Universidad de Concepción

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

Respuestas Biológicas en Peces Nativos por Mezcla Compleja de Contaminantes Químicos en el Río Biobío, Chile Central. Aporte a los Antecedentes Biológicos Necesarios en una Norma Secundaria de Calidad Ambiental

Tesis para optar al grado de

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Mauricio Alejandro Quiroz Jara

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“La naturaleza nunca hace nada sin motivo….”
Aristóteles

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N°	Título	Año	Revista	Indexación	1er Autor (si/no)	Estado
1	Integrated Physiological Biomarkers Responses in Wild Fish Exposed to the Anthropogenic Gradient in the Biobío River, South-Central Chile.	2021	Environmental Management	ISI	Si	Aceptada
2	Reproductive Status of the native fish Percilia irwini along the anthropogenic impact gradient of the Biobio River, South-Central Chile.	2021	Frontiers in Physiology. Research Topic: *Imbalances in the Reproductive Physiology of Aquatic Animals caused by Pollutants*	ISI	Si	Enviada

3	Assessing wild fish exposure to ligands for sex steroid receptors from pulp and paper mill effluents in the Biobio River Basin, Central Chile.	2019	Ecotoxicology and Environmental Safety	ISI	No	Publicada
4	Integrated Physiological Biomarkers Response (IPBR) as A Proposal for the Improvement ff Chilean Secondary Environmental Quality Standards	2021	Freshwater Biology	ISI	Si	Preparación

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2018	VII Congreso SETAC Capítulo Argentino. San Luis, Argentina	Argentina	Integrated Response of Physiological Biomarkers (IRPB) In Two Native Fishes as a Profile Indicator of Biological Effects by Multiple Environmental Stressors.	Oral
2018	SETAC Europe 28th Annual Meeting	Italia	Physiological / Reproductive Status Of Native Fish Exposed To A Complex Chemical Mixture In The Bíobio River, Central Chile.	Oral
2017	12thSETAC Latin America Biennial Meeting	Brasil	Biomarkers In Native Fish Present In The Biobio River, Central Chile, Convergence Effects Of Complex Chemical Mixtures	Póster
2016	XIV Congresso Brasileiro de Ecotoxicologia, ECOTOX 2016	Brasil	Selenium In Gulls: A Review Of Published Data	Póster
2015	SETAC, Latin America 11th Biennial Meeting	Argentina	Estratégias experimentales para la evaluación del riesgo ecotoxicológico en organismos bioindicadores en la Bahía de Concepción, Chile.	Póster

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RESUMEN

El río Biobío es la tercera cuenca más importante del país con una superficie de 24.260Km^2 y abastece de agua potable a cerca de 970.000 habitantes. Numerosas empresas utilizan el recurso hídrico para sus procesos industriales, lo que tiene como consecuencia la eliminación de efluentes sobre su cauce principal generando zonas de contaminación puntual (industrias de la celulosa y el papel, aguas servidas, entre otras) las que, debido a la mejora en sus tecnologías, han presentado un aumento en su productividad en los últimos años. Además, es posible detectar zonas de contaminación difusa, dado por el uso de suelo agrícola y forestal, situación que en conjunto han afectado la biota y calidad del agua principalmente en el tercio inferior de esta cuenca. Los estudios que se han realizado en el río Biobío para detectar el efecto de los efluentes industriales y de plantas de tratamiento de agua servidas sobre los organismos acuáticos, han demostrado efectos desde alteraciones de las funciones metabólicas, activando enzimas de detoxificación, hasta alteraciones de tipo reproductivo. Aunque la mayor parte de estos estudios se han realizado con especies introducidas, y bajo condiciones de laboratorio, los resultados obtenidos por distintos investigadores, dan evidencia de grupos de compuestos que tienen significativos efectos biológicos. Por esta razón, es necesario continuar utilizando herramientas como biomarcadores, debido a que son instrumentos válidos en estudios ecotoxicológicos, para evaluar las respuestas entregadas por peces bioindicadores.

En un marco de evaluación de efectos acumulativos con una aproximación basada en efectos, la importancia de utilizar múltiples biomarcadores en ictiofauna nativa

permite detectar el potencial daño que están produciendo la mezcla compleja de estresores químicos liberados al cauce principal por los efluentes industriales, tratamientos de aguas residuales y/o uso agrícola y forestal en la cuenca. Esta situación adquiere relevancia debido a que la ictiofauna nativa presente en el río Biobío, posee un valor biológico evolutivo que constituye un recurso genético único en el mundo y por lo tanto, representado por un alto endemismo, no sólo a nivel de especies sino también, a nivel de género y familia y por un alto porcentaje de especies con características primitivas. Por esta razón la alteración en los ambientes puede llevar a una disminución de las poblaciones de nuestros peces nativos, y eventualmente a su extinción.

En base a lo expuesto anteriormente, es necesario entender cómo el desarrollo urbano, agrícola e industrial en el río Biobío altera la calidad del agua e impacta el estado fisiológico de la ictiofauna nativa expuesta a distintas condiciones ambientales y estresores de diversa naturaleza, con un gradiente de efectos de mayor intensidad en zonas de contaminación puntual y en un gradiente longitudinal hacia el tercio inferior, respecto a zonas de contaminación difusa; siendo el objetivo de este estudio, establecer e identificar las respuestas biológicas, evaluadas mediante estrategia de biomarcadores en peces nativos, asociadas a mezcla compleja de contaminantes químicos de origen urbano, agrícola e industrial en la cuenca del río Biobío.

Los resultados expuestos en esta tesis de doctorado, dan cuenta de cambios fisiológicos en los peces nativos *Percilia irwini* y *Trichomycterus areolatus*, a lo largo del curso principal del río Biobío. Estos resultados evidencian que los ejemplares presentes en sitios de alto impacto antropogénicos presentan un mayor número de oocitos en la estado primario, con un crecimiento protoplásmico de oocitos primarios significativamente mayor que los sitios de referencia Lonquimay (LQ) y Balsa Caracoles (BC). Este comportamiento también se observa en los oocitos vitelogénicos presentes en la zona de referencia LQ, en comparación con los sitios Puente Coihue (PC) y Santa Juana (SJ), donde se reduce la presencia de ooocitos maduros. El menor número de oocitos vitelogénicos indica un crecimiento gonadal armónico saludable en las áreas de menor intervención antrópica correspondiente a las zonas de referencia (LQ y BC). Además, en el período de otoño (marzo), los oocitos en estado vitelogénico pertenecientes a los sitios PC y SJ evidencian una disminución en crecimiento (diámetro) y frecuencia (número de oocitos por estadío de maduración).

El desarrollo de un índice integrado de biomarcadores fisiológicos (IPBR), permitió evaluar cambios globales de las respuestas biológicas obtenidas y relacionar patrones de efectos con diferentes mecanismos de acción toxicológica en determinadas zonas del río con diferentes perfiles de contaminación. Nuestro índice IPBR indicó la ocurrencia de efectos fisiológicos asociados con un gradiente espacial y temporal, similar al patrón encontrado en biomarcadores moleculares (EROD) e individuales (longitud total, peso total e IGS) medidos en ambas especies. Lo observado en ambas especies estudiadas en el río Biobío mediante IPBR fue influenciada por la perturbación antropogénica provocada por compuestos químicos xenobióticos de diversa naturaleza, sugiriendo un efecto espacial acumulativo debido a la degradación ambiental en el tercio inferior del río Biobío, evidenciando

una disminución de la condición biológica en ambos peces silvestres, y estos cambios manifiestos están relacionados con la alta actividad antropogénica desde Lonquimay hasta los sitios de Puente Coihue y Santa Juana. Los resultados obtenidos a través de IPBR, permiten observar un perfil de efectos fisiológicos en el curso principal del río Biobío y concuerda con el perfil de calidad de agua establecido en esta importante cuenca.

Palabras Claves: Peces nativos; Río Biobio; Reproducción; IPBR; Biomarcadores; Normas de calidad ambiental.

CAPÍTULO I
INTRODUCCIÓN

1.1. ANTECEDENTES GENERALES

El río Biobío es la tercera cuenca más importante del país con una superficie de 24.260Km^2 y abastece de agua potable a cerca de 970.000 habitantes (Figura 1) (Parra *et al*, 2013). Numerosas empresas utilizan el recurso hídrico para sus procesos industriales, lo que tiene como consecuencia la eliminación de efluentes sobre su cauce principal, presentando zonas de contaminación puntual. Respecto a esto, las industrias que en este momento generan un mayor impacto sobre esta cuenca son las industrias de la celulosa y el papel las que, debido a la mejora en sus tecnologías, han presentado un aumento en su productividad desde el año 1990 a 2017, de 324.115 tons/año a 3.815.000 tons/año, de igual manera ha ocurrido con la industria de producción de acero (750 tons/año a 793.757 tons/año) y producción anual de refinería de petróleo (79.417 tons/año a 5.520.322 tons/año), afectando la biota y calidad del agua en el tercio inferior de esta cuenca (Tabla 1) (Parra, 2013).

Por otra parte, en el tercio superior del río Biobío, se presentan efectos de contaminación difusa, principalmente debido a la alta actividad agrícola y forestal. Según indica la DGA (2004) sobre la clasificación de usos de suelo en la cuenca del río Biobío, los terrenos agrícolas, agricultura de riego y plantaciones forestales alcanzan en total un 29% de un total de 2.426.400 hectáreas. Estudios recientes (EULA 2020) indican que en la cuenca del río Biobío, más del 40% de los cambios ocurridos entre 1997 y 2015 fue producto del avance de las plantaciones forestales sobre terrenos agrícolas, matorrales y bosque nativo (Figuras 2 y 3) y donde aún existe incertidumbre respecto a los efectos en este ecosistema acuático debido al escurrimiento lateral en estas zonas y sobre todo al confluir estos eventos de contaminación difusa del tercio superior con la contaminación puntual en el tercio inferior.

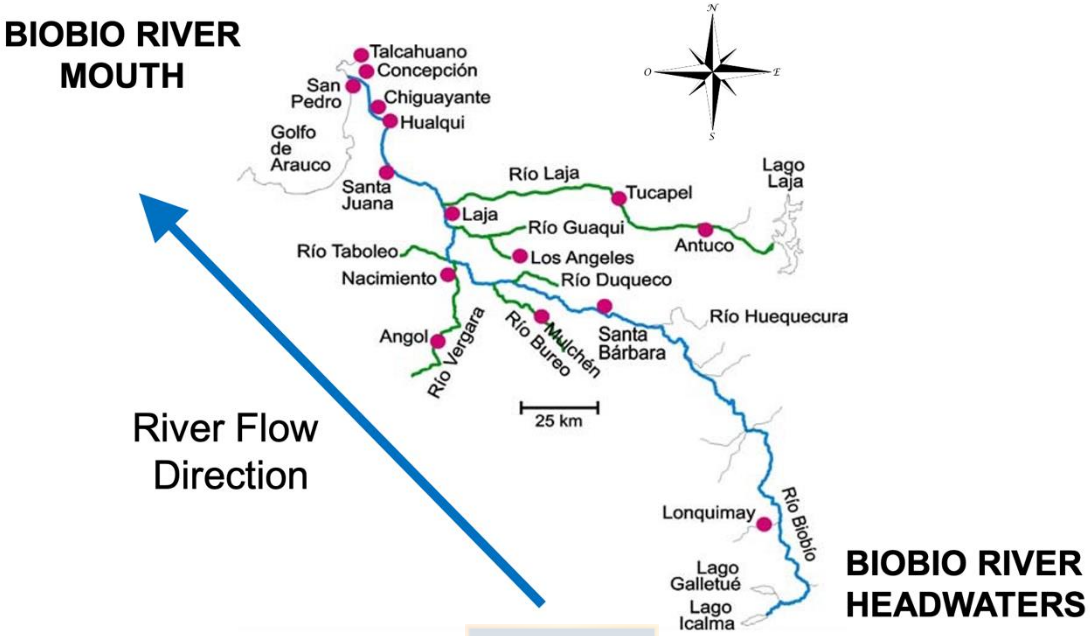

Figura 1. Curso principal del río Biobío y principales centros urbanos adyacentes.

Tabla 1. Resumen Comparativo Situación Ambiental en la Cuenca del Biobío, Chile Central.

Componentes	Unidad	1990	2012	2017	Observaciones
Descargas de Aguas Servidas Con tratamiento	Nº	0	14		El año 1990 existían 31 descargas de aguas servidas, sin tratamiento previo
Cobertura de tratamiento de aguas servidas urbanas	%	0	100	100	(2002 -> 42.7%). Actualmente 100% de aguas servidas urbanas son depuradas previa descarga
Producción Anual de Celulosa	Tons / Año	324.115	**2.049.600**	**3.815.000**	CMPC Celulosa S.A
Q Agua Industrial	m^3 / seg	**1.46**	**3.78**	S/I	
Q RIL		**1.31**	**3.38**		
Producción Anual de Papel	Ton / año	125	136.227	S/I	Papeles Río Vergara S.A*
		73	**114**		Papeles Bio Bío S.A.
Q agua Industrial	m^3 / seg	0.27	0.18	S/I	Papeles Río Vergara S.A*
		0.9	**0.14**		Papeles Bio Bío S.A.
Q RIL	m^3 / seg	0.25	0.17	S/I	Papeles Río Vergara S.A*
		0.08	**0.12**		Papeles Bio Bío S.A.
Producción Anual de Acero	Ton / año	**750**	**1.219.445**	**793.757**	
Refinería Producción Anual ENAP	m^3 / año	79.417	5.520.322	116.000 (barriles/año)	Refinería de Petróleo Crudo en la Región del Biobío
Cobertura Plantaciones Forestales	Hectáreas	578.580	514.765	**902.259**	INFOR, 2020
Cobertura Bosque Nativo Adulto	Hectáreas	367.446	137.524	**348.782**	CONAF, 2011, 2015, EULA 2020
Sistemas Canales de Riego	Hectáreas	180	211.8		
	m^3 / seg	118	158		
Centrales Hidroeléctricas	MW / año	906	2.337	3.500	EULA, 2020

*sin funcionamiento desde 2013. Parra 2013; DGA 2015; CONAMA 2006; INFOR, 2020; MMA, NSCA, 2015; EULA, 2020.

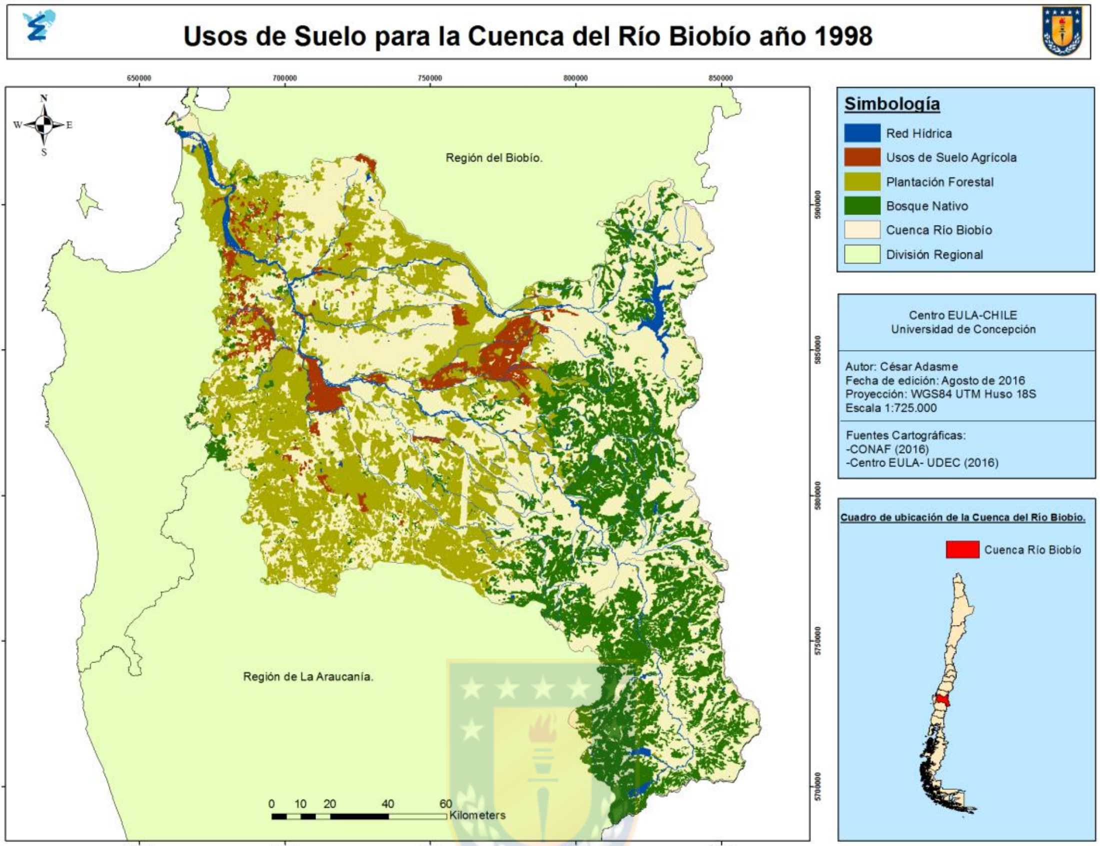

Figura 2. Distribución de uso de suelo para la Cuenca del Río Biobío correspondiente al año 1998.

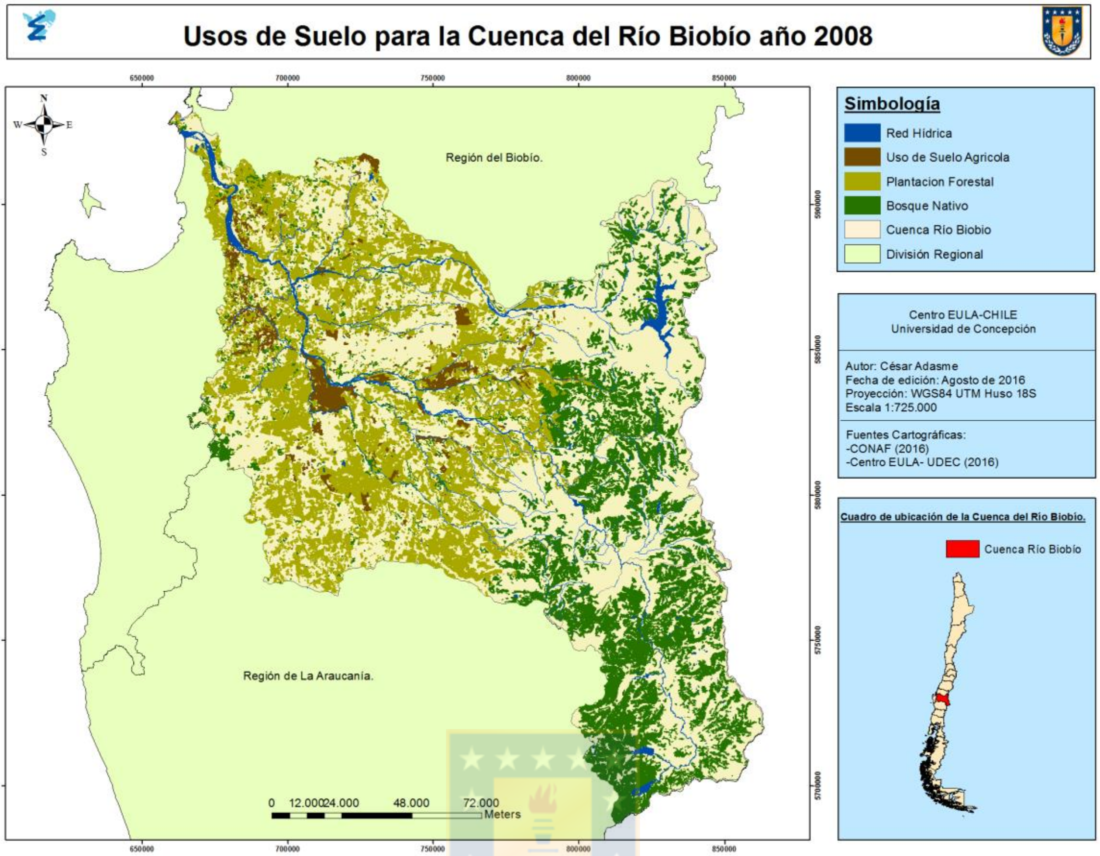

Figura 3. Distribución de uso de suelo para la Cuenca del Río Biobío correspondiente al año 2008.

El creciente desarrollo industrial de nuestro país ha llevado a un aumento de industrias que en su gran mayoría utilizan grandes volúmenes de agua en sus procesos, por lo que generan desechos líquidos que son vertidos a cursos naturales de aguas cercanos. Ejemplos de este tipo de industrias son aquellas ligadas al sector forestal como la industria de celulosa y papel. En relación a este sector empresarial, la Región del Biobío se caracteriza por ser una zona donde la actividad forestal aumenta su nivel productivo año a año, como también en términos económicos y sociales, y el proceso de expansión de este tipo de industria implica un impacto ambiental debido a las modificaciones del entorno natural (Orrego *et al*, 2005a,b; Chiang *et al*, 2010, 2012).

Dentro de la diversidad de actividades urbanas e industriales anteriormente mencionadas, las que presenta potencialmente el mayor impacto ambiental sobre los ecosistemas acuáticos, en términos de efectos puntuales, corresponde al impacto de aguas servidas, y la de producción de pulpa de celulosa, ésta última debido a su método de producción, donde la madera utilizada como materia prima pasa por una serie de procesos físico- químicos (Karrasch *et al*, 2006). Diversos estudios han detectado efectos biológicos producidos por los compuestos presentes en este tipo de efluentes, los que han permitido un gran número de investigaciones en el campo de la ecotoxicología.

Producto del fuerte impacto ambiental de estas industrias sobre el medio ambiente, y sobre todo en los ecosistemas acuáticos, estas empresas han tenido que invertir en tecnología para disminuir el efecto de su sistema de producción, lo que se ha visto reflejado en el uso de sistemas aeróbicos y la conversión de tecnologías de blanqueo en las que se suprime el uso del cloro, llamadas libre de cloro elemental (ECF). (Van Den Heuvel M., Ellis R., 2002).

1.2. Uso de Biomarcadores

Los efluentes de este tipo de industrias involucran una mezcla de sustancias orgánicas e inorgánicas, muchas de las cuales han permanecido desconocidas y/o no han sido químicamente identificadas, las cuales pueden presentar un impacto en el ambiente (Soimasuo *et al*., 1998). Se ha demostrado, entre otros aspectos, que la exposición a esta mezcla química compleja produce problemas reproductivos en varios vertebrados mediante un efecto disruptivo del sistema endocrino (Wells & Van Der Kraak, 2000; Orrego et al, 2019; Barra et al, 2021).

Históricamente, se ha verificado en peces que la exposición a efluentes de celulosa conduce a un estado de estrés con alteraciones directas en su salud (Adams *et al*., 1992). El primer caso de androgenización ambiental, producto de este tipo de efluentes, fue detectado en 1978 en una población de pez moquito (*Gambusia holbrooki*) en un lago de Estados Unidos, donde las hembras presentaban características externas de machos (Jenkins *et al*., 2001); sin embargo, también existen registros de alteraciones en machos (Larsson *et al*., 2000). Por otro lado, estas alteraciones relacionadas al estrés en las funciones bioquímicas y fisiológicas preceden, generalmente, a efectos de mayor impacto en los niveles de población y comunidad, y por lo mismo a un mayor tiempo de respuesta (Fossi, 2000).

Esta necesidad de evaluar y detectar cuál es el impacto de la acción antrópica sobre los ecosistemas acuáticos, ha permitido utilizar cambios en los organismos tanto bioquímicos, fisiológicos e histológicos para poder determinar el resultado de una exposición frente a un xenobiótico o una mezcla de ellos. Estos cambios han sido denominados biomarcadores, es decir, aquella variación bioquímica, celular, fisiológica o de comportamiento, que puede ser medida en un tejido, en un fluido biológico, organismo o población y que proporciona evidencia de exposición a, y/o efectos de uno o más contaminantes (Depledge, 1995).

Durante los últimos años se ha intensificado la búsqueda de métodos de estudio innovadores tendientes a detectar los impactos sobre los ecosistemas acuáticos provocados por la contaminación derivada de las actividades humanas, con el objetivo de diseñar e implementar medidas preventivas. En las últimas décadas, el uso y desarrollo de biomarcadores ha cobrado un interés creciente a fin de evaluar el riesgo (probabilidad de producir efectos adversos) de una sustancia química potencialmente tóxica. Los estudios realizados mediante la utilización de biomarcadores, específicamente a nivel bioquímico- celular, donde es posible inferir sobre lo que está ocurriendo a nivel organísmico, permite evaluar lo que eventualmente ocurrirá a nivel poblacional y por supuesto a nivel de comunidad (Moore *et al*., 2004).

La gran fortaleza de esta metodología, radica en el hecho que se elimina las limitaciones de las estimaciones tradicionales de contaminación ambiental, como las estimaciones de residuos químicos en muestras biológicas y por sobre todo relaciona los efectos con su posible causa.

Junto con los biomarcadores, la necesidad de contar con bioindicadores apropiados, que relacionen la causa con el efecto observado, se ha hecho evidente ante la presencia de compuestos con actividad hormonal, capaces de alterar la fisiología reproductiva normal en comunidades expuestas a ellos (Orrego et al, 2005; Chiang *et al*, 2011; 2012, Quiroz-Jara et al, 2021, Barra et al, 2021).

Durante la última década ha crecido el interés sobre cuál es el efecto a nivel reproductivo (desarrollo gonadal) que tienen los contaminantes presentes en los sistemas acuáticos que actúan como disruptores del sistema endocrino, con el propósito de utilizar estas como un indicador temprano de impacto de las actividades industriales que vierten sus efluentes a los sistemas acuáticos y potencialmente afectan las poblaciones naturales (Dube *et al.*, 2001; Rasmussen *et al*, 2004; Moore *et al*., 2004). Esto se sustenta en el hecho que durante los estados de desarrollo temprano, los peces son altamente sensibles a perturbaciones hormonales, dando como resultado reversión sexual, desarrollo gonadal temprano y atresia temprana de los ovocitos, que pone de relieve la enorme plasticidad y vulnerabilidad de la gónada como órgano centinela (Papoulias *et al*, 2000; Gimeno *et al*, 1998). La inducción de vitelogenina en peces, representa uno de los biomarcadores más sensibles y válidos para la individualización del efecto estrogénico en organismos ovíparos, en especial en organismos que se encuentran directamente expuestos a los efluentes de la industria de la celulosa (Sepúlveda *et al*, 2003).

Los estudios realizados en esta área, señalan que la disrupción endocrina ocurrida en peces nativos, producto de efluentes de diversa naturaleza, se ha deducido por un desarrollo gonadal anormal, retraso de la edad de madurez y alteración de los niveles de hormonas estereoidales. También se ha reportado disminución en la secreción de gonadotropinas, que participan en la gametogénesis y estereidogénesis (GtH I) y en la maduración de la gametogénesis (GtH II), así como depresión en la síntesis de esteroides ováricos (EPA, 1997). Las hembras presentan un menor número de huevos maduros, mientras que en machos una menor expresión de las características sexuales secundarias (Arcand-Hoy & Benson, 1998).

1.3. Antecedentes de Impactos en el Río Biobio

Respecto a la Región del Biobío, el río Biobío es la tercera cuenca hidrográfica más grande del país y es la principal fuente de abastecimiento de agua potable para cerca de 970.000 habitantes, de los cuales 745.000 habitantes, se abastecen de aguas superficiales y 225.000 habitantes de aguas subterráneas (Parra *et al*, 2013); es un recurso hídrico para los procesos industriales (forestales, agrícolas, celulosa y papel, producción de acero, refinería de petróleo, entre otras) adyacentes al río y, es receptor de los efluentes de este tipo industrias, entre otras, con una distribución aproximada del uso del recurso de 93% para riego, 1.5% como fuente de agua potable y 5.5% de uso industrial (Valdovinos & Parra 2006).

El río Biobío recibe actualmente la descarga de diversas industrias, lo que corresponde a fuentes de estrés químico sobre todo de compuestos xenobióticos de carácter estrogénico, neuromoduladores y clastogénicos, que actúan como disruptores de los procesos homeostáticos de la biota presente en el río Biobío (Orrego *et al*, 2005a,b; 2019; Inzunza *et al*, 2005; Alonso et al, 2017). Las características de los efluentes de las industrias dependen de factores como: la materia prima, tecnologías de producción, y sistemas de tratamiento de efluentes.

Estimaciones sobre las descargas industriales en el curso principal del río Biobío serían de aproximadamente 11,5 m^3/s, un valor 10 veces mayor que las descargas urbanas (1,6 m^3/s) liberadas en el río (Valdovinos & Parra 2006). Por otra parte, en Chile, el 83% de la producción nacional de celulosa es generado por industrias adyacentes a la cuenca del río (Gaete *et al*., 1999).

Estudios realizados mediante la implementación de biomarcadores en esta cuenca, han detectado la presencia de compuestos xenobióticos que afectan los organismos que habitan en este río. En un estudio realizado por Fossi *et al* (1995) utilizando la inducción de la actividad de la Citocromo P450 (medida mediante la actividad de la etoxiresofurina-O- detilasa, EROD) determinó una alta actividad EROD e inhibición de la Acetilcolinesterasa, en peces nativos y en aves en la zona de Concepción, indicando una fuerte relación exponencial inversa entre ambos resultados. Por otro lado, Gavilán *et al*. (2001) en un estudio realizado en la desembocadura de tres ríos de la VIII Región (Biobío, Itata y Tubul) utilizando peces como bioindicadores para evaluar la exposición de compuestos organoclorados, y otros compuestos como PCBs, PAHs, dioxinas, entre otros, detectó una alta actividad EROD en el río Biobío, y una fuerte inhibición de la acetilcolinesterasa en el río Itata. Este último resultado se debe a la gran actividad agrícola y forestal utilizada en tierras adyacentes a dicha cuenca.

Otros estudios confirman la presencia de compuestos que alteran el metabolismo normal de peces presentes en la cuenca del río Biobío, donde nuevamente se evidencia actividad EROD en hígado de peces, esta vez posiblemente debido a la presencia de dioxinas y furanos (Orrego *et al*., 2005a). Por otro lado, el mismo autor (Orrego *et al*., 2005b) en un experimento para determinar el efecto de los efluentes de la industria de la celulosa y el papel expuso a peces inmaduros de la especie *Oncorhynchus mykiss* a sedimentos del río que fue divido en tres zonas de descarga de efluentes de estas industrias (Preimpacto, Impacto y Postimpacto). Luego de 29 días de exposición los efectos fueron evidentes en peces de las zonas de impacto y postimpacto, debido a los altos niveles EROD, así como los altos niveles de vitelogenina detectados también para estas dos zonas. Al realizar los análisis histológicos del tejido gonadal, se logró evaluar una inducción de la maduración gonadal en hembras juveniles.

Por otra parte, estudios de genotoxicidad realizados en eritrocitos de trucha arcoíris (*Oncorhynchus mykiss*) han revelado que existe un claro daño genotóxico, medido mediante el test Cometa o SCGEA (Single Cell Gel Electrophoresis Assay), debido principalmente a la alta concentración de Hidrocarburos Aromáticos Policíclicos (PAHs) en los sedimentos en la zona de la desembocadura del río Biobío (Inzunza *et al*., 2005).

Asociado a lo anterior, los contaminantes emergentes, considerados como compuestos pseudo-persistentes provocan efectos adversos y no del todo conocidos en el ambiente (Kuster *et al*, 2008). Esta situación que aborda los principales estudios científicos sobre efectos en los cuerpos de agua en los últimos años (Barceló, 2003), adquiere relevancia debido a la continua liberación de este tipo de compuestos mediante efluentes de plantas de tratamiento de aguas servidas. Diversos estudios han reportado la presencia de estos contaminantes emergentes de tipo micro-contaminantes orgánicos (OMP) en el tercio inferior del río y sin capacidad de eliminación de ellos en las plantas de tratamientos (Rozas *et al*, 2016), como también se han registrado efectos fisiológicos de este tipo de contaminantes en peces en laboratorio e *in situ* en el tercio superior del río Biobío, en las ciudades de Santa Bárbara y Los Ángeles (Saavedra, 2015).

Estos antecedentes confirman que existen alteraciones, bioquímicas, celulares, metabólicas fisiológicas, entre otras, en los peces presentes en el río Biobío, producto de la mezcla química compleja vertida en el cauce principal.

Los estudios mencionados en párrafos anteriores, respecto al efecto difuso generado por actividad agrícola y forestal, así como el efecto puntual debido a la actividad industrial en la longitudinal del río Biobío, además de actuar como receptor de efluentes domésticos, han generado una alteración de la calidad del agua y de la integridad biológica y ecológica de este sistema acuático (Habit *et al*, 2006; Karrasch *et al*, 2006; Valdovinos & Parra, 2006; Orrego *et al*, 2005; Lara et al, 2003, 2009; Echeverría et al, 2007, Little et al, 2009). Este impacto se observa con el cambio de paisaje, por modificación de uso de suelo, que repercute en una disminución del bosque nativo, y un aumento de plantaciones forestales y actividad agrícola (Figuras 2 y 3). Los estudios que se han realizado en el río Biobío para detectar el efecto de los efluentes de diferentes tipos de industrias en sus procesos sobre los organismos acuáticos, han demostrado efectos desde alteraciones de las funciones metabólicas, activando enzimas de detoxificación, hasta alteraciones de tipo reproductivo. Aunque la mayor parte de estos estudios se han realizado con especies introducidas, y bajo condiciones de laboratorio, los resultados aún no demuestran si los efectos observados corresponden a un tipo de contaminante o a una mezcla compleja de compuestos xenobióticos, o si se presenta una sinergia entre la mezcla de contaminantes de origen puntual y difuso, e incluso, cómo las respuestas moleculares y celulares detectadas, afectan la fisiología de la ictiofauna nativa presente en el río.

La importancia de la utilización de múltiples biomarcadores para realizar estudios de efectos en la ictiofauna nativa se debe a que esta fauna presenta un valor biológico evolutivo, constituyendo recursos genéticos únicos en el mundo y, presenta un alto endemismo, no sólo a nivel de especies sino también a nivel de género y familia (Arratia, 1981; Campos *et al*., 1993). Por esta razón la alteración de su biotipo puede llevar a una disminución de las poblaciones de nuestros peces nativos, y eventualmente a su extinción.

Según informa Chiang *et al*, (2010), las especies nativas sólo se han estudiado en los últimos 25 años. Su biología es en general poco conocida, por lo que el principal desafío en el desarrollo de programas de monitoreo estandarizados en sistemas acuáticos chilenos, es una mejor comprensión de la biología de las especies de peces nativos. La documentación sobre la variabilidad natural dentro de valores definidos, aún es escasa, por lo tanto, es difícil estimar una causa/efecto, para comparar las respuestas de las poblaciones de peces nativos expuestos a los compuestos químicos de diversa naturaleza presentes en el río Biobío. Estos vacíos en los datos biológicos básicos en ictiofauna nativa, aumenta la dificultad de la evaluación de las respuestas a un nivel de organización biológica molecular y celular, obtenidas mediante biomarcadores bioquímicos o moleculares, de exposición y/o de efecto (por ejemplo, Vitelogenina, EROD, Cometa, Histología, entre otros). De esta forma, los límites y las metodologías de detección temprana para las especies nativas deben ser aplicables a un modelo local y regional, y deben ser calibrados con las respuestas de otras especies obtenidas mediante esta metodología.

1.4. Evaluación de Efectos Acumulativos

Las diversas actividades en la cuenca del río Biobío, presentes en el espacio y en el tiempo, podrían potencialmente haber generado efectos sumatorios, sinérgicos y/o acumulativos en los peces presentes en esta cuenca. Según reporta Dubé y colaboradores (2012), mediante una estrategia de más de 20 años de estudio, la evaluación de estos efectos acumulativos, corresponde al proceso de evaluar sistemáticamente los efectos, como resultado de incremento, acumulación y la interacción de estresores presentes en un sistema. Así, el propósito de cualquier evaluación de efectos acumulativos es determinar inicialmente si existe un impacto por un desarrollo existente (o de una condición existente), y luego determinar o predecir la extensión del impacto que estará asociada con un desarrollo futuro.

Desde esta perspectiva, y considerando la evaluación de efectos acumulativos, se han propuesto dos aproximaciones para evaluar el impacto de diversos estresores en los sistemas acuáticos. En primer lugar, la aproximación basada en estresores enfatiza el desarrollo de un modelo conceptual, basado en la interacción de estresores con componentes ecosistémicos valorados (por ejemplo paisaje, fauna nativa). Esta aproximación está basada en integradores a larga escala de respuestas biológicas (diversidad, abundancia) y utiliza datos disponibles conducentes a desarrollar un entendimiento consensuado de un ecosistema a través de los múltiples interesados. Esta aproximación se focaliza en potenciales impactos específicos relacionados a un desarrollo propuesto (Munkittrick *et al*, 2000). Sin embargo, si bien es relevante examinar el potencial de impactos conocidos de nuevos estresores, el proceso de estimar el impacto de un desarrollo futuro en un sistema, cuando el estatus existente y sensibilidad del sistema es desconocido, se hace aún más difícil y genera aún más incertidumbre.

Una segunda aproximación denominada “aproximación dirigida a efectos” intenta definir el “estado ambiental acumulado”, es decir, el estado existente de un ecosistema que ha integrado todos los estresores presentes, con el objetivo de reducir el impacto de las modificaciones existentes y predecir y prevenir impactos de la adición de nuevos proyectos de desarrollo a los sistemas acuáticos.

Según indican Munkittrick *et al*, (2000), Dubé *et al* (2003, 2010, 2013) y Squires & Dubé (2012), esta aproximación, implica medir el estado ambiental acumulado de un sistema y trata de identificar 1) si el rendimiento está por debajo del nivel esperado en sitios comparables y 2) qué factores impiden el desempeño normal. El análisis depende de los organismos residentes que integran el lugar de las condiciones ambientales y los estresores. Así, la predicción de impactos futuros sólo puede ser posible cuando existe un entendimiento básico del sistema, y la extrapolación a otros cuerpos de agua requerirá un entendimiento básico de los procesos que estructuran esos sistemas.

Desde esta perspectiva, es necesario considerar que la cuenca del río Biobío, representa la base natural de uno de los más importantes centros de desarrollo económico del país. Los sectores productivos más relevantes y dinámicos corresponden al sector forestal, sector agropecuario (localizado principalmente en las regiones de Ñuble y Bío Bío), sector industrial (industrias de la celulosa y el papel, industrias metalúrgicas, químicas, refinería del petróleo, industria de la curtiembre y textiles) y el sector hidroeléctrico que constituye la principal fuente de suministro de energía eléctrica a nivel nacional, que en conjunto implica cerca del 50% del PGB regional (Parra *et al*, 2013); y potencialmente nuevos proyectos industriales en un futuro cercano (Tabla 1).

Considerando estos antecedentes, al tomar en cuenta una evaluación de efectos acumulativos con aproximación basada en efectos, es posible realizar una evaluación de los aspectos biológicos, que es independiente del tipo de estresor y del desarrollo *a priori* de las vías de respuesta de los potenciales estresores. El estado ambiental acumulado corresponde a la suma de los impactos de todos los estresores químicos presentes en el sistema. Si los organismos residentes crecen, se reproducen y sobreviven a tasas óptimas, es posible asumir que las condiciones existentes no limitan su desempeño. Mientras que si los organismos residentes están limitados en su desempeño, en términos de crecimiento, reproducción o sobrevivencia, los factores ambientales que limitan su condición biológica, pueden ser utilizados como un foco para el análisis de riesgo (Munkittrick *et al*, 2000; Dubé *et al*, 2003, 2010, 2013; Squires & Dubé, 2012).

1.5. Normativa Ambiental para el Río Biobio

En base a lo anterior, y respecto a la cuenca del río Biobío, la actual norma secundaria de calidad ambiental para la protección de las aguas continentales superficiales (2015), indica en sus consideraciones, numeral 3 (tres) que el agua constituye el recurso esencial para la conservación y preservación de los ecosistemas acuáticos, y por su parte, requiere la mantención de las condiciones naturales del medio que hacen posible la óptima evolución y desarrollo de las especies y los ecosistemas que lo conforman; continuando en su numeral 4 (cuatro) que éstas normas tienen la función de mantener o mejorar la calidad de las aguas de la cuenca, y así conservar los ecosistemas acuáticos o preservar los ecosistemas acuáticos y sus servicios ecosistémicos. Sin embargo, como indica el contenido de esta norma, existen intervenciones antrópicas que han generado riesgos para la protección y conservación de esta cuenca en particular, con fuentes de contaminación puntuales y difusas que vierten a cuerpos receptores de agua. Respecto a esto, la norma define en su Título II, Artículo Nº5, niveles de calidad, haciendo sólo referencia a parámetros fisicoquímicos, y definiendo la calidad del agua sólo respecto a valores de concentraciones que no sobrepasen los establecidos, sin considerar los potenciales efectos biológicos que la mezcla compleja de contaminantes químicos pudiera tener sobre los peces nativos presentes en el río, para ser evaluados de forma temprana. De este modo es posible, confirmar el efecto sobre las poblaciones de peces y sobre el estado fisiológico de los peces que habitan en el río Biobio, a través de trabajos de estudio sobre efectos biológicos de diversos autores (Orrego et al, 2005; 2009; 2019, Habit et al, 2006; Quiroz-Jara et al, 2021).

Como se ha mencionado en párrafos anteriores, los biomarcadores proporcionan información valiosa en estudios de campo y son utilizados para medir una amplia gama de respuestas fisiológicas tempranas por exposición a compuestos químicos, a nivel bioquímico, celular y en tejidos (Oliveira *et al*, 2011; Seabra *et al*, 2014; Colin *et al*, 2016; Capela *et al*, 2016). Es por esta razón que son una herramienta útil para el monitoreo de efectos biológicos por contaminación. Sin embargo, el potencial que presenta esta herramienta se encuentra limitado, debido a la falta de un análisis estadístico integrado. Para esto es necesario mejorar la interpretación entre los diferentes biomarcadores multinivel, utilizados para determinar los efectos en peces centinelas, y relacionarlos a los estándares de calidad definidos por la normativa chilena. De esta forma, es posible evaluar las variaciones globales de las respuestas obtenidas, mediante un índice integrado de Biomarcadores de Respuestas Fisiológicas (IPBR), que permita relacionar patrones de efectos con distintos mecanismos de acción toxicológica (Beliaeff *et al*, 2002; Sanchez *et al*, 2013), en áreas determinadas con diferentes perfiles de contaminación, y de esta forma, cumplir lo establecido por la normativa chilena, en lo referente a calidad de agua, además de conservar y preservar los ecosistemas acuáticos.

En relación a los antecedentes planteados, la fauna íctica en el río Biobío, representa un acervo genético propio de nuestra zona biogeográfica. Por esta razón la alteración de su biotopo ha dirigido una disminución de las poblaciones de nuestros peces nativos (Habit *et al*, 2006), y podría llevar potencialmente a su extinción. En base a lo argumentado en párrafos anteriores, y los antecedentes históricos recopilados respecto a la alteración de la calidad del agua y el efecto sobre la integridad biológica y ecológica del río Biobío, el uso de una batería de biomarcadores dentro de un marco de evaluación de efectos acumulativos con aproximación basada en efectos, permite evaluar el potencial impacto sobre la ictiofauna nativa presente en la cuenca, debido a la mezcla compleja de compuestos químicos presentes por contaminación puntual y difusa liberados al sistema, potencial efecto sobre ictiofauna que corresponde a un hotspot de biodiversidad; aspectos y antecedentes biológicos no considerados por la actual Norma Secundaria de Calidad Ambiental para la protección de las de Aguas Continentales Superficiales del Río Biobío.

HIPOTESIS

Dado el creciente desarrollo industrial a lo largo de la cuenca del río Biobío, y los diversos focos de contaminación puntual y difusa, es necesario entender Cómo el desarrollo urbano, agrícola e industrial en el río Biobío altera la calidad del agua e impacta el estado fisiológico de la ictiofauna nativa expuesta a distintas condiciones ambientales y estresores de diversa naturaleza.

Los efectos producidos por los estresores de diversa naturaleza en especies nativas presentes en los ríos de Chile, no son del todo claros. Las nuevas tecnologías implementadas para tratamiento de efluentes como para el tratamiento de aguas residuales, han resultado en la eliminación de compuestos altamente tóxicos y carcinogénicos (e.g. eliminación de dioxinas, ácidos resínicos). No obstante muchos de los compuestos vertidos en los efluentes actúan como disruptores hormonales y/o neuroendocrinos y alteran así funciones metabólicas, fisiológicas, e incluso el sistema reproductivo, generando procesos de androgenización en hembras y/o feminización en machos, así como también eventos dishomeostáticos con posible alteración del comportamiento.

Es por esta razón que, continuando con trabajos anteriores sobre los efectos en peces presentes en el río Biobío, donde se ha detectado una inducción en la maduración sexual de hembras juveniles, aumento de actividad enzimática antioxidante, y de enzimas de biotransformación hepática, como también aumento de la inhibición de Acetilcolinesterasa con consecuencias en la modulación neuromuscular, efectos atribuibles a descargas industriales, urbanas (contaminación puntual) y también a actividad agrícola y forestal (contaminación difusa), se propone la siguiente hipótesis en un marco de investigación:

La mezcla compleja de estresores químicos, por convergencia de diversos focos de contaminación puntual y difusa en la longitudinal del río Biobío, genera efectos expresados como respuestas biológicas adversas de mayor intensidad en peces nativos en el tercio inferior del cauce principal.

Predicciones:

1. La exposición crónica de la ictiofauna nativa a la mezcla de compuestos químicos de origen industrial y agrícola, en el tercio inferior de la cuenca, generan alteraciones fisiológicas medibles mediante medición de índices Fisiológicos y Reproductivos como inducción de actividad enzimática EROD en tejido hepático y alteración del índice Gonadosomático.

2. La exposición crónica de la ictiofauna nativa a compuestos de origen urbano, agrícola e industrial, en el tercio inferior de la cuenca, producen diferentes alteraciones histológicas medibles en gónadas, branquia, riñón e hígado, alterando el estado fisiológico de los ejemplares analizados.

3. La intensidad de las respuestas obtenidas por biomarcadores, depende de la intensidad de los estresores presentes en áreas de contaminación química puntual y difusa.

OBJETIVOS

Objetivo General:

Establecer e Identificar las respuestas subletales, evaluadas mediante estrategia de biomarcadores en peces nativos, asociadas a mezcla compleja de contaminantes químicos de origen urbano, agrícola e industrial en la cuenca del río Biobio

Objetivos Específicos:

1. Caracterizar efectos biológicos por estresores presentes en zonas de contaminación puntual y difusa en la longitudinal del río Biobio.
2. Evaluar y comparar batería de biomarcadores a nivel bioquímico (EROD), Fisiológicos (Factor de Condición, Índice Hepatosomático, Índice Gonadosomático), Reproductivos (Madurez Gonadal en relación a talla de peces) e Histología (alteraciones histopatológicas en Branquia e Hígado), en peces nativos
3. Diferenciar y asociar las respuestas obtenidas mediante biomarcadores a los distintos tipos de estresores químicos de origen puntual y difuso
4. Establecer un Índice Fisiológico Integrado de Biomarcadores (IPBR) aplicable a Normas Secundarias de Calidad Ambiental.

Estructura Organizativa de la Tesis

Esta Tesis está estructurada en Introducción, Desarrollo (sobre la base de cuatro capítulos), Discusión General y Conclusiones Finales.

El primer capítulo corresponde a una introducción que incluye conceptos generales sobre el estado de arte del río Biobio. El primer capítulo incluye también la hipótesis y los objetivos general y específicos y un apartado explicativo sobre la organización del trabajo.

El segundo capítulo incluye los resultados obtenidos de los análisis de los efectos reproductivos en *Percilia irwini* a lo largo de un gradiente de impacto antropogénico en el río Biobío. Este capítulo fue enviado a la revista Frontiers in Physiology (Mauricio Quiroz-Jara, Silvia Casini, Maria Cristina Fossi, Ricardo Barra & Juan F. Gavilán. 2021. Reproductive Status of the Wild Fish *Percilia irwini* in an anthropogenic impact gradient in the Biobio River, South- Central Chile).

El tercer capítulo corresponde a los resultados del análisis de la batería de biomarcadores utilizada y la generación de un Índice Fisiológico Integrado de Biomarcadores (IPBR), para determinar perfil de estado fisiológico por impacto antropogénico a lo largo del río Biobío. Este capítulo ha sido publicado en la revista Environmental Management. (Mauricio Quiroz-Jara, Silvia Casini, Maria Cristina Fossi, Rodrigo Orrego, Juan F. Gavilán & Ricardo Barra. 2021. Integrated Physiological Biomarkers Responses in Wild Fish Exposed to the Anthropogenic Gradient in the Biobío River, South-Central Chile. Environmental Management 67, 1145–1157. https://doi.org/10.1007/s00267-021-01465-y).

El cuarto capítulo corresponde a los resultados de las diferentes campañas de monitoreo (Otoño – Primavera, 2017) para las especies *Percilia irwini* y *Trichomycterus areolatus*, y la integración del índice Fisiológico Integrado de Biomarcadores (IPBR), para comparación de las diferentes respuestas con una variante temporo-espacial: *"Integrated Physiological Biomarkers Response (IPBR) as a proposal for the improvement of Chilean Secondary Environmental Quality Standards."* Mauricio Quiroz-Jara, Rodrigo Orrego, Verónica Delgado, Juan F. Gavilán & Ricardo Barra.

Esta Tesis concluye con la Discusión General y las Conclusiones finales extraídas de los resultados obtenidos.

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CAPÍTULO II

REPRODUCTIVE STATUS OF THE NATIVE FISH *PERCILIA IRWINI* ALONG THE ANTHROPOGENIC IMPACT GRADIENT OF THE BIOBIO RIVER, SOUTH - CENTRAL CHILE

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ABSTRACT

The Biobío river Central Chile is the third most crucial basin in Chile for human use and its ecosystem services, and a hot spot for freshwater biodiversity. It is possible to detect diffuse contamination areas by urban, agricultural, and forestry land and several environmental conditions, including complex chemical stressors that impact the physiological/reproductive state of native ichthyofauna in the Biobio River. The present study describes the histological features of the oocitary development of *Percilia irwini*, endemic species of Chilean continental waters, along the Biobio river in two seasons, autumn and spring. We describe four oocitary growth stages development: Primary, Previtellogenic, Vitellogenic, and Mature stage (n=4587 cells). At the lower third of the Biobío River, *P. irwini* exhibits the significant lowest body weight and body length and an increase in the Gonad-somatic Index (GSI) in autumn and spring and an increase in the number of oocytes in the first maturity stages evidence an active reproductive process in both seasons. These work outcomes agree with previous studies regarding the occurrence of hormonally active compounds that converge in the lower third of the Biobio river and physiological effects in native fish in Chile. These findings reinforce the need to understand the current effects of anthropogenic stressors in the Biobío River native fish fauna.

Keywords: Histology; Wild fish; Gonad maturity; GSI; Reproductive effects.

1. INTRODUCTION

Anthropogenic activities can impact aquatic ecosystems becoming a mayor treat to water-dwelling organism such as fish populations. Contaminants present in aquatic environments are of mayor concern as they can compromise fish reproductive success by severely affecting general condition, growth, behavior, gonadal and gamete structures, production and hatchability of eggs and embryo-larval survival (Munkittrick et al, 1994; McMaster et al, 1996; Kovacs et al, 1996, 1997; Hamilton et al., 2016; Orrego et al., 2021).

For years, many of these reproductive alterations reported in freshwater fish in Chilean aquatic environments have been mainly associated with urban and industrial effluents that have deteriorated the quality of rivers (Barra et al., 2021). A series of natural compounds (e.g., steroids, phytoestrogens, and mycotoxins) or synthetic (e.g., industrial chemicals, pesticides, and their metabolites) detected in Chilean rivers (Alonso et al, 2017) are widely described as endocrine disruptors chemicals (EDCs), because their ability to modulate the endocrine system (León-Olea et al, 2014), interfering the synthesis, secretion, transport, binding or excretion of hormones affecting fish reproduction (Gimeno et al, 1996; Crips et al, 1997; Milestone et al., 2012; Orrego et al, 2005; 2017; 2019).

The first evidence of reproductive effect related to EDCs in chilean wild fish reported by Chiang et al. (2011) showed evidence estrogenic effects at different of biological organization levels from molecular to population in *Percilia gillissi* and *Trichomycterus areolatus* downstream of the pulp mill effluent discharge in the Itata River (Central Chile), with an induction of gonadal 17b- estradiol production, higher frequency of oocytes in advanced stages of maturation and subsequent increase in female gonads size. Even though there has been an increased interest for assessing the reproductive effect in Chilean native fish species (Barra et al., 2021), including genetic biomarkers evaluation (Ali et al., 2020), the existing knowledge about their basic reproductive biology is still limited (Aedo et al, 2009, Habit et al, 2006a,b,c; Chiang et al, 2010; 2014), which has prevented further progress in understanding the real reproductive status of native fish populations. Knowing the series of events involve in the normal development of native fish gonads over a reproductive period, is essential in order to evaluate the severity of effects related to environmental contamination (Parker et al, 1985) especially in small fish species models Gibbons et al.(1998a,b).

In particular, the Biobío river (central Chile, Figure 1) in the last 30 years has presented a series of growing uses that have led to a detriment in water quality (Habit et al., 2006a,b; 2007; Figueroa et al., 2013, Parra et al, 2013), especially in the middle-lower stretch where urban and industrial discharges have caused the incorporation of a variety of organic micropollutants OMPs (Rozas et al, 2016), including persistent organic pollutants like pesticides and emerging contaminants (personal care products) via domestic and municipal wastewater discharge, irrigation and runoff in agriculture and forestry activities, and leaching into groundwater by authorized and unauthorized landfills (Rozas et al, 2016). Although, new effluent treatment technologies have been implemented in both domestic and industrial wastewaters, reproductive effects, continue to be detected along the river (Orrego et al, 2019; 2021; Martyniuk et al, 2020; Barra et al., 2021).

Around 15 years of research have shown reproductive effects in non-native fish model (hatchery reared rainbow trout) evidencing the degree of contamination of Biobío river. From induction of gonad maturation, increased level of plasma vitellogenin (VTG) and gonadosomatic index, to increase the level of endogenous estrogens (estradiol) by induction of cyp19 aromatase gene expression, among others (Orrego et al., 2005, 2007, 2009, 2012, 2019), mostly related to pulp mill effluent discharge areas. However, for native species such as *Percilia irwini*, only laboratory exposure assays to industrial and domestic wastewater have evidenced the relative induction of phosphoproteins-like VTG as reproductive effect (Bahamonde et al., 2019). Due to the convergence of several sources of pollution along the River's length, the complex mixture of chemical stressors that may be responsible for reproductive effects in native fish populations, this study aimed to understand how the high degree of anthropogenic intervention present in Biobio River that change water and ecosystem’s quality, influence the reproductive status of a native fish species *Percilia irwini* under natural condition. To achieve this goal, the normal oocitary development of *Percilia irwini* will be described using histology and autofluorescence analysis, in order to subsequent evaluate the reproductive performance of this specie along the space-temporal gradient of anthropogenic impacts in the river.

2. METHODS

2.1. Study Area

The Biobio river belongs to a hydrographic basin of Andean origin with an area of 24.369km^2, and a length of 380km (Figure 1). Rivers characteristics presents a shape defining two sectors: 1) Initial up-river rhithron zone with a high slope, volcanic soils, mainly native forest cover vegetation, high rainfall and snowfall, high solar radiation and a river network with more than 5000 tributaries first-order rivers, and 2) down-river low-slope Potamon zone without snowfall and predominantly a vegetation cover of introduced species such as pine and eucalyptus.

Five sampling sites were select in the Biobio river from its source to the mouth (36º42´ - 38º49´) to determine if there are impacts in the reproductive physiology of *Percilia irwini* in two seasons (Figure 1). Reference Area**:** Lonquimay (LQ) and Balsa Caracoles (BC), upstream of the Biobio River. These sites present low anthropic impacts, surrounded by native forest vegetation. Middle Area**:** Rucalhue (RC) downstream of the Angostura hydroelectric plant, the third hydroelectric power station present in the Biobío river. This site has relicts of native forest and forest plantations. High Impact Area**:** Puente Coihue (PC) downstream of urban effluents and pulp and paper mill industries. PC has a high predominance of forest plantations associated with the river sector associated with some agricultural activities. Santa Juana sampling site (SJ) presents high anthropic impacts as the convergence area of the several industrial and urban activities present upstream of the Bíobio River.

2.2. Fish Collection

Ethical Statement.

All protocols used in this study were reviewed and approved by the Ethics Committee of the Universidad de Concepción, Chile (Aut. No. 01-2016).

Wild fish's reproductive status was assessed during autumn low flow (March 2017) and spring high flow (September 2017) seasons, in order to evaluate the reproductive responses of fish in these two different scenarios. Fish were captured following the longitudinal gradient of the Biobio River, from the rhithron zone to the potamon zone, selecting sites with similar microhabitat characteristics using a backpack electric fishing equipment (Halltech Electrofisher, Canada) and a blocking net (6 mm mesh opening) were used in riffles (0.3-0.1 m/s, 0.4-0.1m depth) with a bed of boulders and gravel (~15cm diameter) and rocks. Fish were collected, focusing on adult *Percilia irwini* and due to the lack of secondary sexual characteristics, a maximum of 10 adult fish were selected at each site, with sizes greater than 35 mm following previous studies (Chiang et al, 2011). The gonads of female fish were weighted and fixed instantly in the Buoin solution for further analysis. For descriptive purposes, physiological index such as Condition Factor (K=[Total Weight(g)/Total Lenght^3]*100) and Gonadosomatic Index (GSI= [gonad weight(g)/ Total Weight(g)]*100) were calculated.

2.3. Preparation and Analysis of Histological Cuts

The histological analyses were carried out in the Department of Cell Biology of the Faculty of Biological Sciences at the Universidad de Concepción. After the fixation time (48 hours), gonads samples were washed with 70% ethanol to eliminate the Buoin solution (three times for 15 minutes). The samples passed through a dehydration battery with different solutions of ethanol (70% - 99%) and chloroform, to finally infiltrate the samples in liquid paraffin at 58 °C, for two hours, repeating this step twice. After infiltrating the samples, they included in paraffin (Histosec®, MerckMillipore) for a minimum time of 24 hours. The embedded tissue was sectioned (thickness, 7mm) in a manual rotation microtome (Jung). Due to the small size of the gonads of *Percilia irwini*, the entire gonad was embedded in paraffin and cut by a manually rotating microtome. The paraffin was removed by washing with Xylol and stained with Hematoxylin and Chromotrope (0.5%).

A total of 4587 gonad cells were counted (~110 cells per fish) and cell and nuclear oocytes diameter were measured with an optical microscope with a previously calibrated micrometric eyepiece, to take a photograph in an OLYMPUS BX41 microscope with OLYMPUS U-YVO.5XC-2 digital camera attached, at the Microscopy Laboratory of the Department of Applied Ecotoxicology of the Università di Siena, Italy. The proportion of cells in the distinct maturation stages for the different sites along the Biobio River were assigned according to a defined scale for *Percilia irwini* (M. Quiroz, Department of Cell Biology, Faculty of Biologic and Molecular Sciences, University of Concepción, *unpublished data*) and following published studies of other teleost fish (Huaquin et al, 2002) (Table 1).

2.4. Confocal Microscopy

Autofluorescence analysis by confocal microscopy was performed at the Advanced Microscopy Center (CMA) of the Universidad de Concepción, Chile.

Random samples of *Percilia irwini* female gonads previously fixed in Buoin solution were selected and subjected to agarose wash to perform 30μm thick cryostat sections subsequently. The samples were analyzed in a confocal microscope (LSM 780), and excited by the laser to obtain images by autofluorescence. The images of the obtained female gonad samples were 3D reconstructed, in order to observe in detail, the oocytes structures maintaining its physical characteristics (dimension, volume, and shape).

2.5. Statistical Analysis

Data analysis was performed by RStudio, R Software Package (Core Team 2017), and presented as means with a standard error of the mean (± SE). Biological responses between sites were examined for normal distribution (Shapiro–Wilks test) and, if necessary, log-transformed to avoid non-normality. One-way analysis of variance - ANOVA was used to evaluate statistically significant differences for oocytes diameters, and confirmed by a multiple comparisons Tukey *posthoc* test ($p < 0.05$). Each season was analyzed separately.

Pearson Correlations were carried out to determine the relationship between total body length and body weight with the library "car" available on R software (RStudio Team 2020). Multiple linear regression was carried out to determine the variables that directly influence the GSI value to explain reproductive alteration processes. These data were analyzed by season (Autumn and Spring).

All the statistical assumptions were previously verified for Multivariate Analysis of Variance (MANOVA), using the libraries "car," "akima,", “carData”, “car”, “tidyverse”, “ggplot2”, “MASS”, “dplyr”, “mvoutlier”, “mvnormtest”, “reshape”, “WRS”, “stats” available on R software (RStudio Team 2020). MANOVA was employed to determine differences in physiological variables (GSI, total body length, total body weight) with the Reference sample site (LQ) for both sample periods (autumn and spring, respectively). Due to the unbalanced gender proportion observed and the non-significant influence of gender observed with GSI, their statistical analysis was carried out, pooling females and males together. Heatmap was performed by package “stats” version 3.5.0 in RStudio.

3. RESULTS

3.1. Stages of Gonadal Maturity

There are no significant morphological differences in the cytoplasm among oogonia and oocytes in primary stages. The principal difference is given mainly by the cellular and nuclear diameter in the development of these oocytes stages (Figure 2). From previtellogenic oocytes to oocytes in a mature stage, this growth process is accompanied by active incorporation of yolk, in addition to the development of microstructures in the oocyte envelope (Figure 2), which allow distinguishing between the different stages of gonadal maturity in *Percilia irwini* (Table 1).

3.1.1. Primary Stage

A simple layer of follicular cells delimits the oocytes corresponding to the primary state. From a general point of view, these oocytes have a spherical shape and present a highly homogenous and basophilic ooplasm. The core has a large diameter and is central. Besides, nucleoli begin to appear at the periphery of the nucleus and are associated with the inner side of the nuclear envelope (Figure 2).

During this stage of growth, the oocytes of *P. irwini*, like that of other teleosts, increase their cell diameter. This growth from approximately 0.052 mm to 0.096 mm, is accompanied by a decrease in the nucleus/cytoplasm ratio (Selman et al, 1989) (Figure 3). Primary oocytes present a highly basophilic and homogenous ooplasm (Figure 2).

As these oocytes grow, they present a simple layer of follicular cells externally (Figure 2). Distinguishing a nucleus of more significant size in a central position and surrounded by nucleoli, whose envelope presents invaginations and forms the germinal vesicle (McMillan, 2007). The high frequency of nucleoli involved in the synthesis of rRNA for the production of proteins necessary for the development of oocyte (Begovac & Wallace, 1998). Primary stage presents a higher frequency of oocytes (protoplasmic growth) compared to all others stages (Figure 3C).

3.1.2. Previtellogenic Stage

In this stage, the oocytes are delimited by a single layer of follicular cells (Figure 2). As in the previous state, nucleoli are found in the periphery of the nucleus and associated in the internal face of the nuclear envelope; however, comparatively smaller in proportion than in the primary stage (Figure 3C). The nucleus is still prominent and centrally positioned. These oocytes have an ovoid shape, and larger diameter (0.136 – 0.196 mm) than the oocytes described in the primary stage (Table 1).

On the other hand, the ooplasm presents irregularities due to the beginning of the incorporation of yolk (Figure 2). Oocytes in previtellogenic stage have got a defined layer of follicular cells, the onset of yolk micropinocytosis that gives an irregular shape to the ooplasm and an increase in the number of nucleoli associated with the inner side of the nuclear envelope (Selman et al, 1989; McMillan, 2007).

The core remains in a central position, and its envelope is highly irregular (Figure 2). The presence of these components corresponds to a cortical alveolus stage (Selman et al, 1982; 1986; 1989; 1991; Begovac et al, 1989). These would be attached to the membrane and could be formed by endoplasmic reticulum and the Golgi complex, containing glycoproteins synthesized within the oocyte (Selman et al, 1982; 1986; 1989; Ulrich, 1969). As the incorporation of yolk increases, the cortical alveoli are segregated towards the periphery, the follicular cells increase, and it is at this stage where one develops more, and corresponds to the micropillar cell, which will later form the micropile (Selman et al, 1982).

3.1.3. Vitellogenic stage

During vitellogenesis, the radiated area appears bi-laminate (10 - 38 μm thick) and nucleus is progressively displaced towards the animal pole (Thorsen & Fhyn, 1996) (Figure 2). The active incorporation of exogenous vitellogenin into the oocyte is responsible for the increase in the oocyte diameter (Figure 3A), giving a highly irregular shape to the ooplasm (Figure 2). This active micropinocytosis by the oocyte and sequestration to the ooplasm in the form of granules is the most particular cellular event of vitellogenesis (Lange et al, 1982, 1983; Thorsen & Fhyn, 1996) especially in freshwater fishes (Lange et al, 1983). In teleost fishes, the yolk granules ,or platelets, may contain subunits of crystals formed by lipoviteline-phosvitin complexes, suggesting a reserve of essential nutrients not available in fresh water (Thorsen & Fhyn, 1996).

3.1.4. Mature Stage

Mature oocytes, are characterized by their highest development, with diameters reaching 0.664 mm (one-way analysis of variance – ANOVA, p-value < 0.01), an oocyte cover of up to 60 μm (Table 1), composed of the zona radiata (internal) and cells of the theca (thin outer cellular layer) (Figure 2).

The ooplasm is observed fragmented due to the coalescence of the yolk granules and platelets, although without forming a homogeneous structure (Figure 2). Also, the cortical alveoli move towards the periphery.

3.2. Oocitary Cover

Primary oocytes in the early stages of development, are surrounded by a cell border, consisting of a single layer of follicular cells (Figure 2). In this structure it is not possible to observe channels commonly present in other teleost fishes, formed to trap microvilli that extend perpendicularly to the envelope, giving a striated pattern characteristic of the radiated zone (McMillan, 2007).

The development of oocytes implies a greater complexity in the morphology of the cover. The previtellogenic oocytes present a thin outer layer of follicular cells (Figure 2), and a homogeneous and translucent layer named "Z1". In stages of more complex development (vitellogenic stage), the radiated zone is divided into a thick inner layer and an outer layer formed by a single layer of teak cells. This outer layer has a thickness between approximately 10 - 38 µm (Table 1).

The thickness of the radiated zone, such as the degree of coalescence of yolk in mature oocytes, suggests a higher density of mature oocytes. Also, a lower dispersal capacity of the eggs and a possible lower fecundity of this species in comparison with other species with pelagic eggs (Craik & Harvey, 1986; Thorsen & Fhyn, 1996; McMillan, 2007;).

3.3 Individual fish reproductive performance in the Biobio River

Total body length and total body weight were significantly correlated (Adjusted R-squared = 0.81, $p < 0.01$). Mean lengths and weights in females and males were generally constant (Table 2). Individual Condition Factor (K) values in females were 0.73 to 1.76. Individual K values in males were 0.82 to 1.76. Statistical differences were in condition factor in females of *P. irwini* to reference site LQ in autumn and spring (MANOVA $p < 0.05$). Mean Condition Factor values for all fish males and females were 1.17 ± 0.17 (Table 2) and the value for this index not differed among sites.

Total body length and total body weight have a significant decrease in the lower third of the Biobío river at Puente Coihue (PC) and Santa Juana (SJ) sites during autumn (March) and spring (September) (Figure 4). SJ have the smallest specimens compared to the reference sites LQ and BC (ANOVA, Tukey $p <0.01$).

As stated above, Condition Factor values (K) not differed among sites for female and male, where mean K values were 1.17±0.17 (Table 2). Conversely, significant difference were observed in the females *Percilia irwini* GSI (One-way analysis of variance - ANOVA, Tukey *post-hoc* test, $p<0.05$). The values of the GSI increase in the lower third of the Biobío river (Table 2) specially during spring season. The increase of this index is related to the body weight, the gonad weight and a decrease of body length,, which are constantly smaller at the lower third of the river (Table 2, Figure 4). The high presence of oocytes in primary, previtellogenic and mature stages, are evidence of an induction process in the gonadal maturation of these specimens, towards the lower third of the Biobío river (Figure 5A, 5B). According with previous research, the spawning period of this species corresponds to the summer months (November - January), with maximum ovarian development in November (Habit et al, 2006b; 2006c; 2007; Ruiz, 1996; Ruiz and Marchant, 2004).

3.4. Female gonad histology

When determining the physiological variables that could best explain the variation of the GSI in females of *Percilia irwini*, between the different sampling sites, the multiple linear model indicates that 86% of the variability observed in GSI is given by the variables length, weight, gonad weight, and K (Adjusted R-squared = 0.81, $p < 0.01$). The analysis of the female gonadal development of *Percilia irwini* in autumn (March) and Spring (September) presents a statistically significant difference among the sites in the lower third of the Biobío river respect to the reference sites LQ and BC (Figure 6A, 6B).

Diameter of Primary stage oocytes, do not present statistical difference along the sample sites, however the previtellogenic and mature oocytes have a smaller diameter in Puente Coihue (PC) and Santa Juana (SJ), compared to Balsa Caracoles (BC) and Lonquimay (LQ), both reference sites (One-way analysis of variance - ANOVA, Tukey *post-hoc* test $p < 0.01$) (Figure 5 A, B).

During the autumn season (March, figure 5A), the oocyte in the primary stage does not show differences in diameter between the sites. However, the oocyte in the previtellogenic stage shows a difference in diameter concerning the LQ and BC reference sites (Figure 5A). These oocytes have a larger diameter, a situation that coincides with the diameters of the oocytes in the Vitellogenic and Mature stage (One-way analysis of variance - ANOVA, $p < 0.01$). On the other hand, it is possible to observe that in the SJ site, the diameter of the oocytes in the Vitellogenic stage is smaller compared to the other stations (One-way analysis of variance - ANOVA, $p < 0.01$), and the analyzed specimens do not present oocytes in the mature stage (Figure 5A, B).

There is a synchronous proportion in oocytes in different stages in Lonquimay zone (LQ) (Figure 6 A, B). However, is observed that the presence of oocytes in the lower third of the river correspond to protoplasmic growth of oocytes in primary stages, and a decrease in the proportion of oocytes in the mature stage. This change in the proportion of oocytes is associated with a reduction of cell diameter in all oocytes development growth stages describes for *Percilia irwini* (Figure 5 A, B).

In both sampling periods (autumn and spring) at the different study sites, immature oocytes presented a high proportion (Figure 6 A, B). During autumn, a high oocyte protoplasmic growth occurred in the RC, PC, and SJ stations (Figure 6A). Previtellogenic oocytes increase in number downstream Biobio River. Mature oocytes are in a high proportion in the RC and PC sites (Figure 6A). In the spring season, a high proportion of oocytes in the primary stage is observed in all the monitoring sites; however, there is an increase at the RC and SJ sites (Figure 6B). The mature stage oocytes show an increase in proportion towards the PC and SJ sites, while the specimens analyzed in the reference site do not present oocytes in this stage of growth (Figure 6B).

Regarding the Spring season (September), differences are observed in the diameter of oocytes in the primary stage (One-way analysis of variance - ANOVA, $p < 0.01$) (Figure 5B). The analyzed specimens show a greater body length and total body weight (Figure 4); however, there is evidence of a significant increase in the diameter of oocytes in the advanced vitellogenic and Mature stage (Figure 6B,). However, there are no mature oocytes at the LQ reference site, where fish do not present an advanced level of maturity despite finding larger specimens.

Carrying out a comparative and integrated analysis of the distribution of all the oocyte's diameters measured in both seasons (autumn and winter), the association between the diameter of oocytes measured in both seasons is observed. Cluster associates oocytes of autumn, spring and another cluster associates previtellogenic oocytes during autumn, related with mature oocytes from the spring season. This result accounts for the diameter of mature oocytes at the time when Percilia begins its reproductive process. In addition, results confirm the relationship that exists in the increase in oocyte diameters at the PC and SJ sampling sites (Figure 7).

4. DISCUSSION

This study shows evidence of how the high degree of anthropogenic intervention present in Biobio River, previously reported to change its water and ecosystem's quality, influence the reproductive status of adult females of *Percilia irwini* under natural condition.

The data obtained indicate that the high anthropogenic impacted areas in the river (RC, PC, SJ) have the highest number of oocytes in the primary stage (autumn season), with more significant protoplasmic growth of primary oocytes than the reference sites. This pattern is also observed in vitellogenic oocytes present in reference zone LQ, compared to the PC and SJ sites, where the presence of mature oocytes is reduced. The lowest number of vitellogenic oocytes would demonstrate healthy harmonic gonadal growth in the areas with less anthropic intervention. Furthermore, during the autumn period (March), the vitellogenic oocytes belonging to the PC and SJ sites show a decrease in growth (diameter) and frequency (number of oocytes by stages).

This species shows a maximum gonadal development at the reference sites during October and November (Habit et al, 2006a,b; 2007; Ruiz, 1996; Ruiz and Marchant, 2004; Chiang et al, 2011), so the pre-spawning period should occur during early spring (September). Our results conform to what was indicated by Habit et al. (2006) who suggested a partial type spawning related to an asynchronous oocyte development, and also consistent with those determined in the *P. gillissi* congener species (Chiang et al, 2011), which suggests maximum gonadal development in the spring-summer period.

The alterations observed at reproductive level in individuals downstream the LQ and BC reference sites, during both seasons, could be responsible for the differences in the size structure of the male and female of the *P. irwini* population, in which there is a temporal and spatial trend towards smaller sizes. Previous research indicates a loss in the growth rate of several fish species in areas of low environmental quality and sectors directly affected by industrial and urban wastewaters, and agricultural activities (Gavilán et al, 2001; Habit et al, 2006a,b,c; 2007). Along with these reproductive impacts, a number of metabolic and toxicological effects have been extensively described in the Biobio basin (Barra et al., 2021) to show a pollution gradient that has ended up decreasing the quality of the water and associated environments. Most of those laboratory and semi controlled field experiments were developed using introduced fish model such as juvenile *Oncorhynchus mykiss* (Orrego et al., 2005, 2007, 2009, 2011), or embryo and larvae of *Oryzias latipes* (Orrego et al., 2011, 2021), that allowed to demonstrate mainly estrogenic effect related with early maturation of juvenile females, male feminization, and high embryotoxicity especially in males.

Conversely, as previously indicated, the knowledge about the reproductive effect on Chilean wild fish species, at the scale of basins such as the Biobio river, is scarce or incomplete, In Chilean wild fish spawning usually occurs in late spring and early summer season (Chiang et al., 2010, 2011). The observed effect in *P. irwini* could be related to early spawning and maybe forced by the presence of hormonally active substances in the middle-lower area of the Biobio river (Alonso et al, 2017; Orrego et al, 2019; Rozas et al, 2016). The induction of gonadal maturation in females was detected in our study through the morphological and morphometric characterization of the oocytes of *Percilia irwini*. This morphological change in oocytes maturation is evidence of changes in normal female reproductive development, which could be due to exposure to a complex chemical mixture (including EDCs). Evidence of estrogenic effects has been determined by non-lethal EDC assessment tools in native fish. Thus, Bahamonde *et al*. (2019) demonstrated an increase in VTG-type phospholipoproteins (Vtg-like-phosphoproteins) in mucus on *Percilia irwini* exposed to wastewater from the Biobio River basin under laboratory conditions. Fish exposed to cellulose effluents as well as treated wastewater also showed an increase in these Vtg-like-phosphoproteins, similar to fish exposed to EE2 (Barra *et al*, 2021).

A recently published study (Orrego et al, 2019), in which semi permeable membrane devices (SPMDs) were placed in the Biobio river, downstream of the PC site, indicates the presence of steroids hormones like compounds coming mainly from pulp mill industry discharges, were able to bind with equal affinity to natural sex steroid binding protein (SSBP) and androgen Receptor (AR) (goldfish plasma sex steroid binding protein and testicular goldfish androgen receptors, respectively) demonstrating the presence of EDCs. In this way, it is possible to conclude that the estrogenic compounds in this area of the Biobio river are responsible for the increase in estrogenic activity evidenced as an increase in the Gonadosomatic index and an increase in ovarian follicles in females from *Percilia irwini*. These biological responses are consistent with all previous studies carried out at the Biobio River basin during the las decades (Barra et al., 2021).

Although, these observed changes in gonad maturation evidenced by the increase of vitellogenesis, is related to the hypothalamic-pituitary-gonad axis (Arcand-Hoy & Benson, 1998, Arukwe, 2001) and has been shown in introduced fish species (Orrego et al, 2009; 2019; Chiang et al, 2011; 2012), we do not have sufficient evidence to determine this HPG specific effect, or another toxic-mechanism of action excert over *Percilia irwini* could be responsible. Thus, it is critical to know the normal reproductive and physiological processes and thresholds of native fish in order to better understand short and long-term population effects. Our previous work (Quiroz-Jara *et al.*, 2021) has shown alteration of the general physiological state in in the lower third of the Biobio river, through the use of an integrated physiological biomarkers index. These results show a decrease in total length and total weight and a decrease in the size of the gonad in females. Nevertheless, the results obtained in this study indicate an increased gonad maturation, and a high reproductive activity in both seasons, not necessarily related with significant gonad size (Figures 5, 6 and 7).

The knowledge of the reproductive state of *Percilia irwini* allows us not only to increase its conservation strategies, but also to assess a potential biomarker to determine reproductive physiology alterations in of wild fishes, in order to distinguish cause-effect relationships such as those implemented in developed countries monitoring programs.

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Additional Information

Competing interests

The authors declare no competing financial interests.

The authors declare no competing interests.

Author Contributions

M.Q wrote the main manuscript text, analysis of the data and, prepared figures. J.G and R.B. contributed to the conception and design of the study. R.O, S.C and M.C.F. contributed to the assistance of data analysis and writing the manuscript.

Table 1 - Principal characteristics of the oocyte development from *Percilia irwini*[a].

STAGE	Cytoplasmic Diameter (mm)	Nuclear Diameter (mm)	Oocitary Cover	Morphological characteristics	Description of vitellogenesis	Nucleoli
Primary	0.076 ± 0.02	0.03 ± 0.02	Externally delimited by a single layer of follicular cells	Spherical cell. Highly basophilic ooplasm. Cortical alveoli	No presence of yolk granules	Arranged at the periphery of the nucleus and associated with the internal face of the nuclear envelope
Pre-vitellogenic	0.166 ± 0.03	0.06 ± 0.03	Delimited by a thin layer of follicular cells	Ooplasma of irregular appearance due to the beginning of the incorporation of vitello	Beginning of pinocytosis of Vitello	Arranged at the periphery of the nucleus and associated with the internal face of the nuclear envelope. Comparatively in smaller proportion
Vitellogenic	0.332 ± 0.07	0.08 ± 0.02	Oocyte cover thicker. Formed by two layers (radiated area). 10 - 38 μm thick	Ovoid cell. Irregular ooplasm of spongy aspect due to the massive incorporation of vitello. A core of irregular shape and displaced towards the periphery	It activates pinocytosis of Vitello	At the periphery of the nucleus. Comparatively in smallest proportion
Mature	0.526 ± 0.138	0.10 ± 0.09	Zona Radiata of greater thickness. A Uniform layer of teak cells. 20 - 88 μm thick	An ovoid cell of maximum growth and development	Ooplasm is observed fragmented due to the coalescence of the yolk granules and platelets	Not observed by HC stains

[a] Values as mean ± standard deviations diameters of different oocytes stages from *Percilia irwini*

Table 2 - Summary statistics for *Percilia irwini* captured in the Biobio River (Chile), by sex and season

Date	**Sex**	**Site**	**Gonadosomatic Index (GSI)**	**Total body Length (mm)**	**Total body Weight (grams)**	**Gonad weight (grams)**	**Condition Factor (K)**
March 2017	Females	LQ	1.30 ± 0.48 (3)	61.67 ± 6.02 (6)	2.92 ± 0.96 (6)	0.05 ± 0.03 (3)	1.22 ± 0.22 (6)
		BC	0.88 ± 0.75 (6)	59.86 ± 10.61 (7)	2.97 ± 1.69 (7)	0.02 ± 0.01 (6)	1.27 ± 0.08 (7)
		RC	2.13 ± 1.06 (10) *	53.18 ± 9.16 (11) *	1.82 ± 1.13(11) *	0.04 ± 0.02 (10)	1.08 ± 0.14 (11) *
		PC	1.08 ± 0.66 (5) *	53.71 ± 6.97 (7) *	2.04 ± 0.76 (7) *	0.02 ± 0.01 (5)	1.27 ± 0.14 (7) *
		SJ	1.69 ± 1.10 (6) *	52.75 ± 2.82 (8) *	1.69 ± 1.33 (8) *	0.03 ± 0.01 (6)	1.14 ± 0.10 (8) *
March 2017	Males	LQ	0 ± 0 (4)	69.75 ± 4.57 (4)	3.53 ± 0.93 (4)	0.00 ± 0.00 (4)	1.02 ± 0.07(4)
		BC	0 ± 0 (3)	60.33 ± 17.90 (3)	3.13 ± 2.76 (3)	0.00 ± 0.00 (3)	1.21 ± 0.12(3)
		RC	0.92 ± 0.00 (1)	49.00 ± 11.92 (5)	1.68 ± 1.23 (5)	0.02 ± 0.00 (1)	1.21 ± 0.23(5)
		PC	0.31 ± 0.00 (1)	52.63 ± 9.02 (8)	1.75 ± 0.79 (8)	0.01 ± 0.00 (1)	1.17 ± 0.30(8)
		SJ	0.69 ± 0.22 (3)	56.86 ± 4.02 (7)	2.13 ± 0.45 (7)	0.02 ± 0.01 (3)	1.15 ± 0.08(7)
Sept 2017	Females	LQ	2.41 ± 0 (2)	48.00 ± 2.82 (2)	0.98 ± 0.27 (2)	0.03 ± 0.02 (2)	0.87 ± 0.08 (2)
		RC	3.36 ± 2.50 (6) *	52.50 ± 3.33 (6) *	1.55 ± 0.28 (6) *	0.06 ± 0.04 (6)	1.06 ± 0.08 (6) *
		PC	8.05 ± 6.92 (2) *	50.75 ± 10.31 (4) *	1.56 ± 0.83 (4) *	0.09 ± 0.13 (4)	1.12 ± 0.07 (4) *
		SJ	5.94 ± 4.68 (10) *	46.20 ± 4.52 (10) *	1.02 ± 0.43 (10) *	0.07 ± 0.08 (10)	0.99 ± 0.11 (10) *
Sept 2017	Males	LQ	0.71 ± 0.38 (4)	55.60 ± 5.59 (5)	1.70 ± 0.53 (5)	0.94 ± 0.00 (1)	0.95 ± 0.09 (5)
		RC	2.44 ± 0.00 (1)	50.75 ± 7.14 (4)	1.43 ± 0.73 (4)	1.06 ± 0.08 (6)	1.03 ± 0.08 (4)
		PC	3.49 ± 1.48 (5)	55.75 ± 6.63 (8)	1.89 ± 0.76 (8)	1.12 ± 0.07 (4)	1.05 ± 0.12 (8)
		SJ	3.46 ± 1.49 (4)	50.67 ± 3.08 (6)	1.36 ± 0.27 (6)	0.99 ± 0.11 (10)	1.04 ± 0.06 (6)

Values are mean ± standard deviations (*n*). Values marked with an asterisk (*) present a significant difference in the MANOVA (p<0.01) with reference LQ Site.

Figure Captions

Fig. 1. Biobio River Basin showing sites where *Percilia irwini* were collected, upstream areas (Reference sample sites: LQ-BC), middle Area (RC), downstream Area (PC-SJ). Red dots indicate the leading urban areas adjacent to the main course of the Biobío River. Icons (brown) indicate the primary industrial sources contiguous to the main course of the Biobio river.

Fig. 2. Photomicrographs HC Stains and Confocal Photomicrographs of transverse sections of ovaries of *P. irwini*; Primary, Previtellogenics, Vitellogenic and Mature stage oocytes; N: nucleus, NC: nucleoli, NL: Nuclear limit, YG: yolk granules, OE: oocyte envelope, TH: Theca; ZR: Zona Radiata.

Fig. 3. A) Total oocyte diameters, B) total nucleus oocyte diameters, and C) total oocyte number, for the gonadal maturity stages determined for *Percilia irwini* (Primary), Previtellogenic (Pvtg), Vitellogenic (Vtg) and Mature. Bars indicate standard error. Letters (a-d) indicate a statistically significant difference between the different stages (ANOVA p-value < 0.01, post-hoc Tukey's HSD).

Fig. 4. Distribution of total body length in millimeters, total body weight in grams of *Percilia irwini*. A-B) Autumn (March); C-D) Spring (September). Letters (a-c) indicate statistically different groups (One-way ANOVA, $p < 0.05$), confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$).

Fig. 5. A) Diameters of different stages of oocyte development at different sites at de Biobío River during autumn (March). B) Diameters of different stages of oocyte development at different sites at de Biobío River during Spring (September). Letters (a-c) indicate statistically different groups (One-way ANOVA, $p < 0.05$), confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$). Bars indicate standard error.

Fig. 6. A) Total number of oocytes in different development stages at different sites at de Biobío River during autumn (March). B) Total number of oocytes in different development stages at different sites at de Biobío River during Spring (September). Reference sample sites: LQ-BC; middle Area: RC; downstream Area: PC-SJ.

Fig. 7. Hierarchical clustering for the oocites diameters values in *Percilia irwini* (Pi) showing the relationship between seasons and among sites along the Biobío river LQ, BC, RC, PC, SJ.

Figure 1.

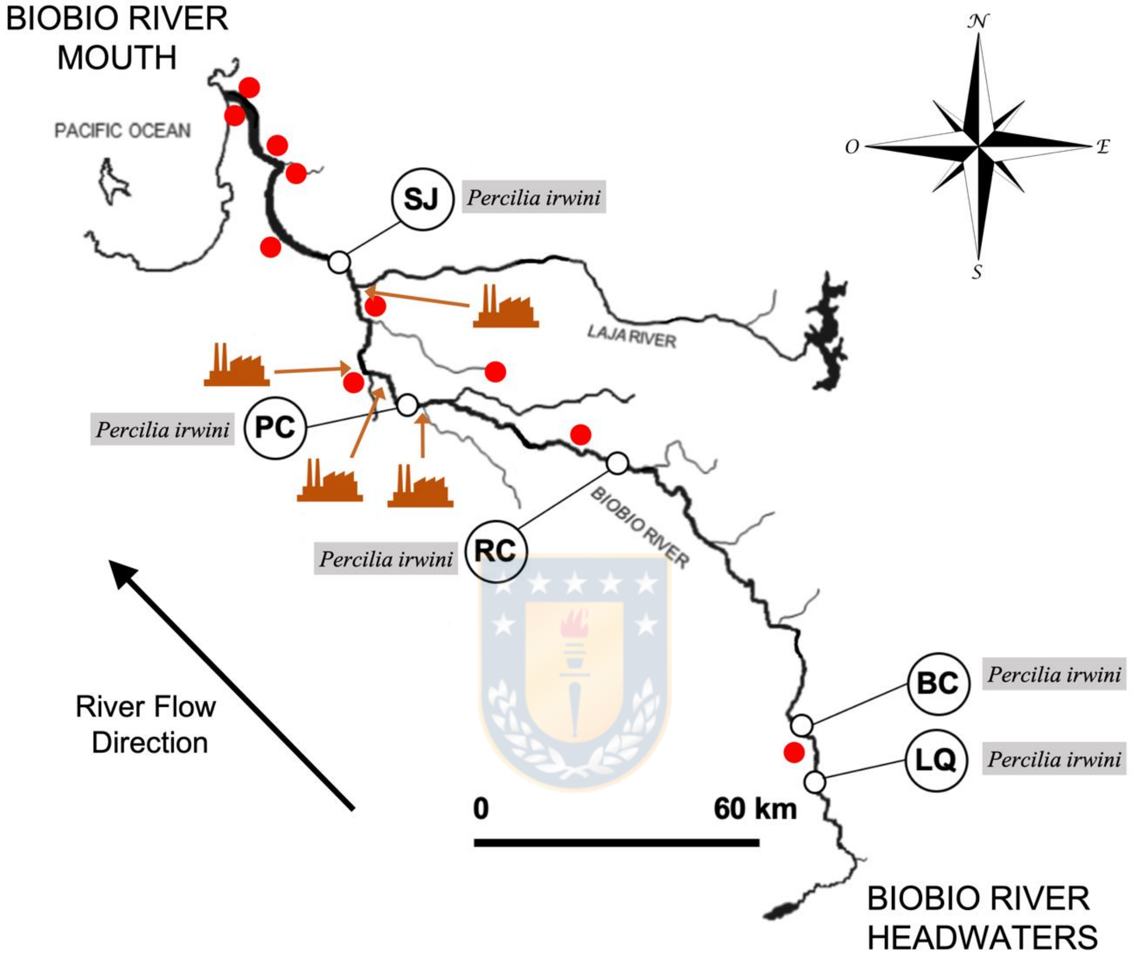

BIOBIO RIVER MOUTH
PACIFIC OCEAN
N
O
E
S
SJ
Percilia irwini
LAJA RIVER
Percilia irwini
PC
Percilia irwini
RC
BIOBIO RIVER
BC
Percilia irwini
LQ
Percilia irwini
River Flow Direction
0
60 km
BIOBIO RIVER HEADWATERS

Figure 2.

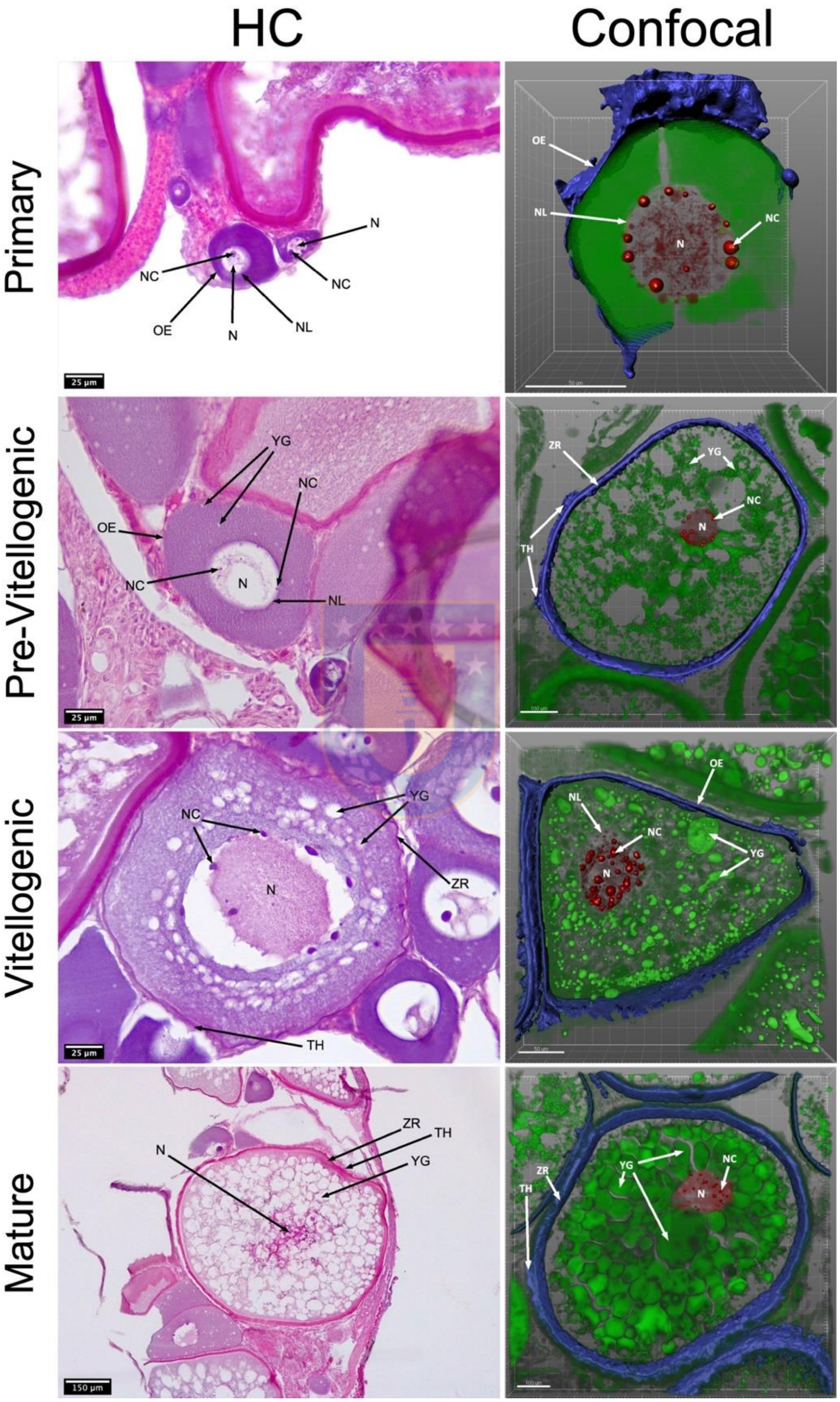

HC
Confocal
Primary
NC
OE
N
NL
N
NC
25 µm
OE
NL
NC
N
50 µm
Pre-Vitellogenic
YG
NC
OE
NC
N
NL
25 µm
ZR
YG
NC
N
TH
100 µm
Vitellogenic
NC
YG
ZR
N
TH
25 µm
OE
NL
NC
N
YG
50 µm
Mature
ZR
TH
N
YG
150 µm
ZR
TH
YG
NC
N
100 µm

Figure 3.

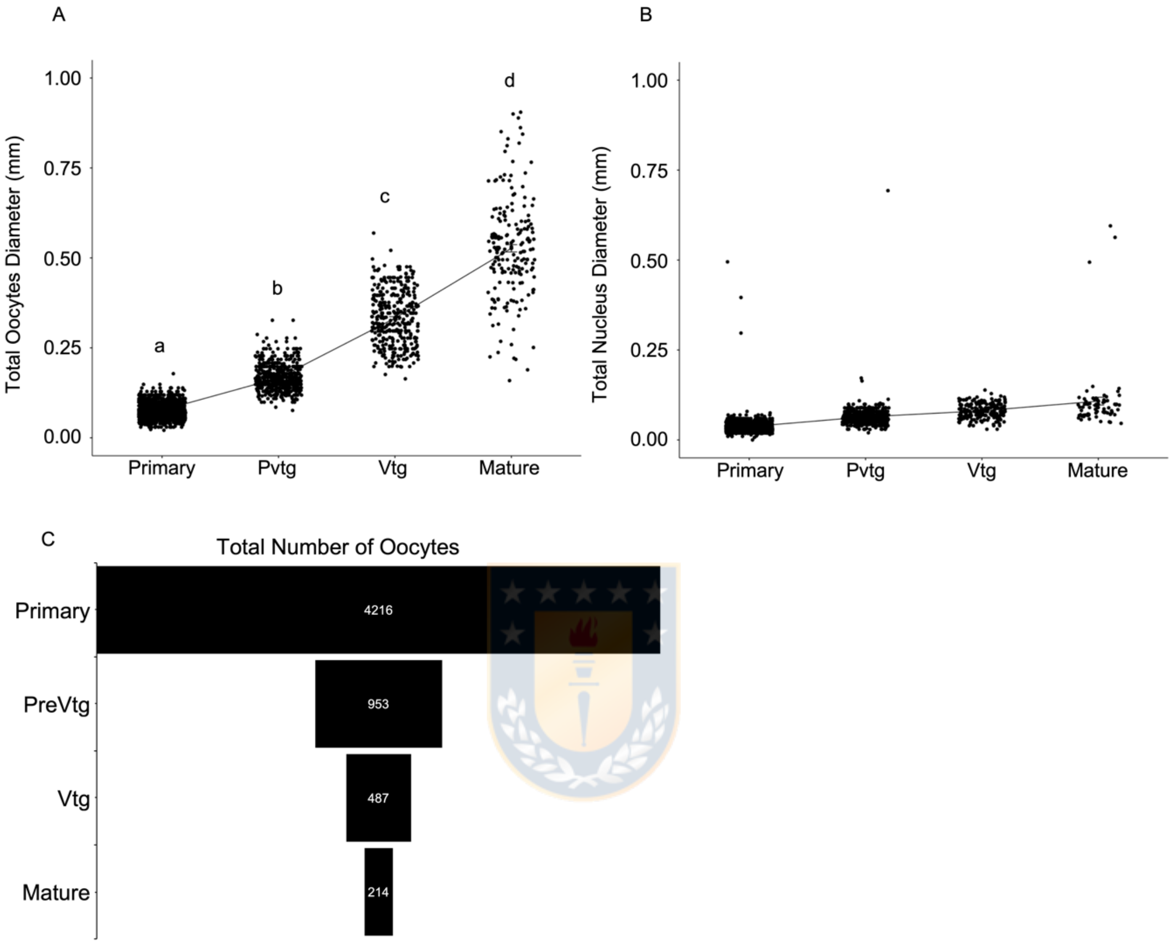

A
B
C
Total Oocytes Diameter (mm)
Total Nucleus Diameter (mm)
1.00
0.75
0.50
0.25
0.00
a
b
c
d
Primary
Pvtg
Vtg
Mature
Total Number of Oocytes
Primary
PreVtg
Vtg
Mature
4216
953
487
214

Figure 4.

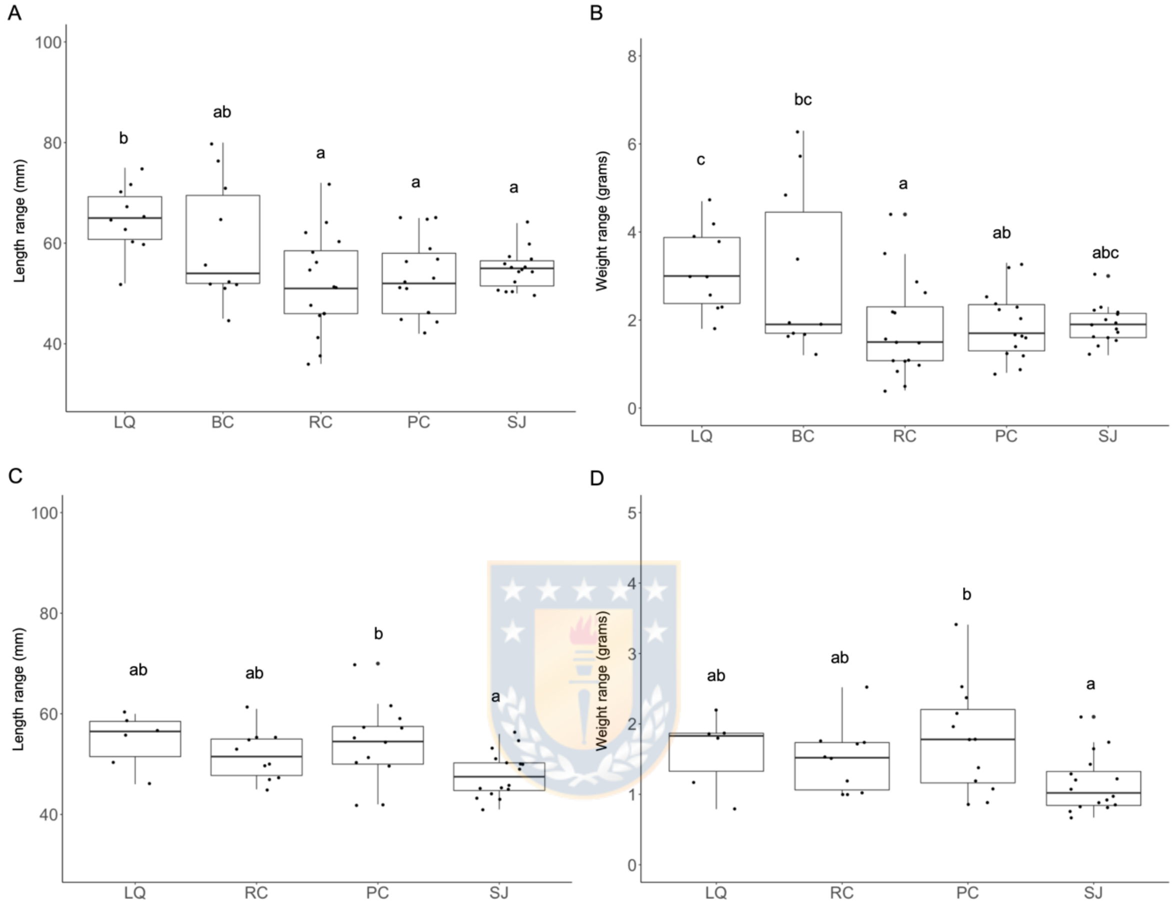

A
Length range (mm)
100
80
60
40
b
ab
a
a
a
LQ
BC
RC
PC
SJ
B
Weight range (grams)
8
6
4
2
0
c
bc
a
ab
abc
LQ
BC
RC
PC
SJ
C
Length range (mm)
100
80
60
40
ab
ab
b
a
LQ
RC
PC
SJ
D
Weight range (grams)
5
4
3
2
1
0
ab
ab
b
a
LQ
RC
PC
SJ

Figure 5.

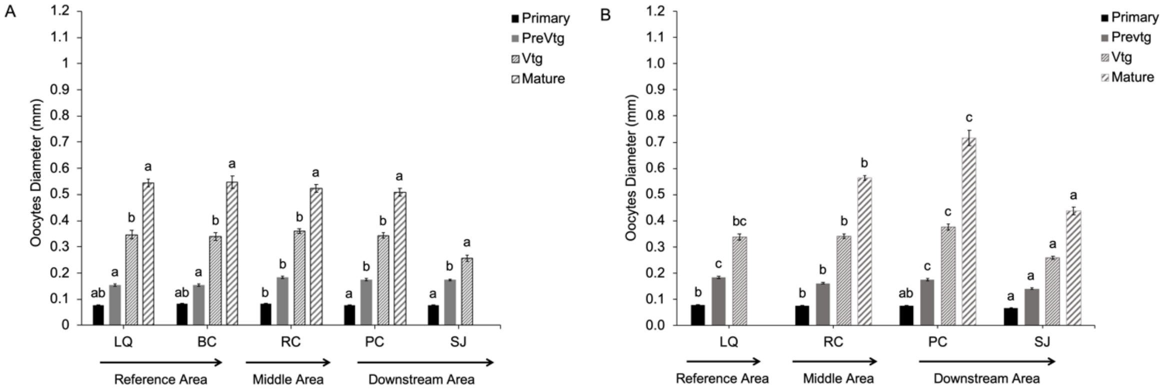


Figure 6.

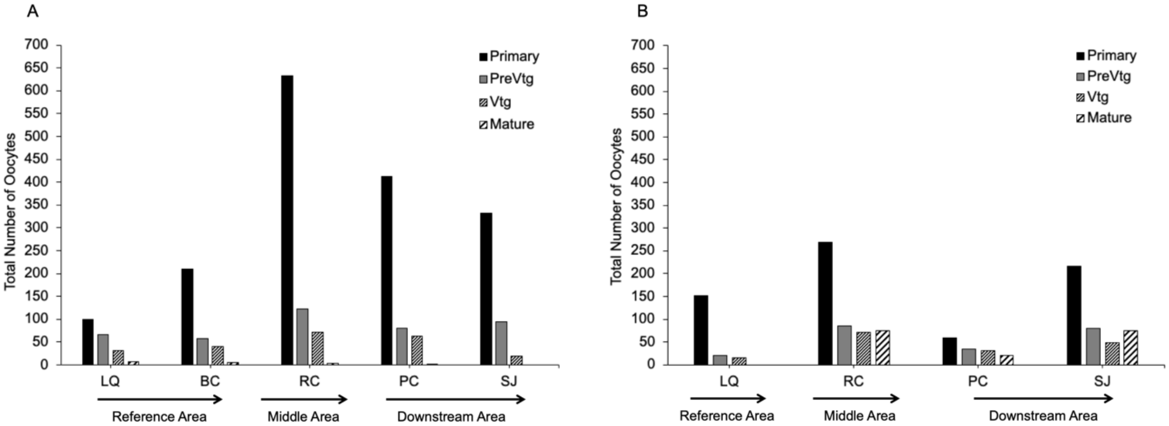
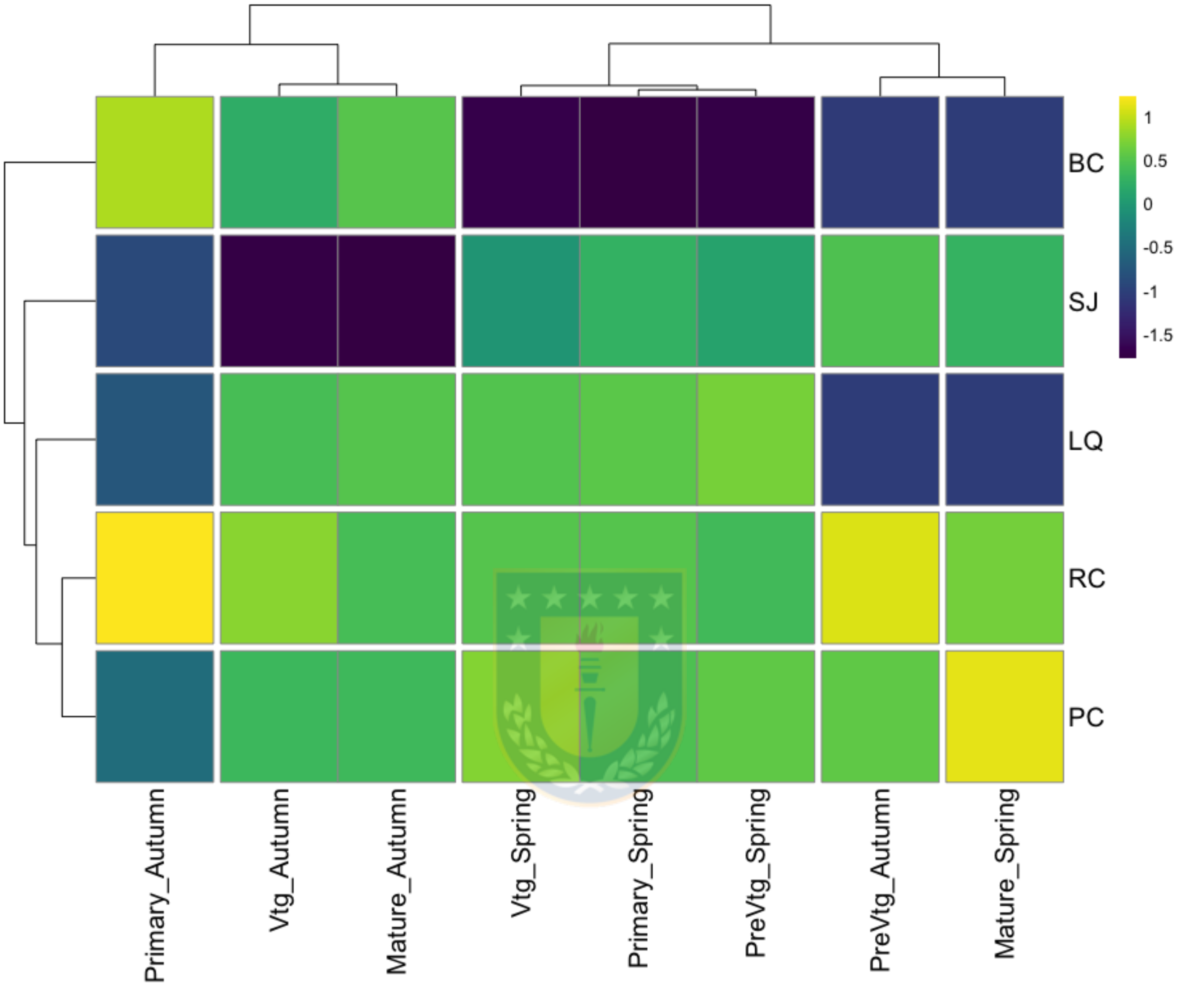


Figure 7.



1
0.5
0
-0.5
-1
-1.5
BC
SJ
LQ
RC
PC
Primary_Autumn
Vtg_Autumn
Mature_Autumn
Vtg_Spring
Primary_Spring
PreVtg_Spring
PreVtg_Autumn
Mature_Spring

CAPÍTULO III

INTEGRATED PHYSIOLOGICAL BIOMARKERS RESPONSES IN WILD FISH EXPOSED TO THE ANTHROPOGENIC GRADIENT IN THE BIOBÍO RIVER, SOUTH-CENTRAL CHILE

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Declarations

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Additional Information

Competing interests

The authors declare no competing financial interests.

The authors declare no competing interests.

Ethics approval

All the methods used in the present study followed relevant guidelines and regulations. Also, the competent authority (Ethics Committee of the Universidad de Concepción, Chile) approved the experiment and protocols of the present study.

Contributions: M.Q wrote the main manuscript text, analysis of the data and, prepared figures. J.G and R.B. contributed to the conception and design of the study. S.C; M.C.F., and R.O. contributed to the assistance of data analysis and writing the manuscript.

Abstract

To evaluate the physiological state of the wild fish inhabiting the Biobío River in South-Central Chile, susceptible to the chemical contamination from different sources, biochemical and physiological biomarkers were applied to wild fish *Percilia irwini* and *Trichomycterus areolatus in situ*. Fish caught in the Biobío river in low, medium, and high anthropic impacts areas, with different pollution degrees along the river. Ethoxyresorufin O-O deethylase (EROD) activity, were evaluated in fish liver. Length, Weight, Gonad weight and Liver weight, Physiological Index, and gill and liver histopathology were conducted. Physicochemical parameters (pH, Temperature, Conductivity, and TDS) were measured at each sampling site. The results indicated a deteriorating condition in the biological parameters of both species in a high anthropic zone. Fishes show an increase in physiological indices and EROD liver activity, agreeing with previous studies supporting evidence of reproductive change development as we move downstream the river. Also, an increase in histopathological lesions towards the lower third stretch of the Biobío River. The Integrated Index of Physiological Biomarkers (IPBR) indicated that sites located in the high impact area (P.*irwini*: BC: 4.09; RC: 3.38; PC: 3.50; SJ: 2.34 and *T.areolatus* BC: 6.06, PC: 5.37; SJ: 5.42) have the most detrimental environmental quality, compared to reference area. The integrated biomarker analysis demonstrates that the alterations observed are related to the high anthropic activity levels downstream from the sites with the least intervention, demonstrating that the IPBR used is a complementary tool for studies of the Environmental Effects Monitoring-approach.

Keywords: Wild Fish; Biomarkers; Physiological Index; Anthropogenic effects

1. INTRODUCTION

In recent decades, water resources have increased dramatically, both for urban, agricultural, and industrial use, generating various sources of point and diffuse pollution (Habit *et al.* 2006a; Figueroa *et al.* 2013; Alonso *et al.* 2017). This development implies a deterioration of water quality and alteration of the ecology of various aquatic systems. The development of water quality criteria to protect aquatic ecosystems is scarce, aggravating the situation even more. The scientific community must protect the ecosystems of developing countries by providing quality criteria based on ecological and toxicological knowledge (Alonso *et al.* 2017).

In South-Central Chile, in particular, the Biobío river, the effects on water quality and alteration of the ecology of the freshwaters systems is relevant given an exponential urban, agricultural and industrial growth that uses the water resource and several ecosystem resources (Habit *et al.* 2006a; Figueroa *et al.* 2013). The intensive use of the Biobío river's water resources in the last 20 years led to a detriment in water quality, especially in the lower stretch where the various urban and industrial discharges associated with this basin converge. The high impact in the Biobío river has possibly caused the incorporation of multiple organic micropollutants (OMPs). These compounds include persistent organic pollutants like pesticides and emerging contaminants (e.g., pharmaceuticals and personal care products) via domestic and municipal wastewater discharge, irrigation and runoff in agriculture and forestry activities, and leaching groundwater by authorized and unauthorized landfills (Rozas *et al.* 2016). These OMPs pollutants have hydrophobic and hydrophilic properties and are present in several environmental compartments and bioaccumulated in plants and animals (Rozas *et al.* 2016).

The Biobío river basin represents the natural base of one of the most critical economic development centers in Chile (Karrash *et al*. 2006; Parra *et al*. 2013; Orrego *et al*. 2019). The most relevant and dynamic productive sectors correspond to the forestry area, agricultural sector, industrial sector (pulp and paper mills, metallurgical, chemical industries, oil refinery, tannery, and textiles industries) (Karrash *et al*. 2006; Parra *et al*. 2013; Orrego *et al*. 2019). The hydroelectric sector constitutes the primary source of electricity supply at the national level, which implies about 50% of the regional GDP (Parra *et al*. 2013); and potentially new industrial projects in the future. Chilean pulp and paper industries each produce more than 1000t/day of pulp from pine and eucalyptus in the BioBío region, discharging their treated effluents into the lower stretch of the Biobío river (Orrego *et al*. 2019).

Initial evidence for biological effects in fish on the Biobío river was reported by others authors (Orrego *et al.* 2005a, 2005b). The authors evidenced in the fish rainbow trout (*Oncorhynchus mykiss*) using a caging strategy downstream of four Chilean mills effluents' discharge and exposed to sediments collected in those areas (Orrego *et al*. 2005a; Inzunza *et al.* 2006). These studies exhibit strong estrogenic effects (plasma vitellogenin induction, increase in gonad size, and mature ovarian follicles) in immature females fish (Orrego *et al*. 2005a, 2005b). These reproductive effects on fish associated with the industrial process have also been confirmed under laboratory and field works in the Itata river (Chiang *et al.* 2010, 2012, 2014). Recently studies of the presence of bioactive substances in the Biobío river were examined by deploying Semi-Permeable Membrane Devices (SPMDs) upstream and downstream of 4 pulp mills effluent discharge. The evidence indicated the occurrence of estrogenic type compounds associated with reproductive effects like an increase in Gonadosomatic Index (GSI), Induction of liver CYP4501A1 enzymes (EROD activity) in pulp mill effluent discharge impacted sites (Orrego *et al.* 2019).

The occurrence of several chemicals in aquatic systems has been well documented worldwide and is one of the main issues at local, regional, national, and global levels (Cerejeira *et al*. 2003; Konstantinou *et al*. 2006). Quantitative and qualitative analyses of these aquatic systems have allowed regulating the emissions of various environmental stressors legally; however, they have not necessarily revealed the potential biological impact on ecosystems. Thus, multidisciplinary and integrative studies, including different levels of biological organization, appear as necessary to harmonize the environmental laws with the health of the impacted ecosystems.

Biomarkers provide valuable information in field studies and are used to measure a wide range of early physiological responses by exposure to chemical compounds at the biochemical, cellular, and tissue levels (Oliveira *et al*. 2011; Seabra *et al.* 2014; Colin *et al.* 2016; Capela *et al.* 2016). Biomarkers being useful tools for monitoring the biological effects caused by chemical contamination. The advantage of histopathology as a biomarker lies in its intermediate location at the biological organization level (Bernet *et al*. 1999). Histological changes appear as a medium-term response to sub-lethal stressors, and histology provides a rapid method to detect irritants' effects, especially chronic ones, in several tissues and organs (Bernet et al., 2004, Colin et al., 2016).

Nevertheless, the lack of an integrated biomarker analysis has limited the potential use of these tools. Hence, to improve the interpretation between the different multilevel biomarkers used to determine the effects on sentinel fish and relate them to the quality standards defined by Chilean environmental regulations is necessary. In this way, it is possible to evaluate the global changes of the responses obtained through an integrated response of physiological biomarkers (IPBR), which allows relating effect patterns with different mechanisms of toxicological action (Beliaeff *et al*. 2002; Sanchez *et al*. 2013; Murussi *et al*. 2015), in certain areas with different pollution profiles. In this way, it is possible to cover specific gaps present in the legislation like Secondary environmental quality standards for Biobio River. Furthermore, effectively comply with what is established by environmental regulations regarding water quality criteria and conserving and preserving aquatic ecosystems (General Bases of the Environment, Chilean law Nº19300). Wild fish fauna in the Biobío River represents a genetic heritage of this biogeographic zone. The alteration of its biotype has led to a potential decrease in their populations, and areas directly affected by mill discharges and urban impact in the Biobío river have been continually associated with changes in wild fish abundance, diversity, and reproductive effects (Habit *et al*. 2006; Chiang *et al*. 2011; Orrego *et al*. 2019).

This study aimed to analyze several biomarkers in the two native fish species *P.irwini* (common name Carmelita) and *T.areolatus* (common name Bagre) along the main course Biobío River, in order to assess their physiological status in a gradient of anthropic intervention. We propose an IPBR as a tool to compare the physiological state and potential biological effects in these wild fish, along a gradient of impacts in the length of the Biobio river. These two selected species have characteristics sought as indicator species in freshwater systems of central Chile as described by Chiang *et al*. (2012). *Percilia gillissi* (and its congeneric species *P. irwini*, restricted to the Biobío River basin) and *T.areolatus* meet our study strategy requirements. *T. areolatus* has a wide abundance and geographic distribution, has a benthic and benthophagous life strategy, with closer contact with the sediment. *P. gillissi* also has a wide distribution. It is found preferentially in rithron environments (Source) and swimming at mid-water. Its diet is mainly benthophagus. Other authors (Chiang *et al*. 2012) showed that this species shares the spawning season with *T. areolatus* and overlapping with its congeneric species (*P. irwini*).

2. MATERIAL AND METHODS

2.1 Description of Study Area

The study area is located in the Biobio River Basin, South-Central Chile. Biobio basin has 24,369 km^2 and a length of 380 km (Figure 1). The Biobio river belongs to a hydrographic basin of Andean origin. Presents a shape defining two sectors: 1) Rhithron zone (Source) with a high slope, volcanic soils, vegetation cover formed by native forest, high rainfall and snowfall, high solar radiation, and a river network with more than 5000 first-order rivers, and 2) low-slope Potamon zone (lower course), without snowfall and predominantly a vegetation cover of introduced species (pine and eucalyptus).

Socio-economically, the Biobio River system and its tributaries and the lakes in its catchment area are among the most important river systems in Chile (Karrash *et al*. 2006). The Biobio River is also one of the most severely polluted water bodies in Chile (Karrash e*t al*. 2006).

All physicochemical parameters (pH, Temperature, Conductivity, Total Dissolved Solids) were measured immediately after sampling using WTW meters (Hanna HI 9829).

Fish were caught at five locations (on March 2017) along the river from its source to the mouth (36º42´ - 38º49´) to assess and compare the physiological status and biological alteration of the wild fishes (Figure 1):

Reference Area (upstream of the Biobio River): Site 1 Lonquimay (LQ) and Site 2 Balsa Caracoles (BC). These sampling sites present low anthropic intervention, surrounded by native forest vegetation.

Middle Impact Area: Site 3 Rucalhue (RC), located downstream of the Angostura hydroelectric plant, is the third hydroelectric power station present in the Biobio river. This zone has relicts of native forest and forest plantations.

High Impact Area: Site 4 Puente Coihue (PC) is located downstream of urban effluents and pulp and paper industries. PC has a high predominance of forest plantations associated with the river sector associated with some agricultural activities. Site 5 Santa Juana (SJ) presents high anthropic intervention and corresponds to the convergence sector of the several activities present upstream of the Biobio River.

2.2. FISH COLLECTION

2.2.1 Ethical Statement.

The study protocol was reviewed and approved by the Ethics Committee of the Universidad de Concepción, Chile (Aut. No. 01-2016).

Wild fish's health status was assessed during the autumn season (March 2017) and post-spawning season. Two fish species (from a total of 18 native species) were collected considering their abundance in the study areas. Small size (less than 10 cm) and relatively limited mobility: *Percilia irwini*, a benthic-pelagic species inhabiting relatively shallow waters (n=66), and *Trichomycterus areolatus* a benthic species (n=40).

The fish sampling was carried out following the longitudinal gradient of the Biobio River, from the Source (Rhithron zone) to the Lower course (Potamon zone) (Figure 1). Due to the lack of secondary sexual characteristics for *P. irwini and T.areolatus*, a maximum of 10 adult fish were selected at each site, with sizes greater than 35 mm based on previous studies (Chiang *et al*. 2011).

Fish were captured at sites with similar microhabitat characteristics using a backpack electric fishing equipment (Halltech Electrofisher, Canada). A blocking net (6 mm mesh opening) were used in riffles (0.2-0.3 m/s, 0.2-0.4m depth) with a bed of boulders and gravel (15cm diameter) and shallow riffles (0.1 - 0.2m/s, 0.1 - 0.2 m depth) with a bed of boulders (15cm diameter) and rock. Size, sex, body length, and body weight was immediately measured. Dissected liver tissues were stored in liquid nitrogen and then transported to the Biomarkers Laboratory of EULA-Center (University of Concepción) for further analysis. Gill, liver, and gonads were also immediately extracted and stored in Bouin solution for histological analysis.

Physiological indexes were calculated based on the morphometric information such Condition Factor (K=[Total Weight(g)/Total Lenght^3]*100); Gonadosomatic Index (GSI= [gonad weight(g)/ Total Weight(g)]*100); Hepatosomatic Index (HSI=[Liver weight(g)/Total Weight]*100) were calculated.

2.3. Histological Analysis

After taking the subsample of different tissue, the histological analyses were carried out in the Department of Cell Biology of the Faculty of Biological Sciences at the Universidad de Concepción. The gonads were weighed and fixed immediately in Bouin solution for further analysis. After the fixation time (48 hours), the samples were washed with 70% ethanol to eliminate the Bouin solution (three times for 15 minutes). Then, the samples were washed through a dehydration battery with different ethanol solutions (70% - 99%) and chloroform, to finally infiltrate the samples in liquid paraffin at 58 ° C, for two hours, repeating this step twice. After infiltrating the samples, samples were included in paraffin (Histosec®, MerckMillipore) for a minimum time of 24 hours. Seven (7) µm sections made in a manual rotation microtome (Jung). The paraffin was removed by washing with Xylol and stained after washing in a hydration battery (100% to 70% ethanol) with

Hematoxylin and Chromotrope (0.5%). Serial sections of *P.irwini* and *T.areolatus*, liver, and gill were acquired, obtaining 7013 measurements in histological sections with four (4) cuts per slide. The tissues' analysis was performed at the microscopy laboratory of the department of Applied Ecotoxicology of the Università di Siena, Italy, with an optical microscope with a previously calibrated micrometric eyepiece, to take a photograph in an OLYMPUS BX41 microscope with OLYMPUS U-YVO.5XC-2 digital camera attached.

2.4. Histopathological indexes

Once the histological sections have been obtained, Histopathological indexes of gill (Iorg_Gill) and liver (Iorg_Liver) were estimated by semi-quantitative protocol following Bernet *et al*. (1999, 2004). Briefly, alterations classified into four major reaction patterns: RP1, circulatory disturbances (gills: hemorrhage, aneurysm, edema; liver: dilatation of sinusoids, vascular congestion, hemorrhage); RP2, regressive changes (gills: epithelial lifting, lamellar disorganization, and shortening; liver: vacuolar degeneration, nuclear alteration, fibrosis, necrosis); RP3, progressive changes (gills and liver: cell hypertrophy and hyperplasia); and RP4, gill and liver inflammation (leukocyte infiltration). Then, Iorg_Gill and Iorg_Liver were calculated based on two factors: the pathological importance of the lesions (importance factor, W) (range 1–3) and the extension of pathological change (score value, a) from 0 (unchanged) to 8 (extreme occurrence). Finally, a total histopathological index (Iorg) was calculated by adding gills and liver indices of each wild fish. A higher value of the Iorg reflects a more severely affected individual.

2.5. EROD activity

EROD activity was analysed according to Lubert *et al*. (1985) standardized protocol in the floating post mitochondrial supernatant (fraction S9) obtained from livers homogenized in a sucrose buffer (0.1 M, pH 7.5) and centrifuged at 9,000g for 20 min at 48ºC. Fluorimetric analyses were performed using an LS 50B spectrofluorometer (PerkinElmer) for 5 min at 25ºC, using reduced NAPH as the electron donor. Protein analysis was performed in a microplate reader (Baush & Lomb, DNM, 9602G) using a Biuret microplate method that uses bovine serum albumin (Sigma-Aldrich) as a reference material. The EROD activity was expressed as pmol/min/mg protein.

2.6. Integrated Physiological Biomarkers Response (IPBR)

The biomarkers' result was then incorporated into the mathematical model Integrated Biomarker Response (Bealieff and Burgeot 2002, Sanchez *et al*. 2013). The index was calculated based on the log-transformation to decrease its variance and be represented as polar plots. For index calculation, the base value obtained for each biomarker at the Lonquimay site (LQ) was used as a reference value (T0). The data for each biomarker and fish between sampling sites was log-transformed (Yi), and the overall mean (μ) and SD (s) were further calculated. Subsequently, the Yi values obtained for each biomarker standardized by the formula: $Zi = (Yi - \mu) / s$, and the difference between Zi and T0 was used to define the biomarker deviation index (A). Finally, to obtain an integrated physiological biomarker response (IPBR), the absolute value of A (|A|) for each biomarker at each sampling site was added. As physiological responses are used, values below zero (0) indicate a decrease in the fish's physiological conditions, considered a decrease in biological responses.

2.7. Statistical Analyses

Data analysis was performed by RStudio, R Software Package (Core Team, 2017). Data examined by exploratory data analysis and continuous data were examined for normal distribution (Shapiro-Wilks test) and log-transformed to avoid non-normality. One-way ANOVA and Tukey were used to evaluate significant statistical differences between sampling sites, and differences were confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$). The data were compared with the reference site Lonquimay (LQ) and between the downstream sites and each species studied. Multiple linear regression (RStudio Team (2020)) was used to determine the variables that directly influence the histopathological effects. All the assumptions were previously verified to validate the analysis. The Polar Chart was performed by package “plotly” version 4.9.0 and Heatmap by package “stats” version 3.5.0 in RStudio.

3. RESULTS

3.1. Physicochemical Parameters

The physicochemical parameters are listed in Table 1. From the upstream sites Lonquimay and Balsa Caracoles (Reference Area), the conductivity had progressively risen from Rucalhue to Puente Coihue and finally to Santa Juana. The pH decreased from 7.04 to 6.91 at Rucalhue and increased in Puente Coihue (7.56) but was somewhat decreased to 6.35 at Santa Juana, possibly by the Laja river, the main tributary of the Biobío river at that site. The pH recorded the highest value at Puente Coihue, subject to the most significant impact of pulp and paper mill effluents and the last third of the Biobio river. The Temperature parameters also change from initial values of around 16ºC downstream at Puente Coihue and Santa Juana (18.8 and 20.8ºC). These values were high and highest in respect to the reference sites Lonquimay and Balsa Caracoles. The Total Dissolved Solids (TDS) increased downstream from 35-36 mg/L at Balsa Caracoles and Lonquimay to 54.4 to 77.9 mg/L at Puente Coihue and Santa Juana.

3.2 Biological parameters: Length, Weight, Gonad weight, and Liver weight

Total body length and total body weight differed significantly among sites for both species *P.irwini* and *T.areolatus* (Table 2 and 3). Mean length was lowest at sites downstream river Puente Coihue and Santa Juana for both wild fish. The total body weight decreased significantly at Puente Coihue and Santa Juana from 3.16 g to 1.18 g for *P.irwini* and 3.78 g - 2.35 g for *T.areolatus*. The gonad weight values in *P.irwini* were 0.047 g to 0.023 g and 0.047 g to 0.0125 g for *T.areolatus*. For both fish, the gonad's mean weight values decreased downstream from sites Lonquimay and Balsa Caracoles. The liver weight values for *P.irwini* decreased from 0.019 g in Lonquimay to 0.013 g at Santa Juana. On the other hand, for *T. areolatus*, mean liver weight had risen at Puente Coihue to 0.028 g and then decreased to 0.018 g at the Santa Juana site (Table 2).

3.3 Physiological Index: K, GSI, and HSI

Condition Factor (K) mean values, no significant statistical differences were observed among sampling sites along the Biobío river and gender in both fish. The mean GSI values have significant differences between sampling sites in *P.irwini* and *T.areolatus* (Table 2 and 3). Compared with upstream sites, LQ and BC, mean GSI values for *P.irwini* increased from 0.39% to 0.81% in the Santa Juana site. *Trichomycterus areolatus* mean GSI values had a progressively significant decrease downstream at Puente Coihue and Santa Juana sites (1.14 and 0.59%, respectively). Hepatosomatic Index (HSI) not differed significantly between the sites for both species (Table 2 and 3). Mean HSI values for *P.irwini* decreased downstream at Puente Coihue and Santa Juana sites (0.73% and 0.62%). Compared with upstream sites LQ and BC, mean HSI values for *T.areolatus* increased downstream at the last third of the Biobío river in PC and SJ sites.

3.4 Histology: Gill and Liver Histopathology Index

Microscopic abnormalities were observed in the gill and liver for both *P.irwini* and *T.areolatus* (Fig. 2) along the Biobío river and among the sampling sites (Fig 3). Common gill histopathologies in *P.irwini* and *T.areolatus* including regressive changes at the architectural and structural alterations, necrosis in the epithelium, architectural and structural alterations in supporting tissue, progressive changes like hyperplasia in the epithelium and supporting tissue. Regarding the liver histopathologies in *P.irwini* and *T.areolatus*, including architectural and structural alterations, plasma alterations, necrosis in liver tissue, and interstitial tissue, all regressive changes. The Gill organ index (Iorg_Gill) values in *P.irwini* had progressively risen at PC and SJ sites (56.6 and 56.8) compare to upstream sites LQ and BC (35.8 and 40). Moreover, *T.areolatus* has a risen values from LQ (33.8) to PC (70.6), but then decreases to the Santa Juana site (46.6), possibly by the Laja river influence, the main tributary of the Biobío river at that site. Fish captured downstream of the pulp and mill effluent discharge at site PC showed the gill organ index's highest values. The Liver organ (Iorg_Liver) index values in *P.irwini* increase progressively downstream Lonquimay site from 6 to 26 at Santa Juana. The highest values were in PC and SJ (25.5 and 26.2). A significant pattern of increased values is observed in *T.areolatus* downstream from LQ and BC (2 and 12) to PC and SJ sites (21.2 and 20).

3.5. Induction of liver CYP4501A1 enzymes

No statistically significant differences were observed in EROD activity in all the sampling sites along the Biobio river (Table 4) in wild fish *P.irwini* and *T.areolatus*.

Nevertheless is possible to observe an increased pattern of the EROD activity levels in *P.irwini* downstream river at PC and SJ sites (234.31 and 411.91 pmol/min/mg) compared to upstream sites LQ and BC. For *T.areolatus* similar pattern is observed compared with upstream LQ site (82.11 pmol/min/mg) with downstream sites PC and SJ (136.40 and 217 pmol/min/mg). For both species, the last site, SJ, showed the highest increased detected. In comparing the EROD activity of both species, *T.areolatus* showed the highest EROD induction compared to *P.irwini* in fish captured at the Santa Juana site.

3.6 Integrated Physiological Biomarkers Response (IPBR)

The IPBR value was calculated in both wild fish for each sampling site, as shown in Figure 4 and Figure 5. Values of the IPBR for *P.irwini* decrease downstream Lonquimay site to Santa Juana. BC site exposes a higher IPBR value (4.09), followed by RC (3.38), PC (3.50), and SJ (2.34). The variables that most influenced the RC's results were the length, weight, gonad weight, GSI, and HSI. At PC and SJ, the variables most influence the IPBR values were length, weight, gonad weight, Liver, K, GSI, HSI, EROD, and the liver and gill organ index. Compared with the upstream sites LQ and BC, the variables length, weight, gonad weight, and GSI have decreased the IPBR values to Puente Coihue and finally Santa Juana. In contrast, the variables liver weight, K, HSI, EROD, and organ Index has an increase in this value (Figure 4).

Values of the IPBR for *T.areolatus* decrease downstream LQ site to SJ (Figure 5). BC site exhibits a higher IPBR value (6.06), followed by PC (5.37) and SJ (5.42). The variables that most influenced these results at the PC site were the length, weight, and gonad weight. At SJ, the variables that most influence the IPBR values were length, weight, gonad weight, liver, K, GSI, HSI, EROD and liver, and gill organ index. Compared with the upstream sites LQ and BC, the variables length, weight, gonad weight, and GSI have a marked decrease in SJ's IPBR values. Also is necessary to note the EROD, liver, and gill organ index had a risen values at PC and SJ for both species.

The values of the IPBR for both species were evaluated through hierarchical clustering to establish relationships among biomarkers, sampling sites, and wild fish species (Figure 6). The Heatmap based on Euclidian distance shows a high relation between both species and every sampling site, from upstream LQ and BC sites to downstream PC and finally SJ. The Integrated response confers a high relation of length and weight, Gonad weight, liver weight, and GSI, at the LQ and BC declining SJ. On the other hand, the biomarkers such as EROD, IorgGill, and IorgLiver exhibit a significant relationship between fish species in the downstream river (PC and SJ) and few correlations in the upstream river at reference sites LQ and BC.

4. DISCUSSION

This wild fish study exhibit responses addressed by biomarkers related to exposure to multiple environmental stressors that converge towards the lower third of the Biobio River. Physicochemical effects include the increase in conductivity between Lonquimay and Santa Juana, which is likely to be mainly due to the high release of industrial effluents in the middle of the Biobio river, as reported by other authors (Karrash *et al*. 2006). High urban, agricultural and industrial activity along the Biobio river also explain the high TDS levels, which indicates a decrease in Oxygen Solubility (DO) levels.

The principal effect observed due to exposure to a complex chemical mixture in these species at the Biobio river occurred at the physiological level, with responses during the postspawning period. Both species showed a reduction in length and weight from Lonquimay to Santa Juana, as reported by other authors (Habit et al. 2006). Also, both species show an increase in their GSI and HSI.

The physiological results found for both species are in agreement with the results of other authors (Habit *el al*. 2006; Orrego *et al*. 2005, 2009; Inzunza *et al*. 2006; Chiang *et al*. 2011), who indicated a reduction of the length and weight, stimulation of the reproductive system and induction of the EROD activity in areas of more significant anthropic impacts.

Regarding the biological effects determined by molecular biomarkers, the liver activity EROD represents the activity of phase I of the enzymatic biotransformation process mediated by CYP1A. Although these biochemical mechanisms are induced shortly after exposure to the number of compounds such as PAHs, PCBs, and aromatic amines (Jonsson *et al*. 2007) being considered an early response biomarker (Colin *et al*. 2015), the CYP1A also catalyze biosynthesis and degradation of the endogenous molecule (e.g., steroids. Lewis 2004). Moreover, it has been described that the activity of the CYP1A subfamily may be affected by the presence of chemical contaminants in aquatic environments (Van der Oost *et al*. 2003), such as resin acid (dehydroabietic) suggested relating to liver metabolic impairment in fish (Orrego *et al*. 2010) or decrease in EROD activity associated with the presence of organochlorine compounds as described by Couillard *et al*. (2005). The induction of this enzymatic activity has been well documented both in laboratory conditions (effluents and sediments bioassays) and in field conditions by caging or capturing introduced and natural fish species (Barra *et al*. 2001, Inzunza *et al*. 2006, Chiang *et al*. 2011, Orrego *et al*. 2005a, 2005b, 2010, 2019,) at the Biobio river. The effects are related to a series of compounds from polycyclic aromatic hydrocarbon (PAH´s) in sediments to phytosterols in industrial effluents.

The chronic exposure of wild fish to chemical contaminants is likely to induce several lesions in different organs, as observed in gills and liver histopathology. In the tissues analyzed in this work, gills and liver are suitable organs for histological examination to determine the effect of pollution (Colin *et al*. 2016). The gills and liver lesions analysis shows a spatial distribution increasing Iorg_Gill and Iorg_Liver from upstream to the downstream river. These injuries are related to the high presence of contaminants such as polycyclic aromatic hydrocarbon (PHA), polychlorinated biphenyls (PCB), Dichloro-Diphenyl-Trichloroethane (DDT), previously reported in the lower third of the Biobío river (Alonso *et al*. 2017, Orrego *et al*., 2005a, 2005b, 2019).

Studies in the Biobío river (Habit *et al*. 2005, Orrego *et al*. 2005, 2019, Habit *et al*. 2019) documented apparent changes during the last decades in the fish communities in the middle section of the Biobio river. These fish communities' changes are attributed to the increase in human activities throughout the basin, including wastewater and pulp mill discharges (Orrego *et al*. 2005a, 2005b, 2006, Chiang *et al*. 2011); however, no cause-effect relationships were demonstrated.

Although the evaluation of IPBR indicated physiological effects associated with a spatial gradient, similar to the molecular (EROD) pattern and individual (length, weight, GSI) biomarkers measured in both fish species, no direct cause-effect relationship was established. Significant differences between species were related to their habitat, pelagic-demersal for *P.irwini*, and benthic for *T.areolatus*. Similar results were recently informed by Orrego *et al*. (2019) regarding molecular and individual biomarkers associated with endocrine-disrupting substances in pulp mills effluent discharge accumulated through SPMDs deployed in different areas at the Biobío river. However, according to different authors (Munkitrich *et al*. 2000, Chiang *et al*. 2011, 2014, Delfino *et al*. 2016), the interpretation of fieldwork results is always associated with high variability due to the presence of multiple external factors, which can influence the analyzed variables, so its results should be considered with caution.

The physiological condition of native fish in the Biobío River may be affected by point and non-point sources of contamination associated with land use, as documented by Orrego *et al.* (2009). This physiological condition change is widespread in the Andean rivers in Chile, where most land-use activities involve industrialization, agriculture, and river basins urbanization. Several authors (Death & Winterbourn, 1995, Habit *et al*. 2009, Orrego *et al*. 2009, 2019, Chiang *et al*. 2012) indicate that disturbances of human origin, such as the contribution of pollutants of different nature and nutrients, can potentially destabilize freshwater systems and directly affect biological communities in terms of diversity and abundance. These types of effects are evident in the case of the Biobío River, owing to the fact the disturbance of anthropogenic origin has generated direct effects on the native fish communities in the main course of the Biobío River, with characteristics of stressed aquatic ecosystems (Orrego *et al*. 2005a, 2005b, 2006, 2009, Habit *et al*. 2019).

The observed pattern of both fish studied in the Biobío river was influenced by the anthropogenic disturbance caused by xenobiotic chemical compounds of diverse nature, suggesting a cumulative spatial effect due to the environmental degradation at the lower third of the Biobío river. The IPBR showed a decrease in both wild fish's biological condition. These manifest changes are related to the high anthropogenic activity from Lonquimay to Puente Coihue and Santa Juana sites.

5. CONCLUSION

The IPBR index developed in native fish in the Biobío River accounts for the variation of native fish species' physiological state from fewer impact sites to mostly impacted sites. Our spatial investigation was able to detect physiological changes in wild fish in the Biobio river. These changes were associated with the high anthropic impacts at the lower third of the river and complement several Environmental Effects Monitoring-approach studies as a tool for evaluation under the different environmental conditions in freshwater systems in Chile.

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Figure Captions

Figure 1. Biobio River Basin showing sites where *Percilia irwini* and *Trichomycterus areolatus* were collected, Reference Area (LQ-BC sites), middle impact Area (RC site), and high impact area (PC-SJ sites). Icons (brown) indicate the primary industrial sources contiguous to the main course of the Biobio river.

Figure 2. (a) Gill tissue of *Percilia irwini* hyperplasia, aneurisms, necrosis, secondary lamella shortening and fusion. (b) liver tissue of *Percilia irwini* showing steatosis. (c) Gill tissue of *Trichomycterus areolatus* hyperplasia, aneurisms, necrosis, secondary lamella shortening and fusion. (d) liver tissue of *Trichomycterus areolatus*, area of lymphocyte infiltration, steatosis. Bars equal 50 mm. H&C stain.

Figure 3. Histopathological Index value for *Percilia irwini* and *Trichomycterus areolatus* from gill and liver tissues along the Biobio river.

Figure 4. IPBR values for *Percilia irwini* for each collected area in the Biobío river. (a) BC - Balsa Caracoles, (b) RC - Rucalhue, (c) PC – Puente Coihue, (d) SJ – Santa Juana. Biomarkers representation in relation to the reference site LQ – Lonquimay.

Figure 5. IPBR values for *Trichomycterus areolatus* for each area in the Biobío river. (a) BC - Balsa Caracoles, (b) PC – Puente Coihue, (c) SJ – Santa Juana. Biomarkers representation in relation to the reference site LQ – Lonquimay.

Figure 6. Hierarchical clustering for the IPBR values in *Percilia irwini* (Pi) and *Trichomycterus areolatus* (Ta) showing the relationship between biomarkers and among sites along the Biobío river LQ, BC, RC, PC, SJ.

Table 1. Physico-chemical parameters in Biobío river, Chile

Site	pH	Temperature (ºC)	Conductivity (μS cm^{-1})	TDS (mg/L)
LQ - Lonquimay	7.04	18.7	72	36
BC - Balsa Caracoles	7.06	16.1	70.2	35.1
RC - Rucalhue	6.91	17.4	102.6	51.2
PC - Puente Coihue	7.56	18.8	108.8	54.4
SJ - Santa Juana	6.35	20.8	156.8	77.9

Table 2. Arithmetic mean (±SE) of Length, Weight, Gonad weight, Liver Weight and physiological biomarkers K, GSI, HSI in *Percilia irwini* in Biobio river, Chile

Site	Length (mm)	Weight (g)	Gonad weight (g)	Liver weight (g)	K	GSI	HSI
LQ	64.90 ± 6.67(b)	3.16 ± 0.95(c)	0.05 ± 0.03(a)	0.02 ± 0.01(a)	1.14 ± 0.20(a)	0.39 ± 0.67(ab)	0.39 ± 0.37(a)
BC	60.00 ± 12.09(ab)	3.02 ± 1.90 (bc)	0.02 ± 0.01(a)	0.03 ± 0.02(a)	1.25 ± 0.09(a)	0.53 ± 0.72(a)	0.96 ± 0.60(a)
RC	51.88 ± 9.89(a)	1.76 ± 1.12(a)	0.04 ± 0.02(a)	0.02 ± 0.01(a)	1.12 ± 0.18(a)	1.39 ± 1.30(b)	0.91 ± 0.42(a)
PC	53.13 ± 7.86(a)	1.89 ± 0.76 (ab)	0.02 ± 0.01(a)	0.02 ± 0.01(a)	1.22 ± 0.24(a)	0.38 ± 0.63(ab)	0.73 ± 0.50(a)
SJ	54.67 ± 3.92(a)	1.89 ± 0.44(abc)	0.02 ± 0.01(a)	0.01 ± 0.01(a)	1.14 ± 0.09(a)	0.81 ± 1.03(ab)	0.62 ± 0.35(a)
F value	4.913	4.596	1.818	1.216	1.363	2.878	1.662
df	4	4	4	4	4	4	4
p-value	0.001	0.002	0.151	0.315	0.257	0.039	0.171

Note. Means followed by the same letter within a column are not significantly different (analysis of variance $p < 0.05$, Tukey). Also shown are ANOVA *F* values, degrees of freedom (df) and *p*-values.

– = Not applicable

Table 3. Arithmetic mean (±SE) of Length, Weight, Gonad weight, Liver Weight and physiological biomarkers K, GSI, HSI in *Trichomycterus areolatus* in Biobio river, Chile

Site	Length (mm)	Weight (g)	Gonad weight (g)	Liver weight (g)	K	GSI	HSI
LQ	89.60 ± 10.81(ab)	3.78 ± 1.69(ab)	0.05 ± 0.03(c)	0.02 ± 0.01(a)	0.50 ± 0.04(a)	1.23 ± 0.42(c)	0.58 ± 0.34(a)
BC	96.00 ± 12.98(b)	4.65 ± 1.98(b)	0.02 ± 0.01(ab)	0.03 ± 0.02(a)	0.50 ± 0.04(a)	0.31 ± 0.18(a)	0.72 ± 0.21(a)
RC	-	-	-	-	-	-	-
PC	77.70 ± 16.78(a)	3.31 ± 2.04(a)	0.04 ± 0.02 (bc)	0.03 ± 0.02(a)	0.73 ± 0.48(a)	1.14 ± 0.60 (bc)	0.79 ± 0.17(a)
SJ	75.20 ± 11.23(a)	2.35 ± 1.30(a)	0.01 ± 0.01(a)	0.02 ± 0.01(a)	0.51 ± 0.03(a)	0.59 ± 0.28 (ab)	0.76 ± 0.32(a)
F value	5.596	4.14	7.478	2.388	1.634	8.944	1.214
df	3	3	3	3	3	3	3
p-value	0.002	0.012	0.001	0.085	0.2	<0.001	0.319

Note. Means followed by the same letter within a column are not significantly different (analysis of variance $p < 0.05$, Tukey). Also shown are ANOVA *F* values, degrees of freedom (df) and *p*-values

– = Not applicable

Table 4. Arithmetic mean (±SE) of EROD (pmol/min/mg protein) in *Percilia irwini* and *Trichomycterus areolatus* in Biobio river, Chile

	Percilia irwini	*Trichomycterus areolatus*
Site	EROD (pmol/min/mg)	EROD (pmol/min/mg)
LQ - Lonquimay	395.342 ± 161.078 a	82.110 ± 44.275 a
BC - Balsa Caracoles	332.273 ± 343.237 a	116.19 1± 65.499 a
RC - Rucalhue	236.297 – 154.647 a	-
PC - Puente Coihue	234.309 ± 103.587 a	136.405 ± 109.836 a
SJ - Santa Juana	411.908 ± 271.507 a	217.619 ± 157.410 a
F value	2.109	0.944
df	4	3
P *value*	0.094	0.44

Note. Means followed by the same letter within a column are not significantly different (analysis of variance $p < 0.05$, Tukey). Also shown are ANOVA *F* values, degrees of freedom (df) and *p*-values

– = Not applicable

Figure 1.

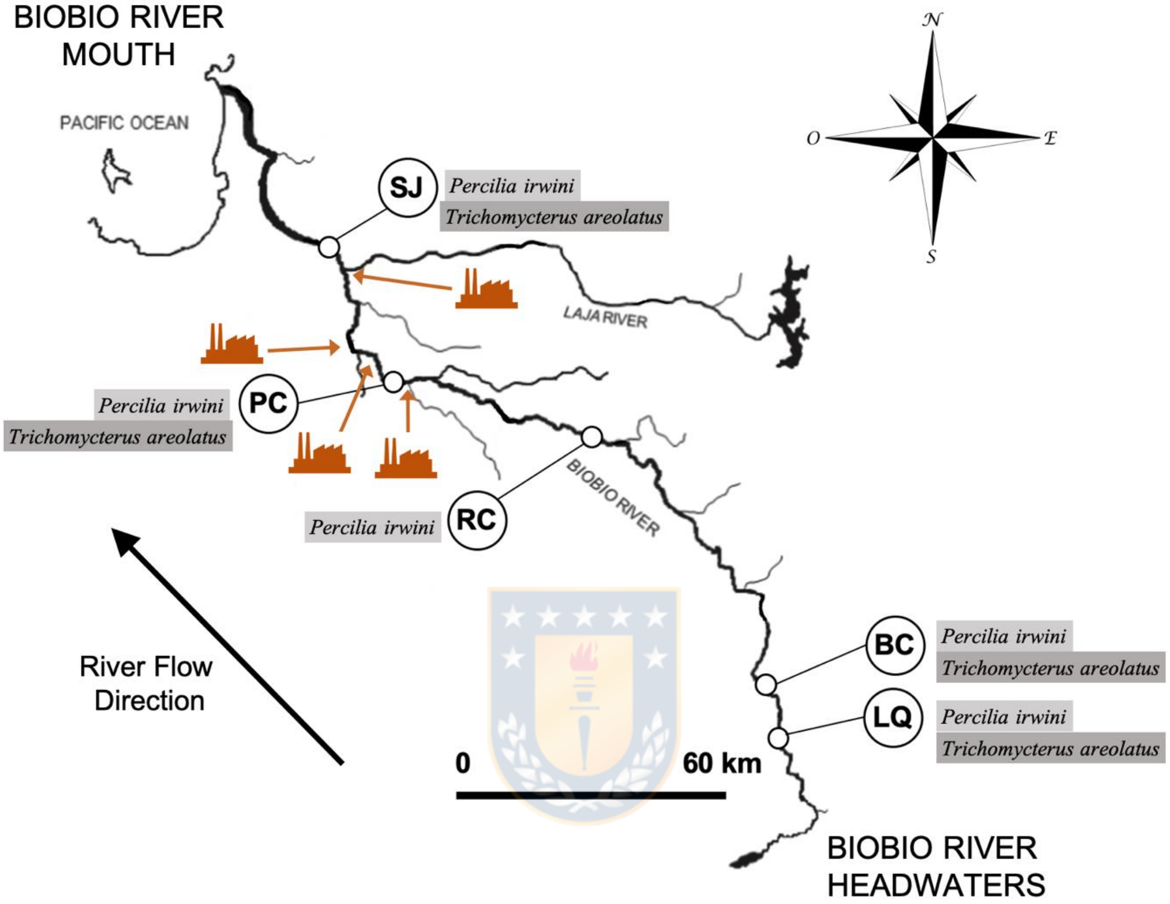

BIOBIO RIVER MOUTH
PACIFIC OCEAN
N
O
E
S
SJ
Percilia irwini
Trichomycterus areolatus
LAJA RIVER
PC
Percilia irwini
Trichomycterus areolatus
RC
Percilia irwini
BIOBIO RIVER
River Flow Direction
BC
Percilia irwini
Trichomycterus areolatus
LQ
Percilia irwini
Trichomycterus areolatus
0
60 km
BIOBIO RIVER HEADWATERS

Figure 2.

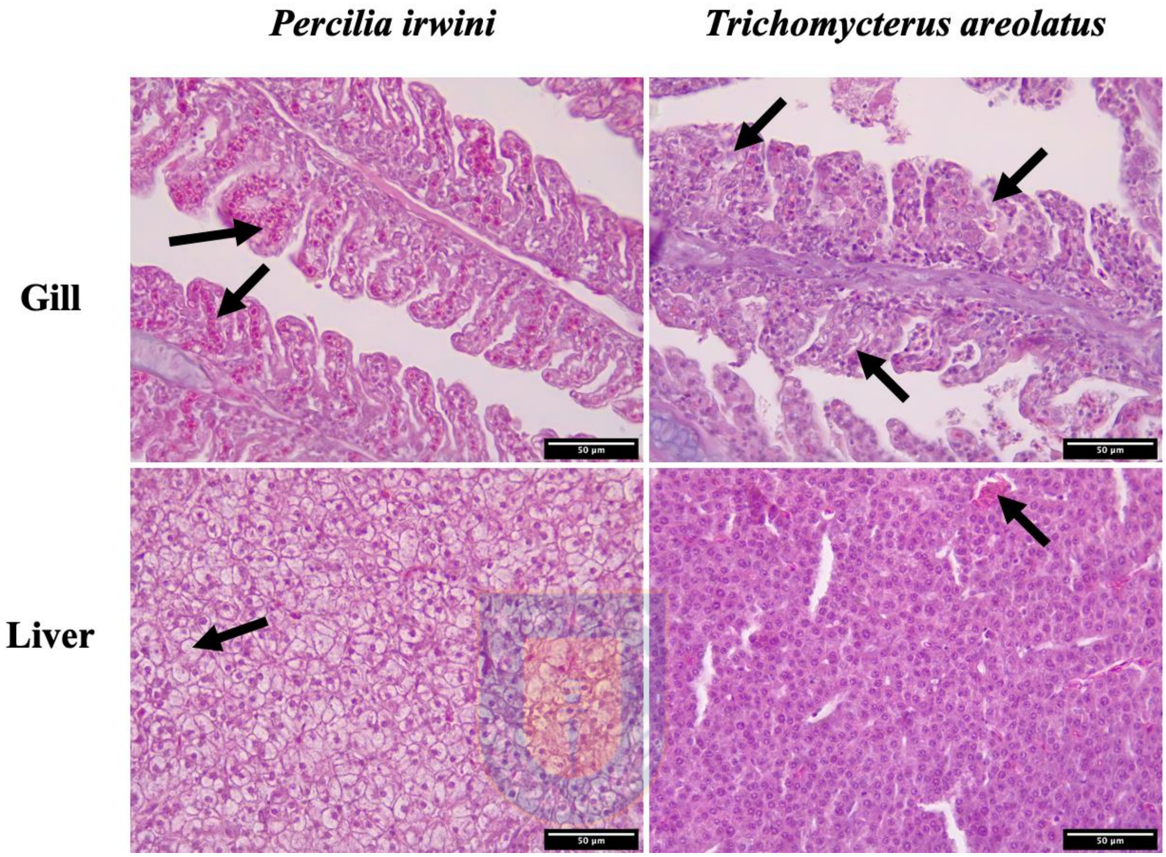

Percilia irwini
Trichomycterus areolatus
Gill
Liver
50 µm
50 µm
50 µm
50 µm

Figure 3.

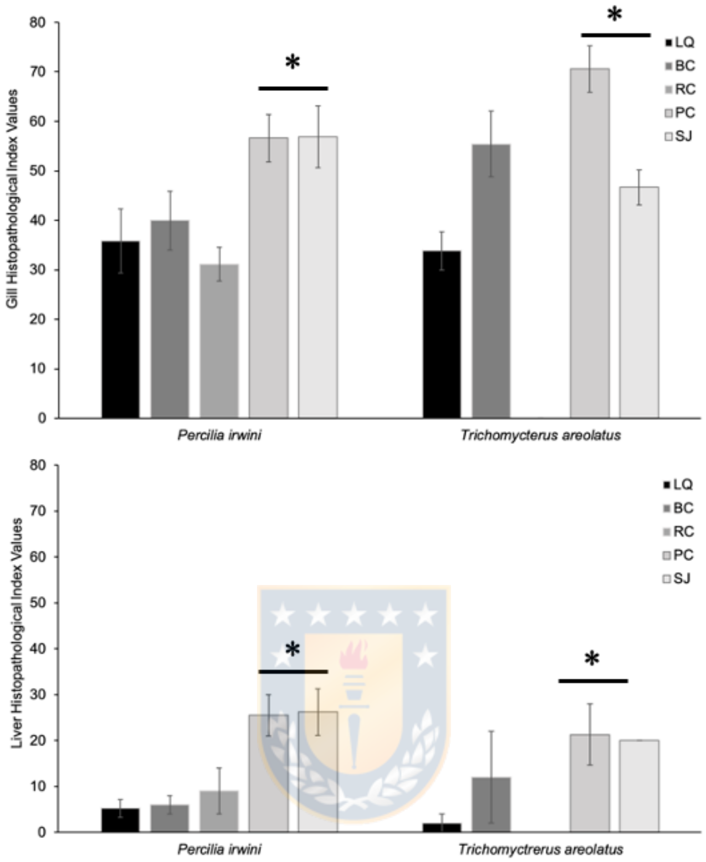

Gill Histopathological Index Values
80
70
60
50
40
30
20
10
0
*
*
■ LQ
■ BC
■ RC
▨ PC
□ SJ
Percilia irwini
Trichomycterus areolatus
Liver Histopathological Index Values
80
70
60
50
40
30
20
10
0
*
*
■ LQ
■ BC
■ RC
▨ PC
□ SJ
Percilia irwini
Trichomyctrerus areolatus

Figure 4.

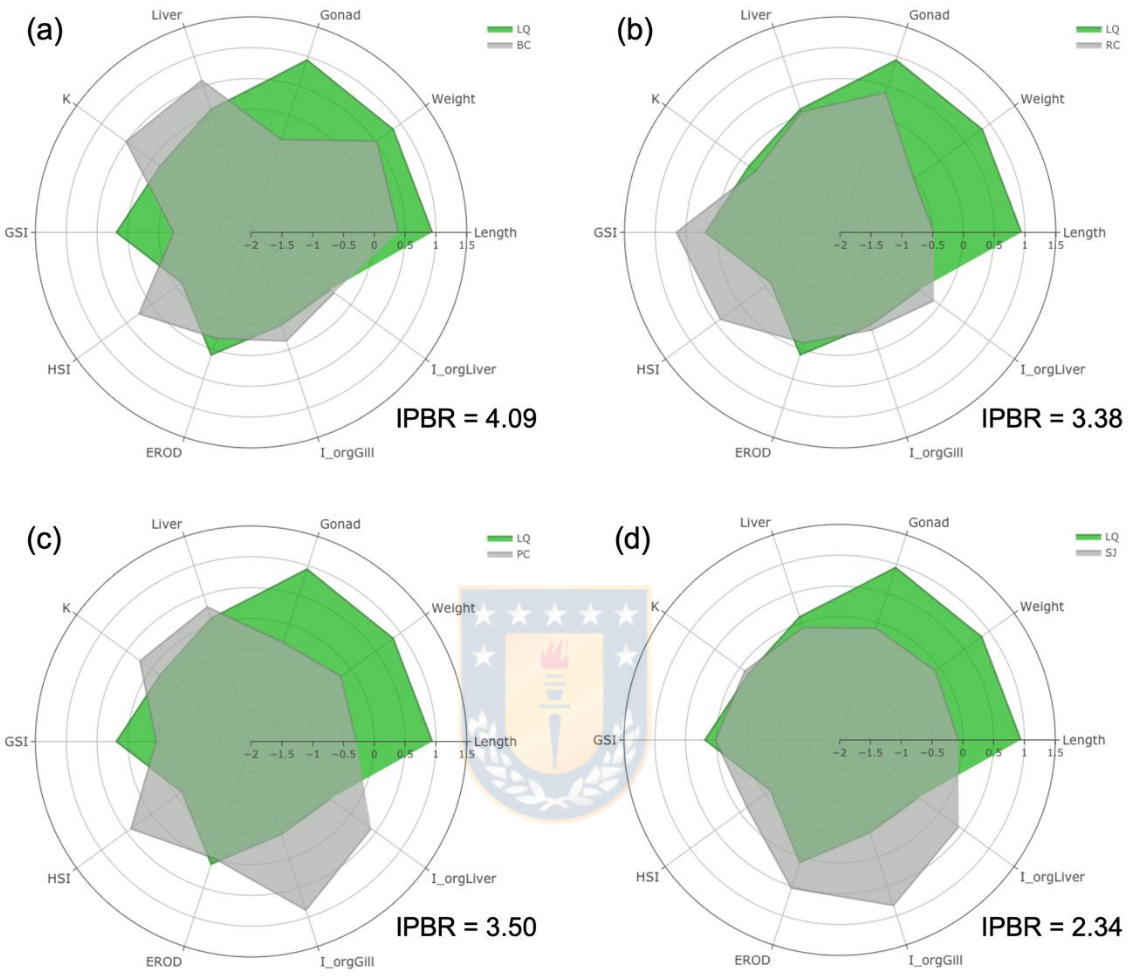

(a)
Liver
Gonad
Weight
Length
I_orgLiver
I_orgGill
EROD
HSI
GSI
K
−2 −1.5 −1 −0.5 0 0.5 1 1.5
LQ
BC
IPBR = 4.09
(b)
Liver
Gonad
Weight
Length
I_orgLiver
I_orgGill
EROD
HSI
GSI
K
−2 −1.5 −1 −0.5 0 0.5 1 1.5
LQ
RC
IPBR = 3.38
(c)
Liver
Gonad
Weight
Length
I_orgLiver
I_orgGill
EROD
HSI
GSI
K
−2 −1.5 −1 −0.5 0 0.5 1 1.5
LQ
PC
IPBR = 3.50
(d)
Liver
Gonad
Weight
Length
I_orgLiver
I_orgGill
EROD
HSI
GSI
K
−2 −1.5 −1 −0.5 0 0.5 1 1.5
LQ
SJ
IPBR = 2.34

Figure 5.

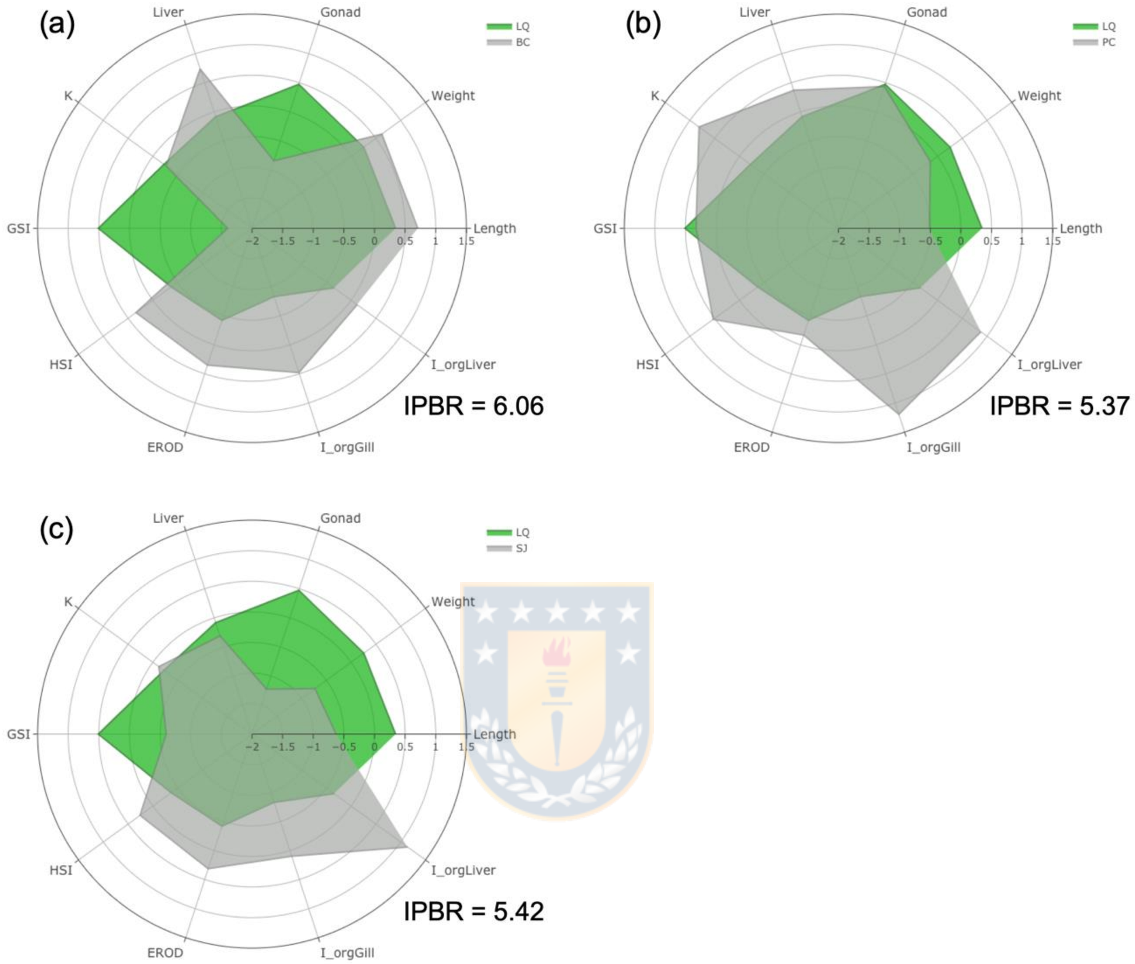
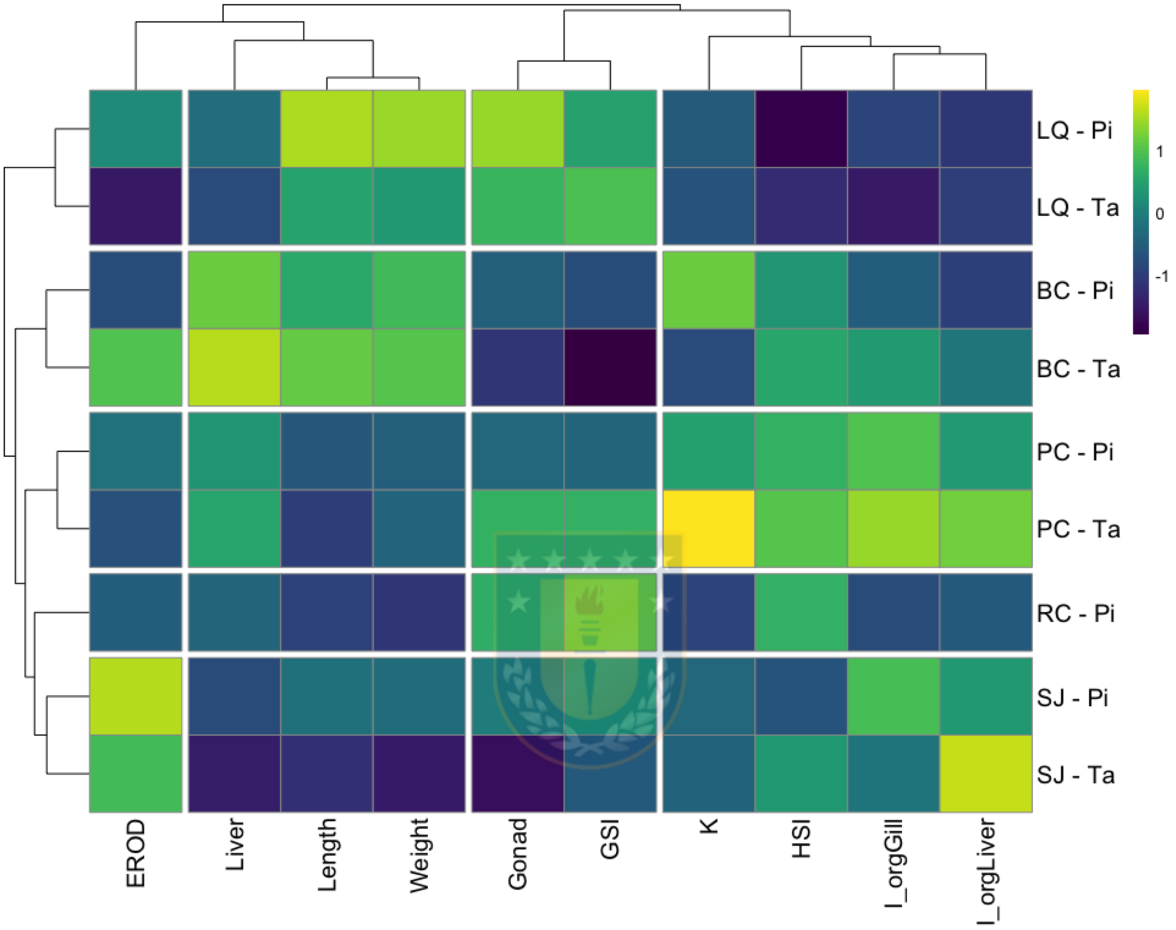

(a)
Liver
Gonad
Weight
Length
I_orgLiver
I_orgGill
EROD
HSI
GSI
K
LQ
BC
−2
−1.5
−1
−0.5
0
0.5
1
1.5
IPBR = 6.06
(b)
LQ
PC
IPBR = 5.37
(c)
LQ
SJ
IPBR = 5.42

Figure 6.


LQ - Pi
LQ - Ta
BC - Pi
BC - Ta
PC - Pi
PC - Ta
RC - Pi
SJ - Pi
SJ - Ta
EROD
Liver
Length
Weight
Gonad
GSI
K
HSI
I_orgGill
I_orgLiver
1
0
-1

CAPÍTULO IV

INTEGRATED PHYSIOLOGICAL BIOMARKERS RESPONSE (IPBR) AS A PROPOSAL FOR THE IMPROVEMENT OF CHILEAN SECONDARY ENVIRONMENTAL QUALITY STANDARDS

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Abstract

The Biobio River water resource is used in many ways, such as drinking water, irrigation, power generation, industrial activities, among others. These anthropogenic pressures caused a deterioration in the quality of the water and generated ecotoxicological impacts. In south-central Chile, in the Biobio river basin, the principal causes of the changes in the landscape have been the opening of agricultural lands and the increase in the forestry activity of exotic species such as *Pinus spp.* and *Eucalyptus spp*., that dominate the landscape. To evaluate the physiological status of native fish inhabiting the Biobío River in South-Central Chile, susceptible to the chemical contamination from different sources, a battery of biochemical and physiological biomarkers was applied to the native fish *Percilia irwini* and *Trichomycterus areolatus* evaluated *in situ*. Fish were caught in the main course of the Biobío river in areas of low, medium, and high anthropic intervention, characterized by different degrees of pollution along the river in autumn and spring season. Physicochemical parameters (pH, Temperature, Conductivity, and TDS) were measured at each sampling site. We propose using an IPBR (Integrated Physiological Biomarker Response) to improve the interpretation between different biomarkers and relate biological responses to the quality standards defined by Chilean environmental regulations. The Integrated Index of Physiological Biomarkers (IPBR) indicated that Puente Coihue and Santa Juana sites have the most detrimental environmental quality for both species. The results indicated a deteriorating condition in the biological parameters of both species in a high impacts anthropic zone.

Keywords: Rivers, Native fish; biomarkers; anthropic impacts; IPBR.

INTRODUCTION

The Biobío River is the third most important basin in Central Chile, with an area of 24.369km^2. The water resource provided by the Biobio River is used in various forms such as drinking water, irrigation, power generation, industrial activities, among others (Parra et al, 2012; Figueroa et al, 2013). This pressure of anthropogenic origin, due to the use of the various ecosystem services, has been continuously expanding since 1990. This pressure has generated a series of ecological and ecotoxicological impacts, such as losing fish diversity, increased nutrient loads and the bioaccumulation of several toxic compounds (Habit et al, 2006a; Oyarzún et al, 2007; Alonso et al, 2017). The replacement of the native forest by forest plantations of *Pinus radiata* and *Eucalyptus spp*. in south-central Chile (Lara et al. 2003, 2010, Echeverría et al. 2007) have consequences as deterioration of ecosystem services as biological diversity (Echeverría et al. 2007, Díaz et al. 2018) (Figure 1). The provision of water in quality and quantity (Oyarzún et al. 2007, Little et al. 2009), the increase in soil erosion, and contrast negative impacts on local communities (Díaz et al. 2018).

Many companies use the Biobio river water resources for industrial processes, which results in the elimination of effluents on their main channel, presenting areas of point and diffuse pollution. Regarding this, the industries that currently generate the greatest impact on this basin are the pulp and paper mill industries, which, due to the improvement in their technologies, have presented an increase in their productivity since 1990, followed by the oil refinery and wastewaters (Orrego et al, 2005a,b; Chiang et al, 2010, 2012, Barra et al, 2021).

The particular geomorphology of Chile has geographic barriers formed by Andes Mountains, the Pacific Ocean and the Atacama desert. It has generated a unique and not very diverse continental ichtyofauna composition, with small body sizes, and high endemism, at the level of species, genus and families (Mann, 1954; Ruiz and Marchant, 2004).

Chilean fish fauna is represented by a reduced number of species 11 families, 17 genera and 46 native species (Arratia et al, 1981; Campos et al, 1993; Valdevenito et al, 1995; Habit et al, 2006b). Native fish fauna in the Biobío River represents a genetic heritage of this biogeographic zone. The alteration of its biotype has led to a potential decrease in their populations, and areas directly affected by mill discharges and urban impact in the Biobío river have been continually associated with changes in native fish abundance, diversity, and reproductive effects (Habit *et al*. 2006a,b; Chiang *et al*. 2011; Orrego *et al*. 2019; Quiroz-Jara et al, 2021).

The use of individual organisms is essential as it allows follow-up studies to develop at both lower biological organizational levels to understand mechanisms (e.g., tissue, physiological, biochemical) as an early response, and at upper organizational levels to understand the ecological relevance (Munkittrick et al, 2000). Biomarkers provide valuable information in field studies and are used to measure a wide range of early physiological responses by exposure to chemical compounds at the biochemical, cellular, and tissue levels (Oliveira *et al*. 2011; Seabra *et al.* 2014; Colin *et al.* 2016; Capela *et al.* 2016). Biomarkers being useful tools for monitoring the biological effects caused by chemical contamination.

The current pattern of species richness, diversity and abundance of fishes in the Biobio river, contrast with the typical pattern of the fish community in riverine systems, where there are an increasing species richness, diversity and abundance from upstream to downstream (Habit et al, 2006a,b; 2007). From this perspective, these authors warns that there is a decrease in population sizes in areas highly intervened by the anthropic action.

Most fish populations in the Biobio river are exposed to a wide variety of man-made chemicals (Alonso et al, 2017; Barra et al, 2021). Often these chemicals are found at concentrations that are not directly toxic to fish. Nevertheless, at sublethal concentrations, exposure can induce harmful effects and potentially impact populations (Habit et al, 2006b). When fish cannot avoid chemical pollutants, certain pollutants activate detoxification mechanisms, and/or individuals may modify their phenotype in response to chemical exposure (phenotypic plasticity) (Hamilton et al, 2016). Understanding exposure effects of man-made chemicals on wildlife populations is the ultimate goal of risk assessment. Considering the antecedents raised, the need to evaluate and detect the environmental impact on aquatic ecosystems has allowed using changes in biochemical, physiological and histological levels in organisms to determine the impacts of exposure to environmental stressors.

The lack of integrated biomarker analysis in rivers has limited the potential use of these tools and the capacity to get early responses. Hence, it is necessary to improve the interpretation between the different multilevel biomarkers used to determine the effects on sentinel fish and relate them to the quality standards defined by Chilean environmental regulations. It is possible to evaluate global changes of the biological responses through an integrated response of physiological biomarkers (IPBR). The IPBR allows relating effect patterns with different mechanisms of toxicological action in specific samples areas and different pollution profiles (Beliaeff *et al*. 2002; Sanchez *et al*. 2013; Murussi *et al*. 2015; Quiroz-Jara et al, 2021). In this way, it is possible to cover specific gaps in the Chilean legislation like Secondary environmental quality standards for Biobio River. Furthermore, effectively comply with environmental regulations established by water quality criteria and conserving and preserving aquatic ecosystems (General Bases of the Environment, Chilean law Nº19300; NSCA 2015).

This study aimed to analyze several biomarkers in the two native fish species *P.irwini* (common name Carmelita) and *T.areolatus* (common name Bagre), along the main course Biobío River in two different seasons (autumn and spring) in order to assess their physiological status in a gradient of anthropic intervention. We propose an IPBR as a tool to compare the physiological status and biological responses in these native fish along a gradient of impacts in the main course of the Biobio river.

2. MATERIAL AND METHODS

2.1 Description of Study Area

The study area is located in the Biobio River Basin, South-Central Chile. Biobio basin has an area of 24.369 km^2, and a length of 380 km (Figure 1). The Biobio river belongs to a hydrographic basin of Andean origin. Presents a shape defining two major sectors: 1) Rhithron zone (Source) with a high slope, volcanic soils, vegetation cover formed by native forest, high rainfall and snowfall, high solar radiation, and a river network with more than 5000 first-order rivers, and 2) low-slope Potamon zone (lower course), without snowfall and predominantly a vegetation cover of introduced species (pine and eucalyptus).

Socio-economically, the Biobio River system along with its tributaries and the lakes in its catchment area is one of the most important river systems in Chile (Karrash *et al*. 2006). The Biobio River is also one of the most severely polluted bodies of water in Chile (Karrash e*t al*. 2006). All physicochemical parameters (pH, Temperature, Conductivity, Total Dissolved Solids) were measured immediately after sampling using WTW meters (Hanna HI 9829).

The fish samples were taken on March and September 2017 at five locations along the river from its source to the mouth (36º42´ - 38º49´) to assess and compare the physiological status and biological alteration of the wild fishes (Figure 1):

Reference Area (upstream of the Biobio River): Site 1, Lonquimay (LQ) and Site 2, Balsa Caracoles (BC). These sampling sites present low anthropic intervention, surrounded by native forest vegetation.

Middle Impact Area: Site 3, Rucalhue (RC), located downstream of the Angostura hydroelectric plant, is the third hydroelectric power station present in the Biobio river. This zone has relicts of native forest and forest plantations.

High Impact Area: Site 4, Puente Coihue (PC) is located downstream of urban effluents and pulp and paper industries. PC has a high predominance of forest plantations associated with the river sector associated with some agricultural activities. Site 5, Santa Juana (SJ) presents high anthropic intervention and corresponds to the convergence sector of the several activities present upstream of the Biobio River.

2.2. Fish Collection

2.2.1 Ethical Statement.

The study protocol was reviewed and approved by the Ethics Committee of the Universidad de Concepción, Chile (Aut. No. 01-2016).

The health status of wild fish was assessed during autumn and spring seasons (March and September 2017), during post and pre spawning season. Two species of fish (from a total of 18 native species) were collected considering their abundance in the study areas, small size (less than 10 cm), and relatively limited mobility: *Percilia irwini*, a benthic-pelagic species inhabiting relatively shallow waters (n=110) and *Trichomycterus areolatus* a benthic species (n=59).

The fish sampling was carried out following the longitudinal gradient of the Biobio River, from the Source (Rhithron zone) to the Lower course (Potamon zone) (Figure

2). Due to the lack of secondary sexual characteristics for *P. irwini and T.areolatus*, a maximum of 10 adult fish were selected at each site, each season, with sizes greater than 35 mm based on previous studies (Chiang *et al*. 2012).

Fish were captured at sites with similar microhabitat characteristics using a backpack electric fishing equipment (Halltech Electrofisher, Canada) and a blocking net (6 mm mesh opening) were used in riffles (0.2-0.3 m/s, 0.2-0.4m depth) with a bed of boulders and gravel (15cm diameter) and shallow riffles (0.1 - 0.2m/s, 0.1 - 0.2 m depth) with a bed of boulders (15cm diameter) and rock. Size, sex, body length, and body weight was immediately measured. Dissected liver tissues were stored in liquid nitrogen and then transported to the Biomarkers Laboratory of EULA-Center (University of Concepción) for further analysis. Physiological index Condition Factor (K=[Total Weight(g)/Total Lenght^3]*100); Gonadosomatic Index (GSI= [gonad weight(g)/ Total Weight(g)]*100); Hepatosomatic Index (HSI=[Liver weight(g)/Total Weight]*100) were calculated.

2.5. EROD activity

EROD activity was analysed according to Lubert *et al*. (1985) standardized protocol in the floating postmitochondrial supernatant (fraction S9) obtained from livers homogenized in a sucrose buffer (0.1 M, pH 7.5) and centrifuged at 9,000g for 20 min at 48C. Fluorimetric analyses were performed using an LS 50B spectrofluorometer (PerkinElmer) for 5 min at 25ºC; using reduced NAPH as the electron donor. Protein analysis was performed in a microplate reader (Baush & Lomb, DNM, 9602G) using a Biuret microplate method that uses bovine serum albumin (Sigma-Aldrich) as a reference material. The EROD activity was expressed as pmol / min / mg protein.

2.6. Integrated Physiological Biomarkers Response (IPBR)

The result of the biomarkers were then incorporated into the mathematical model Integrated Biomarker Response (Bealieff and Burgeot 2002, Sanchez *et al*. 2013). The index was calculated based on the log-transformation to decrease its variance and be represented as polar plots. For index calculation, the base value obtained for each biomarker at the Lonquimay site (LQ), was used as reference value (T_0). The data for each biomarker and fish between sampling sites was log-transformed (Y_i), and the overall mean (μ) and SD (s) were further calculated. Subsequently, the Y_i values obtained for each biomarker standardized by the formula: $Z_i = (Y_i - \mu) / s$ and the difference between Z_i and T_0 was used to define the biomarker deviation index (A). Finally, to obtain an integrated physiological biomarker response (IPBR), the absolute value of A (|A|) for each biomarker at each sampling site was added. As physiological responses used, values below zero (0) indicate a decrease in the physiological conditions of the fish, considered as a decrease in biological responses.

2.7. Statistical Analyses

Data analysis were performed by RStudio, R Software Package (Core Team, 2017). Data examined by exploratory data analysis and continuous data were examined for normal distribution (Shapiro-Wilks test) and log-transformed to avoid non-normality. One-way ANOVA and Tukey were used to evaluate significant statistical differences between sampling sites, and differences confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$). The data were compared with the reference site Lonquimay (LQ) and between the downstream sites and each species studied. Multiple linear regression (RStudio Team (2020)) was used to determine the variables that directly influence the histopathological effects. All the assumptions were previously verified to validate the analysis. The Polar Chart was performed by package “plotly” version 4.9.0 and Heatmap by package “pheatmap”, “stats” and “ggplot2” in RStudio version 3.5.0.

3. RESULTS

3.1. Physicochemical Parameters

The physicochemical parameters are listed in Table 1. In The Spring and autumn seasons, from the upstream sites Lonquimay and Balsa Caracoles (Reference Area), the conductivity had progressively risen from Rucalhue to Puente Coihue and finally to Santa Juana. The pH decreased from 7.04 to 6.91 in autumn at Rucalhue and increased in Puente Coihue (7.56) but was somewhat decreased to 6.35 at Santa Juana, possibly by the Laja river, the main tributary of the Biobío river at that site. The pH recorded the highest value at Puente Coihue, subject to the most significant impact of pulp and paper mill effluents and the last third of the Biobio river. The Temperature parameters also change from initial values of around 16ºC downstream at Puente Coihue and Santa Juana (autumn: 18.8 - 20.8ºC, spring: 11.8 – 16.1ºC). These values were high and highest respect to the reference sites Lonquimay and Balsa Caracoles. The Total Dissolved Solids (TDS) increased in autumn and spring, downstream from Balsa Caracoles and Lonquimay to Puente Coihue and Santa Juana sites (Table 1).

3.2. Integrated Physiological Biomarkers Response (IPBR)

The biomarkers analyzed in Percilia show significant variation along the river. It is observed that the weight and length biomarkers decrease significantly towards the lower third of the river, decreasing significantly to the LQ reference site. The decrease of these biomarkers befalls in both seasons (Autumn and Spring) (Figure 3). The weight of the gonad also shows a decrease along the river to the LQ reference site. The variable that significantly influences PC and SJ corresponds to EROD activity, which presents an induction towards these stations, associated with an increase in the variable HSI. The increase in EROD activity is significantly higher in the Spring season (Figure 3).

IPBR values were calculated in both wild fish for each sampling site in each autumn and spring season, as shown in Figure 5. IPBR values for P.irwini decrease downstream from the Lonquimay site to Santa Juana. In autumn, the reference site LQ exhibits a higher IPBR value (3.9), followed by BC (3.6), RC (2.9), PC (2.2), and SJ (1.1). In spring, the IPBR values correspond to LQ (3.9), BC (3.6), PC (4.4), and SJ (4.0). The variables that most influenced these results were length, weight, gonad weight, GSI, and HSI. In PC and SJ, the variables that most influenced the IPBR values were length, weight, gonad weight, Liver, K, GSI, HSI, EROD. Compared with the upstream sites LQ and BC, the variables length, weight, and gonads, GSI has a decrease in the values of IPBR value to Puente Coihue and Santa Juana. In contrast, the variables weight of the liver, K, HSI, EROD has an increase in these values (Figure 3).

Biomarkers analyzed in Trichomycterus show a significant variation towards the lower third of the Biobio river. As in Percilia, during the autumn season, it is observed that the variables that significantly influence the physiological state of Trichomycterus correspond to length and weight (Figure 4). It is observed that the weight of the gonad decreases significantly at the SJ sampling site to the LQ reference site. The EROD variable shows a significant induction towards the lower third. As in Percilia, there is evidence of induction of EROD activity, associated with an increase in the physiological parameter HSI. The induction of EROD Activity is significant both in the Autumn and Spring season (Figure 4).

IPBR values for T. areolatus decrease at the LQ site and increase downstream towards the SJ sampling site (Figure 5). In autumn, the reference site LQ exhibits an IPBR value of (2.7), BC (5.5), PC (3.3), and SJ (4.2). In the spring season, the IPBR values correspond to LQ (2.7), PC (4.8), and SJ (5.4). The variables that most influenced these results at the PC site were the length, weight, and weight of the gonads. In SJ, the variables that most influenced the IPBR values were length, weight, gonad weight, liver, K, GSI, HSI, EROD, and liver. Compared with the upstream sites LQ and BC, the variables length, weight, weight of the gonad, GSI has a marked decrease in the values from IPBR to SJ. The variables EROD activity and liver weight presented high values in PC and SJ for both species.

The IPBR values for both species were evaluated by hierarchical grouping to establish relationships between biomarkers, sampling sites, and native fish species (Figure 6). The Euclidean distance-based heatmap shows a markedly high relationship between both species and each sampling site, from upstream sites LQ and BC to downstream PC and finally SJ. Integrated response confers a high length-to-weight ratio, GSI and gonad weight, HSI, and liver weight. On the other hand, the biomarker EROD activity exhibits a significant relationship between native fish species in the downstream river (PC and SJ) and a lower correlation in the upstream river at the LQ and BC reference sites.

4. DISCUSSION

The IPBR has been proposed as a helpful tool to explore and explain different biological responses in native fish along the Biobio river. This wild fish study exhibit responses addressed by biomarkers related to exposure to multiple environmental stressors that converge towards the lower third of the Biobio River.

Physicochemical effects include the increase in conductivity between Lonquimay and Santa Juana, which is likely to be mainly due to the high release of industrial effluents in the middle of the Biobio river, as reported by other authors (Karrash *et al*. 2006). High urban, agricultural and industrial activity along the Biobio river, also explain the high levels of TDS, which in turn indicate a decrease in Oxygen Solubility (DO) levels. These results are associated with the high intervention of the basin, which implies an increase in agricultural and forestry activity. These high impacts involves a high lateral surface runoff that increases suspended solids, mainly in the lower third of the Biobio River. The increase in TDS can potentially alter the gill structure of the organisms and cause alteration in gas exchange, which involves an alteration of the physiological state of the organisms under study (Colin et al, 2016; Quiroz-Jara et al, 2021).

The principal effect observed as a result of exposure to a complex chemical mixture in these species at the Biobio river occurred at the physiological level, with responses during post and pre spawning period. Both species showed a reduction in length and weight from Lonquimay to Santa Juana. Also, both species show an increase in their gonadosomatic and hepatosomatic index.

The physiological responses found for the both species are in agreement with the results of others authors (Habit *el al*. 2006a; Orrego *et al*. 2005a,b, 2009; Inzunza *et al*. 2006; Chiang *et al*. 2010; Quiroz-Jara et al. 2021), who indicated reduction of the length and weight, stimulation of the reproductive system and induction of the EROD activity in areas of greater anthropic impacts (Barra et al, 2021). This information agrees with our results, where EROD activity is induced downstream from the LQ Reference site in both sampling periods in both species.

Studies in the Biobío river (Habit *et al*. 2006a,b, Orrego *et al*. 2005a,b, 2019, Habit *et al*. 2019) documented obvious changes during the last decades in the fish communities in the middle section of the Biobio river. These changes in the fish community are attributed to the increase in human activities throughout the basin, including wastewater and pulp mill discharges (Orrego *et al*. 2005a, 2005b, 2006, Chiang *et al*. 2010); however, no cause-effect relationships were demonstrated. In addition, it is necessary to associate the biological responses obtained with the variables that exceed the levels established by the Chilean environmental regulations (NSCA D.S Nº9 / 2015 MMA).

Although the evaluation of IPBR indicated the occurrence of physiological effects associated with a spatial gradient, similar to the pattern found in molecular (EROD) and individual (length, weight, GSI, HSI) biomarkers measured in both fish species in two seasons, no direct cause-effect relationship was established. Significant differences between species were related to their habitat, pelagic-demersal for *P.irwini*, and benthic for *T.areolatus*. Similar results were recently informed by Orrego *et al*. (2019) regarding molecular and individual biomarkers associated with endocrine-disrupting substances in pulp mills effluent discharge accumulated through SPMDs deployed in different areas at the Biobío river. However, according to different authors (Munkitrich *et al*. 2000, Chiang *et al*. 2010, 2014, Delfino *et al*. 2016, Barra et al, 2021), the interpretation of fieldwork results is always associated with high variability due to the presence of multiple external factors, which can influence the analyzed variables, so its results should be considered with caution.

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The observed pattern of both fish studied in the Biobío river was influenced by the anthropogenic disturbance caused by xenobiotic chemical compounds of diverse nature, suggesting a cumulative spatial effect due to the environmental degradation at the lower third of the Biobío river (Quiroz-Jara et al, 2021; Barra et al, 2021). The

IPBR showed a decrease of the biological status in both native fish in two different seasons, and these manifest changes are related to the high anthropogenic activity from Lonquimay to Puente Coihue and Santa Juana sites.

5. CONCLUSION

The IPBR index developed in native fish in the Biobío River accounts for the variation of the physiological state of native fish species from less impact sites to mostly impacted sites. Our spatial and temporal investigation was able to detect physiological changes in wild fish in the Biobio river. These changes were associated with the high anthropic impacts at the lower third of the river and complement several studies of Environmental Effects Monitoring-approach as a tool for evaluation under the different environmental condition in freshwater systems in Chile.

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Figure Captions

Figure 1. Biobio River Basin showing land use change between A) 1998 and B) 2008.

Figure 2. Biobio River Basin showing sites where *Percilia irwini* and *Trichomycterus areolatus* were collected, upstream areas (Reference sample sites: LQ-BC), middle Area (RC), downstream Area (PC-SJ). Red dots indicate the leading urban areas adjacent to the main course of the Biobío River. Icons (brown) indicate the primary industrial sources contiguous to the main course of the Biobio river.

Figure 3. Polar plots from IPBR values for *Percilia irwini* for each collected area in the Biobío river in both seasons (Autumn and Spring). BC - Balsa Caracoles, RC - Rucalhue, PC – Puente Coihue, SJ – Santa Juana. Biomarkers representation in relation to the reference site LQ – Lonquimay.

Figure 4. Polar plots from IPBR values for *Trichomycterus areolatus* for each area in the Biobío river in both seasons (Autumn and Spring). (a) BC - Balsa Caracoles, PC – Puente Coihue, SJ – Santa Juana. Biomarkers representation in relation to the reference site LQ – Lonquimay.

Figure 5. IPBR values for *Percilia irwini* and *Trichomycterus areolatus* for each area in the Biobío river in both seasons (Autumn and Spring). BC - Balsa Caracoles, RC – Rucalhue, PC – Puente Coihue, SJ – Santa Juana. Biomarkers representation in relation to the reference site LQ – Lonquimay.

Figure 6. Hierarchical clustering for the IPBR values in *Percilia irwini* (Pi) and *Trichomycterus areolatus* (Ta) showing the relationship between biomarkers sites along the Biobío river LQ, BC, RC, PC, SJ, and among seasons (autumn and spring).

Table 1. Physico-chemical parameters in Biobío river, Chile, measured at the time of fish collection in March (autumn) and September (spring) 2017.

Parameters	**LQ**		**BC**		**RC**		**PC**		**SJ**	
	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring
pH	7.04	7.6	7.06	-	6.91	7.7	7.56	7.8	6.35	7.6
Temperature (ºC)	18.7	17.7	16.1	-	17.4	11.1	18.8	11.8	20.8	16.1
Conductivity (μS cm^{-1})	72	36.6	70.2	-	102.6	28.7	108.8	31.5	156.8	41.9
TDS (mg/L)	36	17.9	35.1	-	51.2	14.3	54.4	18.2	77.9	21

Note. LQ – Lonquimay; BC – Balsa Caracoles; RC - Rucalhue; PC – Puente Coihue; SJ – Santa Juana

Table 2. Variables above the levels established by NSCA-BB (D.S. No 9/2015 MMA), for the main course in two consecutive years (2017-2018).

Variable	Unidad	DS 9	BB0	BB3	BB7	BB8
Total Aluminiun	mg/L	Average	0.15	0.08	0.2	0.38
Ammonium	mg N/L	Percentile 85	0.02	0.02	0.02	0.02
AOX	mg/L	Percentile 85	0.01	0.01	0.03	0.02
Chloride	mg/L	Percentile 85	5.9	3.6	3.9	4.1
Fecal Coliforms	NMP/100mL	Percentile 85	4	11	5832	173
Conductivity	mS/cm	Percentile 85	85.9	103	109.1	108.6
DBO5	mg/L	Percentile 85	1.7	1.2	1.3	1.4
DQO	mg/L	Percentile 85	12.3	8.3	9.3	10
Total Phosphorus	mg/L	Average	0.09	0.02	0.04	0.05
Total Iron	mg/L	Average	0.1	0.1	0.3	0.4
Phenol Index	mg/L	Percentile 85	0.002	0.002	0.002	0.002
Nitrate	mg N/L	Average	0.04	0.18	0.17	0.23
Nitrite	mg N/L	Average	0.005	0.005	0.005	0.005
Total Nitrogen	mg/L	Average	0.1	0.2	0.3	0.3
Orthophosphate	mg/L	Average	0.03	0.03	0.03	0.03
Disolved Oxygen	mg/L	Percentile 15	10.3	10	9.3	8.8
pH		Percentile 15	6.8	7	7.2	7.2
TDS	mg/L	Average	3.6	2.2	4	6.4
Sulfates	mg/L	Percentile 85	8.6	7.7	9.1	9.9

*In red variables that consecutively exceed the levels established by Chilean D.S Nº 9/2015 MMA. BB0: Upstream Pangue reservoir; BB3: Puente Coihue (PC); BB7: Between Puente Coihue (PC) and Santa Juana (SJ); BB8: Santa Juana (SJ). EULA, 2020.

Figure 1.

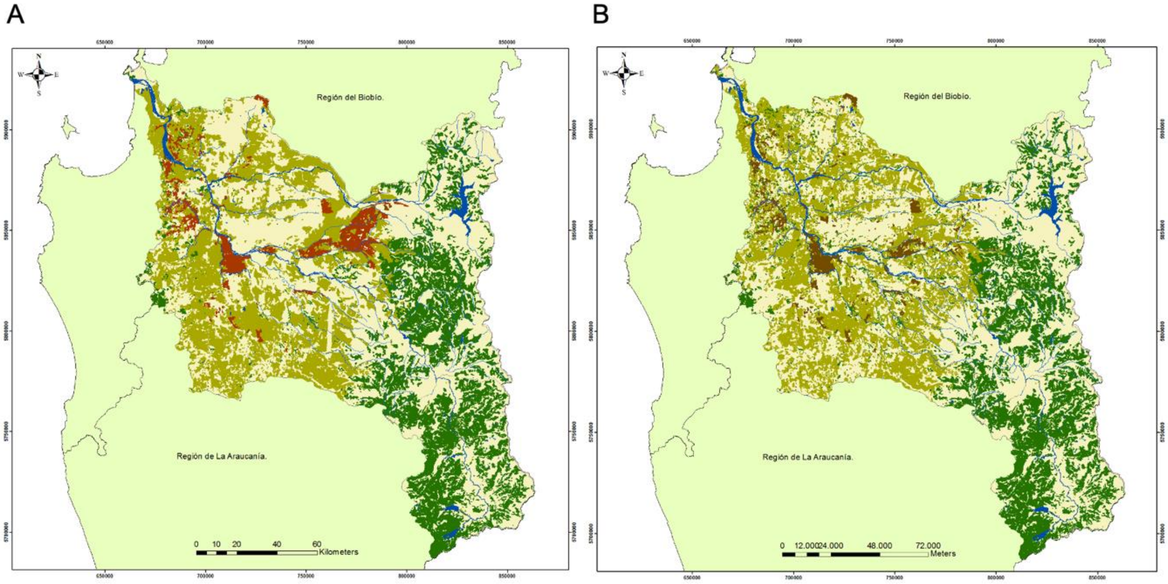

A
B
N
W
E
S
Región del Biobío.
Región de La Araucanía.
650000
700000
750000
800000
850000
5900000
5850000
5800000
5750000
5700000
0 10 20 40 60 Kilometers
0 12.000 24.000 48.000 72.000 Meters

Figure 2.

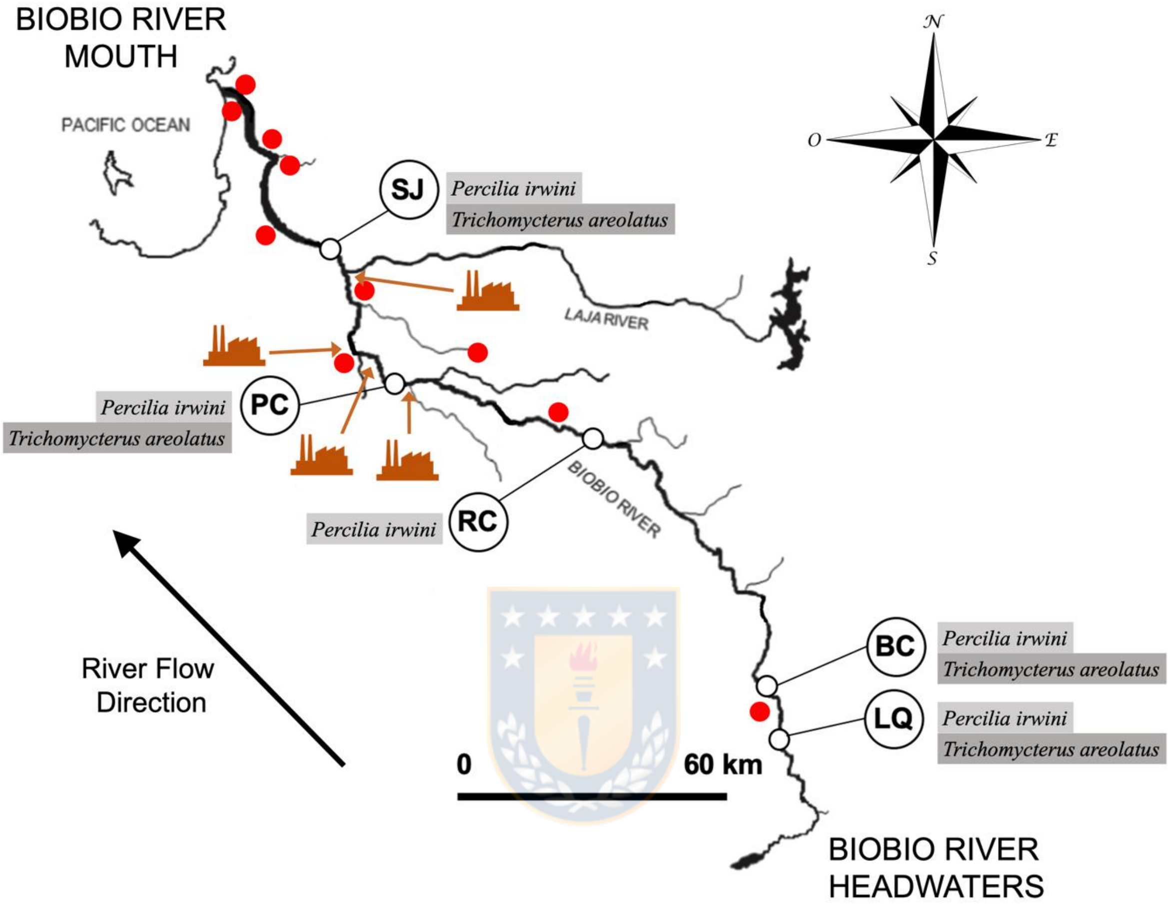
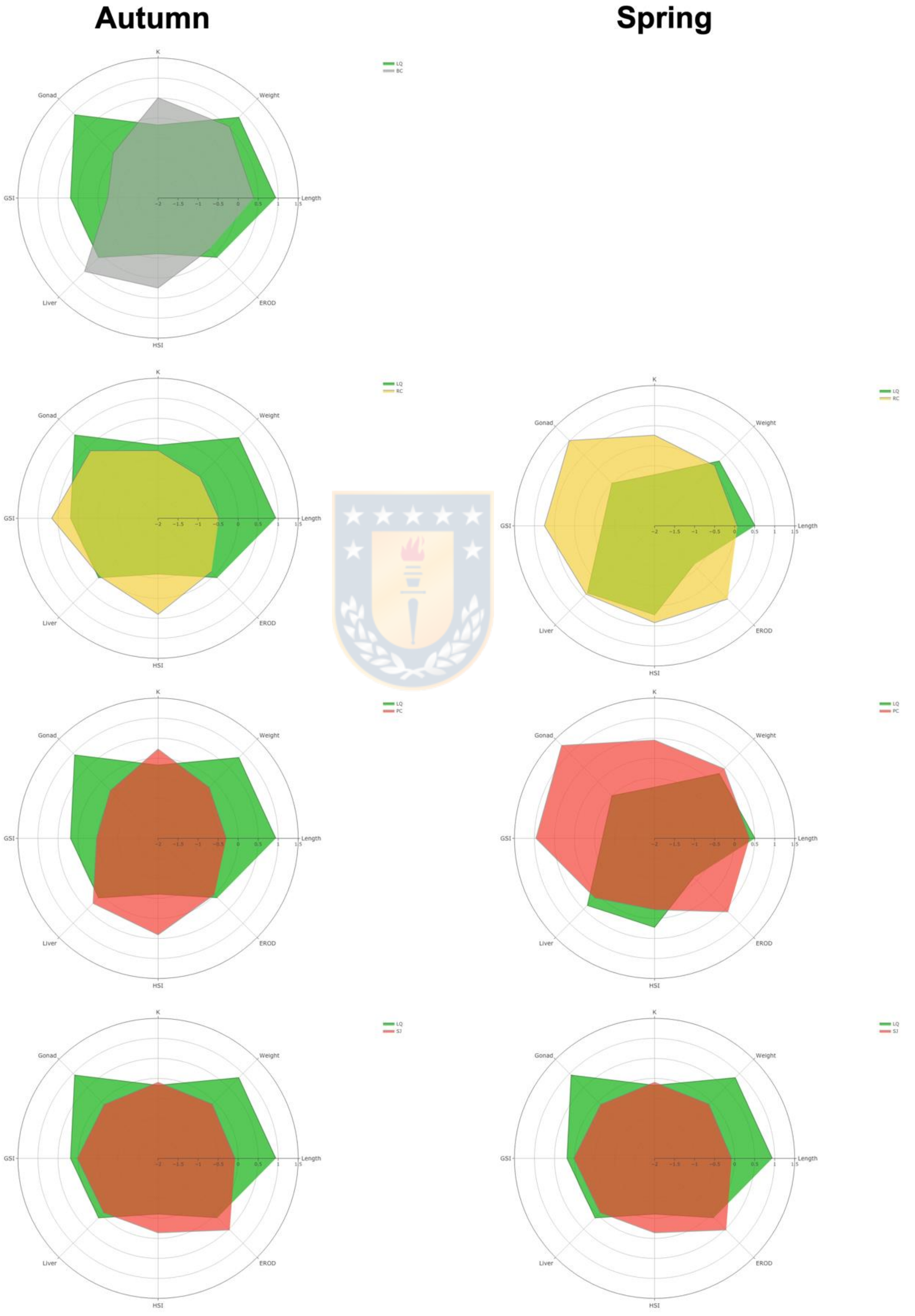
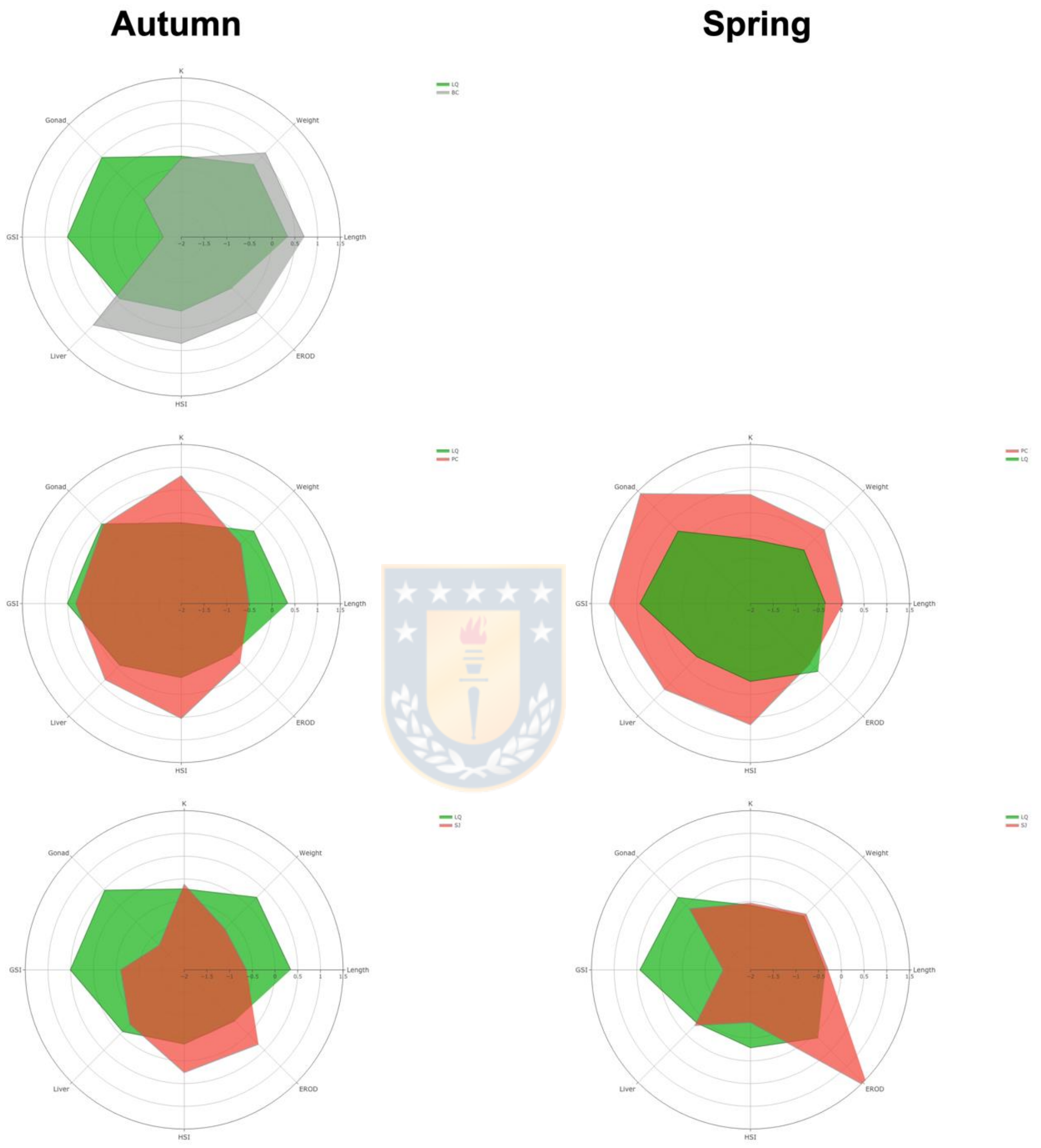

BIOBIO RIVER MOUTH
PACIFIC OCEAN
N
O
E
S
SJ
Percilia irwini
Trichomycterus areolatus
LAJA RIVER
PC
Percilia irwini
Trichomycterus areolatus
RC
Percilia irwini
BIOBIO RIVER
BC
Percilia irwini
Trichomycterus areolatus
LQ
Percilia irwini
Trichomycterus areolatus
River Flow Direction
0
60 km
BIOBIO RIVER HEADWATERS

Figure 3.



Autumn
Spring
K
Weight
Length
EROD
HSI
Liver
GSI
Gonad
−2
−1.5
−1
−0.5
0
0.5
1
1.5
LQ
BC
LQ
RC
LQ
RC
LQ
PC
LQ
PC
LQ
SJ
LQ
SJ

Figure 4.


Autumn
Spring
LQ
BC
LQ
PC
PC
LQ
LQ
SJ
LQ
SJ
K
Weight
Length
EROD
HSI
Liver
GSI
Gonad
-2
-1.5
-1
-0.5
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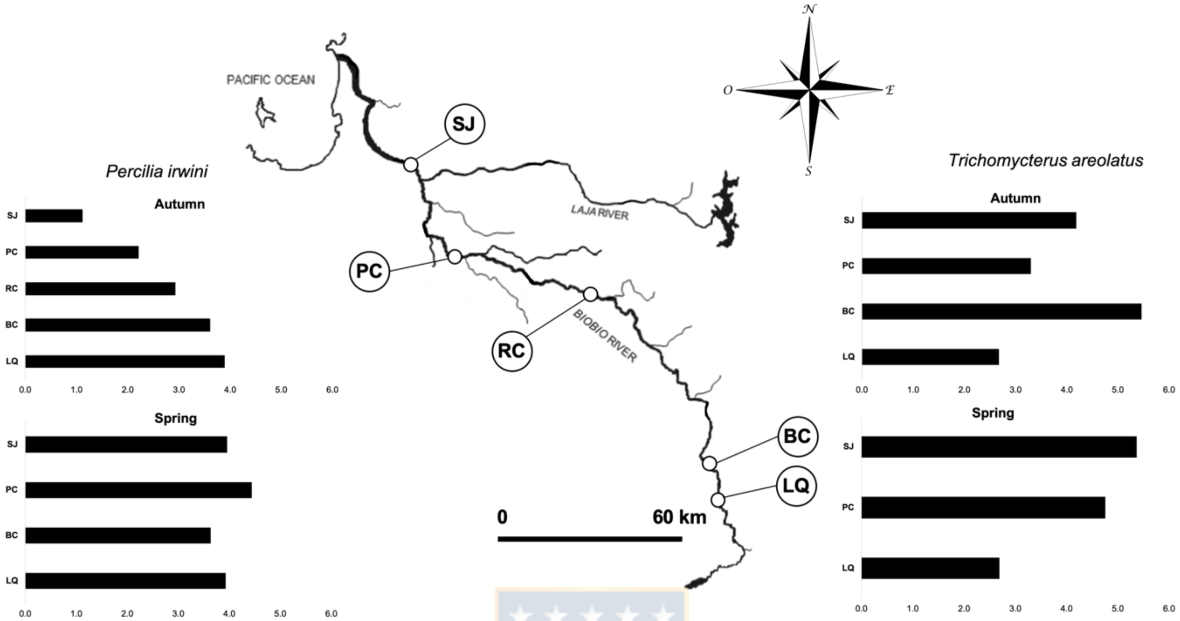
1
1.5

Figure 5.


N
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PACIFIC OCEAN
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RC
BC
LQ
LAJA RIVER
BIOBIO RIVER
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60 km
Percilia irwini
Autumn
SJ
PC
RC
BC
LQ
0.0
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4.0
5.0
6.0
Spring
SJ
PC
BC
LQ
0.0
1.0
2.0
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5.0
6.0
Trichomycterus areolatus
Autumn
SJ
PC
BC
LQ
0.0
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Spring
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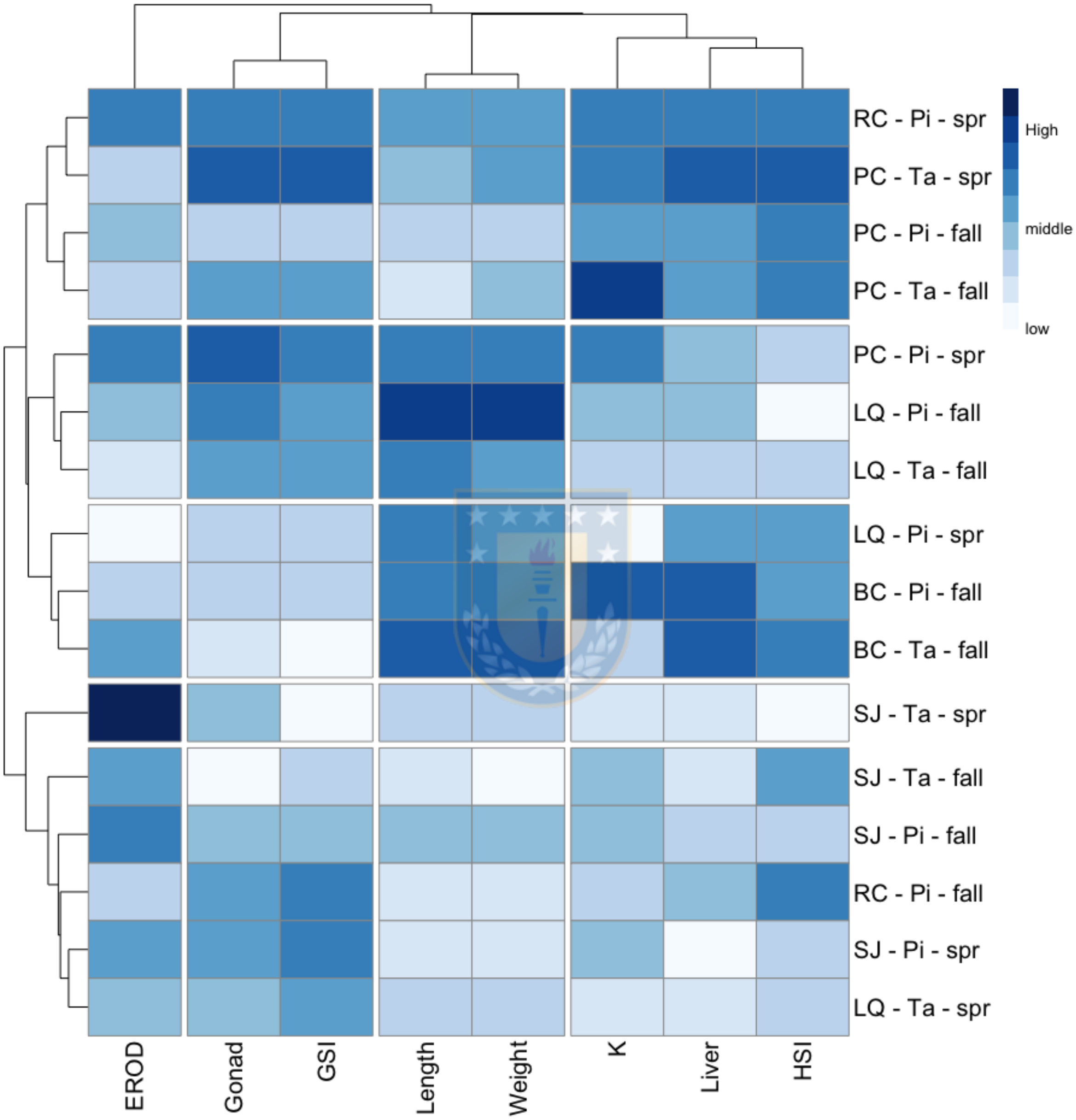
Figure 6.


RC - Pi - spr
PC - Ta - spr
PC - Pi - fall
PC - Ta - fall
PC - Pi - spr
LQ - Pi - fall
LQ - Ta - fall
LQ - Pi - spr
BC - Pi - fall
BC - Ta - fall
SJ - Ta - spr
SJ - Ta - fall
SJ - Pi - fall
RC - Pi - fall
SJ - Pi - spr
LQ - Ta - spr
EROD
Gonad
GSI
Length
Weight
K
Liver
HSI
High
middle
low

CAPÍTULO V

DISCUSIÓN GENERAL

La estrategia utilizada en el desarrollo de esta tesis mediante el uso de bioindicadores (*Percilia irwini* y *Trichomycterus areolatus*), y una batería de biomarcadores, fue diseñada con el propósito de poner a prueba nuestra hipótesis de trabajo, que plantea la necesidad de comprender cómo la mezcla química compleja presente en el río Biobío, dado por el alto impacto antropogénico (urbano, agrícola e industrial), y que convergen hacia el tercio inferior de éste río, generan alteraciones fisiológicas en los organismos analizados. Así, hemos logrado establecer que los organismos presentes en zonas de alto impacto antropogénico, presentan respuestas biológicas significativas, respecto a los organismos que habitan en zonas de menor intervención.

Basados en el concepto de Biomarcador, propuesto por Depledge (1995), se evaluaron múltiples respuestas a distintos niveles de organización biológica, desde el nivel molecular y fisiológico, hasta el nivel reproductivo en peces nativos, respuestas que evidencian efectos biológicos adaptativos, producto de la alta intervención presente a lo largo de la longitudinal del río Biobío, y modificaciones que corresponden a la diferente biología de las dos especies bioindicadoras seleccionadas.

El río Biobío recibe en forma continua la entrada de agentes xenobióticos, por eliminación de efluentes industriales cuyas descargas son tratadas y liberadas directamente al cauce principal, según especifica el Decreto Supremo Nº90, además de los efluentes urbanos (como plantas de tratamientos de aguas servidas) con una alta liberación de compuestos emergentes; y la apertura de tierras agrícolas y aumento en la actividad forestal de especies exóticas como *Pinus spp*. y *Eucalyptus spp*. las cuales dominan el paisaje, generan patrones de cambio en la cobertura de uso de suelo, modificando el régimen de caudal del río, través de aumento de escorrentía lateral superficial, debido a la alta erosión por deforestación de bosque nativo (Little et al, 2009).

Estudios previos realizados en el río Biobío han detectado alteraciones a nivel reproductivo, neuronal, conductual, alimenticio y metabólico. Ensayos ecotoxicológicos, han demostrado que individuos juveniles de *Oncorhynchus mykiss* (trucha arcoiris), expuestos a sedimentos provenientes de zonas de descargas de industrias de celulosa, han tenido respuestas como la inducción de la madurez gonadal, es decir, presencia de oocitos vitelogénicos en organismos juveniles (Orrego et al, 2005).

Numerosos contaminantes emergentes, como productos farmacéuticos utilizados en medicina humana y veterinaria, incluidos medicamentos antiinflamatorios no esteroideos, analgésicos, antibióticos, reguladores de lípidos, hormonas esteroides y fungicidas, han sido detectados en altas concentraciones en ambientes acuáticos chilenos (Chiang, et al, 2012; Cárdenas-Soraca et al, 2020; Barra et al, 2021).

Estos productos químicos y productos de degradación dentro de estas mezclas pueden ser responsables del aumento de la actividad EROD, y trastornos reproductivos, como el mayor grado de maduración gonadal. Por otra parte, como se expone en los Capítulos II, III, y IV, se presenta un aumento de indicadores fisiológicos tales como el Índice Hepatosomático e Índice Gonadosomático.

Durante la última década, ha crecido el interés científico sobre cuál es el efecto reproductivo de diversos factores ambientales estresantes en los sistemas acuáticos. Muchos de estos factores estresantes actúan como disruptores del sistema endocrino. Así, el uso de la gónada adquiere interés como indicador temprano del impacto de las actividades urbanas, agrícolas e industriales en los sistemas acuáticos que potencialmente afectan a las poblaciones naturales (Dube et al, 2001; Rasmussen et al, 2002).

Esto se basa en el hecho que durante las primeras etapas de desarrollo, los peces son altamente sensibles a las alteraciones hormonales, lo que resulta en reversión sexual, desarrollo gonadal temprano y atresia temprana de los ovocitos, lo que resalta la enorme plasticidad y vulnerabilidad de la gónada como órgano centinela.

El conocimiento de la serie de cambios que ocurren en las gónadas de los peces a lo largo del tiempo es fundamental para el conocimiento de la biología reproductiva básica y su uso en algunos métodos de evaluación toxicológica (Parker et al, 1985). De esta forma, Algunos autores (Gibbons et al.1998a,b) señalan que los peces pequeños han sido una excelente herramienta para ser utilizados como modelos para evaluar los efectos de la contaminación ambiental. Por lo tanto, el conocimiento de la biología reproductiva de los peces nativos nos proporciona evidencia para identificar posibles efectos biológicos adversos en su estado fisiológico natural.

Respecto al estado reproductivo de *Percilia irwini*, esta especie muestra un desarrollo gonadal máximo en los sitios de referencia durante octubre y noviembre (Habit et al, 2006a,b, 2007; Ruiz, 1996; Ruiz & Marchant 2004, Chiang et al, 2011), por lo que el período de pre-desove ocurre a principios de la primavera (septiembre). Estos resultados corresponden a lo señalado por otros autores (Habit et al, 2006) y a lo determinado en la especie congénero *Percilia gillissi* (Chiang et al, 2011), lo que sugiere un máximo desarrollo gonadal en el período primavera-verano. *Percilia irwini*, descrita para el río Biobío, Región BioBío, Chile Central, presenta un desove de tipo parcial, relacionado con el modo de maduración ovárica y correspondiente a un desarrollo oocitario asincrónico (Vizziano et al, 1999), donde los ooocitos están presentes en las diferentes etapas de maduración como se establece en la Tabla 1, Capítulo II.

Los datos obtenidos indican que los ejemplares presentes en sitios de alto impacto antropogénicos presentan un mayor número de ovocitos en la estado primario, con un crecimiento protoplásmico de oocitos primarios significativamente mayor que los sitios de referencia Lonquimay (LQ) y Balsa Caracoles (BC). Este comportamiento también se observa en los oocitos vitelogénicos presentes en la zona de referencia LQ, en comparación con los sitios Puente Coihue (PC) y Santa Juana (SJ), donde se reduce la presencia de ooocitos maduros. El menor número de oocitos vitelogénicos demostraría un crecimiento gonadal armónico saludable en las áreas de menor intervención antrópica correspondiente a las zonas de referencia (LQ y BC). Además, en el período de otoño (marzo), los oocitos en estado vitelogénico pertenecientes a los sitios PC y SJ evidencian una disminución en crecimiento (diámetro) y frecuencia (número de oocitos por estadío de maduración).

Las alteraciones fisiológicas reproductivas que observamos a nivel reproductivo en individuos aguas abajo de los sitios de referencia LQ y BC, para ambos períodos de estudio, podrían ser responsables de las diferencias en la estructura de tamaño de los ejemplares machos y hembras de la especie *P. irwini*, en los que existe una tendencia temporal y espacial hacia tamaños más pequeños. Junto a estos impactos reproductivos, puede existir una disrupción metabólica en las hembras, observada como una pérdida de adultos de mayor tamaño, como lo señalan otros autores (Aedo et al, 2009), en estudios previos para la cuenca del Biobío, donde existe una pérdida en la tasa de crecimiento de estas especies en áreas de baja calidad ambiental y sectores directamente afectados por efluentes industriales (Orrego et al, 2005, 2009), urbanos y agrícolas (Habit et al, 2006).

Durante el período post-desove correspondiente a otoño, *Percilia* mostró un aumento en los valores de GSI y el tamaño de las gónadas, aunque las variaciones en el peso de las gónadas en las hembras no fueron significativas. El aumento de IGS observado en los dos períodos de muestreo coincide con el aumento en este valor de índice detectado en estudios previos con respecto a descargas de efluentes industriales aguas abajo de los sitios de referencia (Orrego et al, 2005; Inzunza et al, 2006).

Junto a estos impactos reproductivos, se han descrito ampliamente una serie de efectos metabólicos y toxicológicos en la cuenca del Biobío (Barra et al., 2021) para mostrar un gradiente de contaminación que ha terminado por disminuir la calidad del agua y ambientes asociados. La mayoría de los experimentos de laboratorio y de campo semicontrolados se desarrollaron utilizando modelos de peces introducidos como los juveniles de *Oncorhynchus mykiss* (Orrego et al., 2005, 2007, 2009, 2011), o embriones y larvas de *Oryzias latipes* (Orrego et al., 2011, 2021), que permitió demostrar efecto principalmente estrogénico relacionado con maduración temprana de hembras juveniles, feminización masculina y alta embriotoxicidad especialmente en machos.

Como se indicó anteriormente, el conocimiento sobre el efecto reproductivo de las especies de peces silvestres chilenas, a escala de cuencas como el río Biobío, es escaso o incompleto. En los peces nativos chilenos, el desove ocurre generalmente a fines de primavera y principios de la temporada de verano (Chiang et al., 2010, 2011). El efecto observado en *P. irwini* podría estar relacionado con el desove temprano y quizás forzado por la presencia de sustancias hormonalmente activas en la zona media-baja del río Biobío (Alonso et al, 2017; Orrego et al, 2019; Rozas et al, 2016).

La inducción de la maduración gonadal en hembras se detectó en nuestro estudio a través de la caracterización morfológica y morfométrica de los oocitos de *Percilia irwini*. Este cambio morfológico en la maduración de los ovocitos es evidencia de cambios en el desarrollo reproductivo femenino normal, que podría deberse a la potencial exposición a una mezcla química compleja (incluidos los EDC).

Un estudio publicado recientemente (Orrego et al, 2019), en el que se colocaron dispositivos de membrana semipermeable (SPMD) en el río Biobío, aguas abajo del sitio PC, indica la presencia de compuestos de tipo esteroidal provenientes principalmente de descargas de la industria de las plantas de celulosa, donde estos compuestos pudieron unirse con igual afinidad a la proteína de unión a esteroides sexuales naturales (SSBP) y a receptores de androgénicos (AR) lo que demostró la presencia de EDCs.

De esta manera, es posible inferir que los compuestos estrogénicos en esta zona del río Biobío son responsables del aumento de la actividad estrogénica evidenciado como un aumento del índice Gonadosomático y un aumento de los folículos ováricos en hembras de *Percilia irwini*. Estas respuestas biológicas son consistentes con todos los estudios previos realizados en la cuenca del río Biobío durante las últimas décadas (Barra et al., 2009; Barra et al., 2021).

Respecto al análisis de otros biomarcadores determinados en *Percilia irwini* y *Trichomycterus areolatus*, nuestros resultados exhiben respuestas relacionadas con la exposición a múltiples estresores ambientales que convergen hacia el tercio inferior del río Biobío (Capítulo III). Los efectos asociados a parámetros fisicoquímicos medidos in situ en el río Biobío incluyen el aumento de la conductividad (μS cm^{-1}) entre Lonquimay y Santa Juana, que probablemente se debe principalmente a la alta liberación de efluentes industriales en la zona media del río Biobio, según lo informan otros autores (Karrash et al. 2006). La alta actividad urbana, agrícola e industrial a lo largo del río Biobío, también explica los altos niveles de TDS, que a su vez explica una disminución en los niveles de Oxígeno disuelto (OD) (Tabla 1 Capítulo III; Tabla 1 Capítulo IV). Estos resultados están asociados a la alta intervención de la cuenca, lo que implica un aumento de la actividad agrícola y forestal. Estos altos impactos involucran una alta escorrentía superficial lateral que incrementa los sólidos en suspensión, principalmente en el tercio inferior del río

Biobio. El aumento de TDS puede potencialmente alterar la estructura branquial de los organismos y provocar una alteración en el intercambio de gases, lo que implica una alteración del estado fisiológico de los organismos en estudio (Colin et al, 2016; Quiroz-Jara et al, 2021) (Capítulo III).

El principal efecto observado como resultado de la exposición a una potencial mezcla química compleja en estas especies en el río Biobío ocurre a nivel fisiológico, con respuestas durante el período post desove. Ambas especies evidencian una reducción en longitud y peso desde Lonquimay (Referencia) hasta Santa Juana (zona de alto impacto ambiental).

Los resultados fisiológicos encontrados para ambas especies concuerdan con los resultados de otros autores (Habit el al. 2006; Orrego et al. 2005, 2009; Inzunza et al. 2006; Chiang et al. 2011), quienes indican reducción de la longitud y peso, inducción del en la reproducción e inducción de la actividad EROD en áreas de mayor impacto antrópico.

En cuanto a los efectos biológicos determinados por biomarcadores moleculares, la actividad hepática EROD representa la actividad de la fase I del proceso de biotransformación enzimática mediado por CYP1A. Aunque estos mecanismos bioquímicos se inducen poco después de la exposición a una serie de compuestos como PAH, PCB y aminas aromáticas (Jonsson et al. 2007), la actividad EROD también puede ser considerado un biomarcador de respuesta temprana (Colin et al. 2015), debido a que CYP1A también cataliza la biosíntesis y degradación de la moléculas endógenas como esteroides (Lewis 2004).

La inducción de esta actividad enzimática ha sido bien documentada tanto en condiciones de laboratorio (bioensayos de efluentes y sedimentos) como en condiciones de campo mediante jaulas o captura de especies de peces introducidas y naturales en el río Biobío (Barra et al. 2001, Inzunza et al. 2006, Chiang et al. 2011, Orrego et al.2005a, 2005b, 2010, 2019,), siendo relacionado con la presencia de una serie de compuestos desde hidrocarburos aromáticos policíclicos (HAPs) en sedimentos (Barra et al, 2009) hasta fitoesteroles en efluentes industriales.

La falta de un análisis integrado de biomarcadores ha limitado el uso potencial de estas herramientas. Es por esto que se hace necesario mejorar la interpretación entre los diferentes biomarcadores multinivel utilizados para determinar los efectos sobre un bioindicador y relacionarlos con los estándares de calidad definidos por la normativa ambiental chilena. Sin embargo, como indica el contenido de esta norma, existen intervenciones antrópicas que han generado riesgos para la protección y conservación de esta cuenca en particular, con fuentes de contaminación puntuales y difusas que vierten a cuerpos receptores de agua. Respecto a esto, la norma define en su Título II, Artículo Nº5, niveles de calidad, haciendo sólo referencia a parámetros fisicoquímicos, y definiendo la calidad del agua sólo respecto a valores de concentraciones que no sobrepasen los establecidos, sin considerar los potenciales efectos biológicos que la mezcla compleja de contaminantes químicos pudiera tener sobre los peces nativos presentes en el río, para ser evaluados de forma temprana.

Esta problemática decidimos abordarla mediante el desarrollo de un índice integrado de biomarcadores fisiológicos (IPBR), de modo de evaluar los cambios globales de las respuestas obtenidas que nos permitió relacionar patrones de efectos con diferentes mecanismos de acción toxicológica (Beliaeff et al.2002; Sanchez et al.2013; Murussi et al.2015), en determinadas zonas del río con diferentes perfiles de contaminación.

Esta estrategia nos permitió abordar y cubrir brechas específicas presentes en la legislación chilena como la Norma Secundaria de Calidad Ambiental para aguas superficiales en el Río Biobío. Además, entregar una alternativa para cumplir efectivamente con lo establecido por la normativa ambiental en cuanto a criterios de calidad del agua y conservación y preservación de los ecosistemas acuáticos (Bases Generales del Medio Ambiente, Ley Chilena Nº19300).

Esta estrategia se abordó principalmente en respuesta a que la fauna nativa de peces en el río Biobío representa un acervo genético de esta zona biogeográfica. La alteración de su biotipo ha llevado a una potencial disminución de sus poblaciones, y las áreas directamente afectadas por descargas industriales, efluentes urbanos y agrícolas en el río Biobío se han asociado continuamente con cambios en la abundancia, diversidad y efectos reproductivos en peces nativos (Habit et al. 2006; Chiang et al. 2011; Orrego et al. 2019).

En relación a estos antecedentes, propusimos un IPBR como herramienta para comparar el estado fisiológico y los posibles efectos biológicos en *Percilia irwini* y *Trichomycterus areolatus*, a lo largo de un gradiente de impactos a lo largo del río Biobío, en dos estaciones del año (otoño y primavera), correspondientes a periodos de menor caudal y de aumento de caudal, respectivamente (Capítulos III y IV).

La evaluación realizada mediante IPBR indicó la ocurrencia de efectos fisiológicos asociados con un gradiente espacial y temporal, similar al patrón encontrado en biomarcadores moleculares (EROD) e individuales (longitud total, peso total e IGS) medidos en ambas especies, mas no es posible establecer una relación directa causa-efecto. Las principales diferencias significativas entre las especies se relacionan con el hábitat, pelágico-demersal para *P.irwini* y bentónico para *T.areolatus*, situación que explica la diferencia en las respuestas biológicas obtenidas mediante este índice (Capítulos III y IV). Sin embargo; según diferentes autores (Munkitrich et al. 2000, Chiang et al. 2011, 2014, Delfino et al. 2016), la interpretación de los resultados del trabajo de campo siempre se asocia a una alta variabilidad debido a la presencia de múltiples factores externos, que pueden influir en la variables analizadas, por lo que sus resultados deben considerarse con cautela. Entre estas variaciones se encuentra el cambio de régimen de caudal presente en el río, situación que durante la campaña en terreno realizada en primavera implicó un aumento de caudal, y por lo tanto, un aumento en el factor de dilución en el río, sin embargo, las respuestas (IPBR) obtenidas por las dos especies de biología diferente, da cuenta de los diversos procesos adaptativos a la exposición crónica de una potencial mezcla química compleja (Capítulo IV).

En General, el patrón IPBR observado en ambas especies estudiadas en el río Biobío fue influenciada por la perturbación antropogénica provocada por compuestos químicos xenobióticos de diversa naturaleza, sugiriendo un efecto espacial acumulativo debido a la degradación ambiental en el tercio inferior del río Biobío. El IPBR mostró una disminución de la condición biológica en ambos peces silvestres, y estos cambios manifiestos están relacionados con la alta actividad antropogénica desde Lonquimay hasta los sitios de Puente Coihue y Santa Juana (Capítulos II, III y IV).

CONCLUSIÓN GENERAL

La evaluación y monitoreo de problemas ambientales, sobre todo en importantes sistemas de aguas continentales como la cuenca del río Biobío, deben ser abordados desde una perspectiva integral, para de esta forma entregar respuestas que puedan considerar los tres grandes pilares de las ciencias ambientales: Económico, Ecológico y Social. Esto debido a que estos sistemas entregan importantes servicios ecosistémicos, y la utilización de los recursos debe considerarse desde la sustentabilidad y la sostenibilidad.

Si bien las normativas ambientales, desde el espíritu de la ley, permiten regular de cierta forma la liberación de compuestos de diversa naturaleza, propio de los procesos industriales, e incluso urbanos, el establecer valores de concentraciones de referencia no es suficiente. Esto debido a que cada sistema continental presenta diferente orogénesis, que debe ser considerada por los tomadores de decisiones.

Las respuestas fisiológicas-reproductivas observadas en las dos especies bioindicadoras seleccionadas, dan cuenta de una exposición crónica a una mezcla química compleja. Este proceso se evidencia por la modificación fisiológica respecto a la disminución de la longitud total y el peso total, en pos de mantenerse reproductivamente activos, es decir, destinación de energía para procesos fisiológicos reproductivos, proceso concordante con el alto crecimiento protoplásmico descrito para ambas especies en época de “post desove” y “pre desove” (otoño y primavera respectivamente). Es por este motivo, que el conocimiento del estado reproductivo de *Percilia irwini* y *Trichomycterus areolatus* nos permite no solo incrementar sus estrategias de conservación, sino también evaluar un biomarcador potencial para determinar alteraciones de la fisiología reproductiva en peces nativos, con el fin de distinguir relaciones causa-efecto como los programas de seguimiento implementadas en países desarrollados.

El índice IPBR desarrollado en peces nativos en el río Biobío da cuenta de la variación del estado fisiológico de las especies de peces nativos desde sitios de menor impacto hasta sitios de mayor perturbación antropogénica. Nuestra investigación espacio-temporal logró detectar cambios fisiológicos en *Percilia irwini* y *Trichomycterus areolatus* en el río Biobío. Estos cambios se asocian a los significativos impactos antrópicos en el tercio inferior del río, zona de convergencia de focos de contaminación puntual y difusa. Además el desarrollo de IPBR complementa diversos estudios de Monitoreo de Efectos Ambientales como herramienta de evaluación bajo las diferentes condiciones ambientales en los sistemas de agua dulce en Chile.

En base a la información aportada por esta tesis, podemos aceptar nuestra hipótesis de trabajo, estableciendo que efectivamente se observan alteraciones biológicas adversas en el tercio inferior del río Biobio, debido a la convergencia de una potencial mezcla química compleja y reconociendo las diferencias espaciales de las respuestas medidas mediante batería de biomarcadores, observadas en cinco sitios de monitoreo. Por lo tanto, podemos afirmar que las respuestas biológicas observadas en zonas de alto impacto antrópico se encuentran localizadas y es posible asociarlas espacialmente a la principal área de influencia (tercio inferior).

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