraciones detectadas (17,6–22,9 µg/L) se sitúan dentro del rango de los valores reportados en ecosistemas impactados por descargas urbanas e industriales en otras regiones del mundo (Kunhikrishnan et al., 2015; Kwarciak-Kozłowska & Fijałkowski, 2025), lo que confirma que la liberación de nanopartículas ya no es un fenómeno circunscrito a los países industrializados. Este hallazgo demuestra que los sistemas de tratamiento de aguas residuales no logran eliminar completamente los nanomateriales, permitiendo su ingreso sostenido a los cuerpos de agua (Karkee & Gundlach-Graham, 2024). La morfología observada mediante microscopía electrónica —con tamaños entre 10 y 206 nm y fases de anatasa y rutilo— respalda la coexistencia de fuentes naturales y antropogénicas (S. S. Raut et al., 2024), siendo las descargas domésticas e industriales las principales responsables de la acumulación aguas abajo (Bäuerlein et al., 2017).

En relación con la Hipótesis 1, los resultados obtenidos permiten considerarla confirmada. La presencia de nanopartículas de TiO_2 fue verificada en los distintos puntos de muestreo a lo largo de la cuenca del río Biobío, evidenciando que estos nanomateriales forman parte del sistema fluvial. Además, se observó un incremento de las concentraciones en los tramos inferiores del río, particularmente aguas abajo de asentamientos urbanos y zonas de descarga de aguas residuales, lo que indica una influencia significativa de fuentes antropogénicas en su distribución. Este patrón espacial respalda la hipótesis de que las actividades humanas contribuyen de manera relevante a la presencia y acumulación de nanopartículas de TiO_2 en el sistema fluvial.

Desde una perspectiva ecotoxicológica, el análisis de expresión génica en *Chlorella vulgaris* reveló que incluso concentraciones ambientalmente realistas de TiO_2 son capaces de alterar mecanismos fotosintéticos esenciales. La represión del gen rbcL, involucrado en la fijación de carbono, y la sobreexpresión de psaA, psaB y psaD, vinculados al Fotosistema I, evidencian una respuesta adaptativa de tipo hormético, donde la microalga intenta compensar el estrés oxidativo y mantener el balance energético. Esta plasticidad molecular refleja un ajuste fino frente a la interferencia de las nanopartículas con el transporte electrónico fotosintético, generando un escenario donde la productividad primaria podría verse afectada a largo plazo, con potenciales repercusiones en la base trófica de los ecosistemas.

En organismos consumidores primarios como *Daphnia magna*, los ensayos crónicos mediante exposición trófica confirmaron efectos reproductivos significativos con valores de NOAEC = 8,8 µg/L y LOAEC = 17,6 µg/L, lo que evidencia la posibilidad de impactos ecológicos bajo condiciones reales de exposición. La ausencia de toxicidad aguda, acompañada de una clara afectación crónica, refuerza la importancia de incluir evaluaciones prolongadas y multiescalares en los protocolos regulatorios, ya que las nanopartículas no siempre provocan mortalidad inmediata, pero sí pueden alterar la función ecológica y la estructura poblacional de las comunidades acuáticas.

A nivel conceptual, los resultados de este trabajo ponen en evidencia una brecha importante en el desarrollo y gestión de las nanotecnologías. La industria y la investigación aplicada han priorizado la eficiencia y la innovación funcional de los nanomateriales sin considerar suficientemente su destino final en el medioambiente (Dabare et al., 2025). La persistencia, movilidad y capacidad de bioacumulación de las nanopartículas hacen necesario replantear su diseño bajo principios de "nanotecnología verde" o eco-design, que integren desde su origen criterios de biodegradabilidad y menor impacto ambiental (Al-Awady et al., 2015b). El avance tecnológico no puede desvincularse de la sostenibilidad; la falta de un enfoque de ciclo de vida completo perpetúa la transferencia del riesgo ecológico a los ecosistemas naturales (Mallari et al., 2025).

En este contexto, la normativa ambiental tanto nacional como internacional muestra rezagos significativos. La mayoría de las regulaciones actuales se enfocan en contaminantes clásicos (metales, pesticidas, hidrocarburos), sin contemplar los nanomateriales como categoría diferenciada (Nielsen, 2024; K. Raut & Sharbidre, 2025). Es urgente establecer límites específicos, metodologías de detección estandarizadas y marcos regulatorios que obliguen a la industria a demostrar la inocuidad ambiental de sus productos antes de su comercialización. En Chile, la incorporación de estos criterios dentro de instrumentos como la Evaluación de Impacto Ambiental o las Normas de Calidad Secundaria representaría un avance fundamental hacia una gestión preventiva y no reactiva de los contaminantes emergentes.

Finalmente, las evaluaciones de riesgo ecológico adquieren un rol central para integrar la evidencia generada en este estudio, ya que permiten traducir los resultados experimentales en implicancias ambientales reales (Burgos-Aceves et al., 2025). Los valores de efecto obtenidos para *Daphnia magna* (NOAEC, LOAEC y EC_{50}) se encuentran dentro del rango de concentraciones ambientales medidas en el río Biobío, lo que demuestra que las condiciones naturales actuales ya se acercan o incluso superan los niveles capaces de generar efectos biológicos en organismos sensibles. Este hallazgo es particularmente relevante, pues indica que los ecosistemas fluviales pueden estar sometidos a un estrés crónico continuo, no detectable mediante análisis fisicoquímicos tradicionales, pero sí evidenciado a través de respuestas biológicas subletales.

La coincidencia entre concentraciones ambientales y valores de efecto ecotoxicológico sugiere que la exposición prolongada a nanopartículas de TiO_2 podría alterar procesos ecológicos clave, como la productividad primaria, la eficiencia trófica y la dinámica poblacional de especies filtradoras (Bellingeri, 2025; Yetu et al., 2024). Además, la naturaleza coloidal y reactiva de estas nanopartículas amplifica su riesgo, ya que tienden a agregarse, adsorberse a materia orgánica o asociarse a biofilms, lo que modifica su biodisponibilidad y facilita su transferencia trófica hacia niveles superiores. Por lo tanto, los modelos de riesgo deben considerar no solo la concentración total medida, sino también la fracción realmente disponible para los organismos, ya que esta define el potencial de interacción biológica.

El enfoque integrado propuesto en este estudio que combina evidencia química, biológica y molecular, refuerza la necesidad de avanzar hacia evaluaciones de riesgo basadas en múltiples líneas de evidencia (Weight of Evidence, WoE). Solo mediante este tipo de aproximación es posible establecer márgenes de seguridad ambiental más representativos, expresados en valores de concentración predicha sin efecto (Predicted No Effect Concentration, PNEC) que reflejen el comportamiento dinámico de las nanopartículas en el ambiente. Incorporar estos enfoques en las normativas permitiría desarrollar programas de monitoreo adaptativo y estrategias de gestión más efectivas, orientadas no solo a la detección de contaminantes, sino también a la preservación funcional de los ecosistemas acuáticos frente a los desafíos emergentes de la nanotecnología.

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El presente estudio constituye un avance significativo en la comprensión del comportamiento y los efectos ecotoxicológicos de las nanopartículas de dióxido de titanio (TiO_2) en ecosistemas acuáticos, aportando evidencia inédita para la cuenca del río Biobío. La detección y caracterización de estas nanopartículas, junto con la estimación de sus concentraciones ambientales, confirman que su presencia forma parte del escenario contaminante actual asociado a actividades urbanas e industriales. Este hallazgo demuestra que los nanomateriales ya se encuentran incorporados en los sistemas fluviales y que su liberación al ambiente representa un desafío emergente para la gestión ambiental.

A nivel biológico, los resultados evidencian que concentraciones ambientalmente realistas de TiO_2 pueden alterar la fisiología de los productores primarios. La represión y sobreexpresión diferencial de genes clave para la fotosíntesis en *Chlorella vulgaris* reflejan una respuesta adaptativa frente al estrés oxidativo inducido por las nanopartículas, lo que sugiere posibles efectos en cascada sobre la productividad y estabilidad del ecosistema. De manera complementaria, los bioensayos con *Daphnia magna* demostraron efectos crónicos sobre la reproducción y supervivencia incluso en rangos cercanos a los detectados ambientalmente, lo que refuerza la relevancia ecológica de los niveles observados en el medio natural.

Estos resultados ponen de manifiesto una contradicción en el desarrollo actual de la nanotecnología: el avance en innovación y funcionalidad de los nanomateriales no ha sido acompañado por una reflexión equivalente sobre su destino final y sus impactos ambientales. La ausencia de un enfoque de ciclo de vida completo perpetúa la transferencia del riesgo ecológico hacia los ecosistemas naturales. En este contexto, resulta necesario promover una nanotecnología responsable que incorpore principios de ecodiseño, degradabilidad y trazabilidad desde las etapas iniciales de producción.

Asimismo, la falta de una normativa específica para nanomateriales en Chile y en la mayoría de los países limita la capacidad de control y prevención. Las regulaciones actuales se centran principalmente en contaminantes convencionales y no consideran la complejidad del comportamiento ambiental de los nanomateriales. Por ello, se vuelve prioritario actualizar los marcos regulatorios e incorporar metodologías de monitoreo adaptativo basadas en múltiples líneas de evidencia —química, biológica y molecular— que permitan identificar efectos subletales y anticipar riesgos ecológicos.

Finalmente, las evaluaciones de riesgo ecológico realizadas en este estudio indican que las concentraciones ambientales actuales pueden aproximarse a niveles capaces de generar efectos biológicos medibles, lo que sugiere una reducción en los márgenes de seguridad ambiental. La integración de herramientas experimentales y de modelación permitirá mejorar la estimación de concentraciones predichas sin efecto (Predicted No Effect Concentration, PNEC) y fortalecer la base científica para una gestión preventiva. En conjunto, esta investigación aporta evidencia clave sobre la presencia y los efectos de nanopartículas de TiO_2 en sistemas fluviales, contribuyendo al desarrollo de estrategias científicas y regulatorias orientadas a compatibilizar el avance tecnológico con la sostenibilidad ambiental.

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