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Respuesta metabólica de comunidades microbianas marinas expuestas a Azametifos, pesticida usado en salmonicultura

Tesis para optar al grado de Magíster en Ciencias con mención en Oceanografía

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RESUMEN

La salmonicultura en Chile ha experimentado un significativo crecimiento a partir de la década de los noventa. Sin embargo, la susceptibilidad de los centros de cultivo a los brotes de *Caligus rogercresseyi*, ha dado lugar a importantes pérdidas económicas. Los pesticidas, entre ellos Azametifos, se utilizan para mitigar el impacto de este piojo de mar. Azametifos puede afectar a especies "no objetivo" que viven cerca de granjas de salmón como el plancton microbiano, aunque hay poca información al respecto. El objetivo de esta Tesis fue determinar el efecto de diferentes concentraciones de Azametifos, sobre el metabolismo de comunidades microbianas marinas (<100 μm) en dos zonas contrastantes, una sin presencia de salmonicultura y la otra con un alto nivel de actividad acuícola. Se utilizaron los siguientes enfoques metodológicos: mediciones de la actividad enzimática de β-glucosidasa y Malato Deshidrogenasa (MDH), técnicas fingerprints (EcoPlates para bacterias y la microplaca FF para hongos marinos) y la estimación de la respiración aeróbica microbiana. Se observó una mayor degradación por microorganismos marinos del pesticida Azametifos en la zona donde hay cultivos de salmones (~ 72,4%) en comparación con un área no utilizada para la producción acuícola (~ 26,1%). La respuesta de la actividad microbiana de la β-glucosidasa a Azametifos es muy variable, pero existe una tendencia a actividades más altas en las fracciones de menor tamaño (5-25 μm y 0,22-3 μm) cuando se exponen a altas concentraciones de Azametifos. También se detectó una respuesta muy variable en la actividad microbiana de MDH. Los sustratos de carbono más utilizados por bacterioplancton y hongos marinos son los relacionados con el metabolismo de los carbohidratos. También se determinó un mayor uso de ácidos carboxílicos y aminoácidos en los tratamientos con concentraciones más altas de azametifos. La actividad enzimática, junto con las huellas metabólicas, podrían usarse como un indicador de perturbaciones en comunidades microbianas marinas

ABSTRACT

Salmon production in Chile has increased significantly since the 1990s; however, there have been significant economic losses owing to outbreaks of *Caligus rogercresseyi*. Pesticides, among them Azametthiphos, are used to mitigate the impact of this sea louse. Azamethiphos can affect non-target species living near salmon farms such as microbial plankton, although there is little information about this. The general objective of this study was to determine the effect of different concentrations of the pesticide Azamethiphos on the metabolism of microbial plankton communities (<100 μm) in two contrasting zones, one without salmon farming and the other with a high level of aquaculture activity. Several methodological approaches were used, including the enzymatic activity of β-glucosidase and malate dehydrogenase (MDH), fingerprinting techniques (EcoPlates for bacteria and FF microplate for marine fungi) and the estimation of microbial aerobic respiration. We found greater degradation of the pesticide Azamethiphos in the salmon culture zone (~72.4%) than in an area not previously used in salmon production (~26.1%). The response of microbial β-glucosidase activity to Azamethiphos is highly variable, but there is a tendency toward more activity in the smaller size fractions (5-25 μm and 0.22-3 μm) when exposed to high Azamethiphos concentrations. We also detected a highly variable response in the microbial MDH activity. The most widely used carbon substrates by bacterioplankton and marine fungi are those related to carbohydrate metabolism. We also found increased use of carboxylic acids and amino acids in the treatments with higher concentrations of Azamethiphos. Enzyme activity, together with metabolic fingerprints, could be used as an indicator of perturbations in marine microbial communities.

1. INTRODUCCIÓN

Un crecimiento significativo experimentó la acuicultura en Chile a partir de la década de los noventa (Ibieta *et al.,* 2011; Avendaño-Herrera, 2018), posicionando al país como el segundo productor de salmónidos a nivel mundial, después de Noruega (FAO, 2018). Sin embargo, la susceptibilidad de los centros de cultivo de salmón a los brotes de enfermedades por ectoparásitos, ha dado lugar a importantes pérdidas económicas relacionadas con la disminución de calidad del producto final, crecimiento retardado de los peces parasitados, incremento de la susceptibilidad frente a otros patógenos y costos generados por los tratamientos (Johnson *et al.,* 2004; Costello, 2006; Torrissen *et al.,* 2013; Quiñones *et al.,* 2019; Dresdner *et al*., 2019).

El piojo de mar (*Caligus rogercresseyi*) es uno de los patógenos más importante y de mayor impacto económico para la industria del salmón en Chile (Bravo *et al*., 2015). Con el fin de mitigar este impacto, la industria ha recurrido al uso de pesticidas. Hasta la fecha los productos registrados en el Servicio Agrícola y Ganadero (SAG) son: Benzoato de Emamectina 0,2% (EMB, avermectina), Deltrametrina 1% (piretroide sintético), Cipermetrina 5% (piretroide sintético), Diflubenzurón 80% (inhibidor síntesis de quitina), Azametifos 50% y Lufenuron 10%. Azametifos es un pesticida organofosforado soluble en agua (1,1 g L^{-1}) que permanece en la fase acuosa en el medio ambiente (Burridge, 2013), se descompone principalmente por hidrólisis en agua, con una vida media de 8,9 días (SEPA, 2008). Además de los pesticidas, el peróxido de hidrógeno también se ha utilizado en Chile, especialmente durante la crisis de la ISA en 2007-2010 (Bravo *et al.,* 2010). Recientemente, se han introducido baños de agua dulce en Chile como una herramienta para combatir la caligidiosis, aunque su uso aún se encuentra en etapa experimental en algunos centros de cultivo de salmón. Sin embargo, los baños de agua dulce y peróxido de hidrógeno se usan ampliamente en Noruega y otros países, especialmente porque son alternativas más ecológicas para combatir los piojos de mar que los pesticidas (Hjeltnes *et al.,* 2018).

La liberación de pesticidas se ha identificado como un importante problema ambiental (Nash, 2003; Gebauer *et al.*, 2017), dado que los pesticidas y/o sus intermedios posteriores pueden afectar a especies no objetivo (Rohr *et al.*, 2006; Urbina *et al.*, 2018). En particular, pueden afectar a peces e invertebrados acuáticos que habitan en las proximidades donde se aplican los tratamientos, alterando la estructura de las poblaciones en el entorno inmediato (Johnson *et al.*, 2004; Burridge *et al.*, 2010, 2014; Ernst *et al.*, 2014).

Los microorganismos marinos también pueden transformarse en organismos "no objetivo" del uso de pesticidas. Si bien existen estudios de toxicidad sobre microorganismos, la mayoría de los estudios se han centrado en la degradación microbiana de los pesticidas (DeLorenzo *et al.*, 2001). Debido al uso de pesticidas en la agricultura y la ganadería, se han realizado muchos más estudios sobre los efectos ecotoxicológicos de los pesticidas en los microorganismos del suelo que en las poblaciones microbianas marinas o estuarinas (DeLorenzo *et al.*, 2001; Muturi *et al.*, 2017).

Rain-Franco *et al.* (2018) encontraron que hay un efecto significativo en las comunidades microbianas marinas cuando se aplica un pesticida, mientras que los tratamientos combinados no mostraron un impacto significativo en la producción primaria fotoautotrófica (Rain-Franco *et al.*, 2018). El benzoato de emamectina produce una disminución del 60-90% de la fijación de carbono tanto fotoautotrófica como quimioautotrófica. También se ha detectado que la adición de Azametifos aumenta la tasa de producción primaria *in situ* en la zona norte de la Patagonia Chilena (Rain-Franco *et al.*, 2018).

Un enfoque para estudiar el impacto de compuestos, como Azametifos, sobre microorganismos marinos, es el análisis de posibles alteraciones en sus respuestas metabólicas. En el caso de ecosistemas terrestres, los pesticidas que llegan al suelo agrícola pueden alterar el metabolismo local y/o las actividades enzimáticas de microorganismos (Engelen *et al.*, 1998; Liu *et al.*, 2008; Hussain *et al.*, 2009), teniendo impacto negativo sobre la actividad enzimática de las hidrolasas, oxidorreductasas y actividades deshidrogenasa de comunidades bacterianas (Perucci y Scarponi, 1994; Ismail

et al., 1998; Malkomes y Dietze, 1998; Monkiedje y Spiteller, 2002; Monkiedje *et al.*, 2002, Menon *et al.*, 2005; Cáceres *et al.*, 2009).

La cuantificación de la actividad enzimática extracelular de los microorganismos del suelo, como la β-glucosidasa, se utiliza para evaluar la diversidad funcional, la calidad ambiental y los efectos antropogénicos sobre la salud del ecosistema (Marx *et al.*, 2005; Ai *et al.*, 2012). La actividad de las enzimas deshidrogenasas se utiliza como índice metabólico de fertilización (Skujins, 1973; Parinkina *et al.*, 1994; Alef, 1995; Salazar *et al.*, 2011). La actividad de la enzima intracelular Malato Deshidrogenasa (MDH) se ha propuesto como un indicador de actividad microbiana, dado que refleja la reacción metabólica oxidativa global de la comunidad microbiana (Martínez *et al.*, 2002). Un incremento de la actividad MDH, en los suelos, indica una mayor remineralización de la materia orgánica (Martínez *et al.*, 2002).

Otra herramienta potencial para monitorear los cambios en la diversidad funcional microbiana en suelos y sedimentos estuarinos sometidos a algún tipo de perturbación, es el sistema de microplacas BIOLOG® EcoPlates ™ propuesto por Insam en 1997 para bacterias (Campbell *et al.*, 1997; Staddon *et al.*, 1997; Classen *et al.*, 2003; Calbrix *et al.*, 2005; Echavarri-Bravo *et al*., 2015), y el sistema BIOLOG® FF microplate para hongos (Dobranik y Sak, 1999). Estas técnicas se han utilizado para estudiar los perfiles fisiológicos a nivel comunitario para comprender el funcionamiento de la microbiota que participa en el ciclo del carbono (Schutter y Dick, 2001) y para evaluar los cambios en la diversidad microbiana funcional producida por la adición de antibióticos al suelo (Liu *et al.*, 2012). La pérdida de capacidad de las comunidades microbianas para mantener funciones como la uniformidad catabólica o el uso uniforme del sustrato, son indicadores de disminución de la salud del suelo (Chapman *et al.*, 2007).

El objetivo principal del presente estudio es determinar los efectos del pesticida Azametifos en el metabolismo de las comunidades de plancton microbiano en dos áreas contrastantes, una donde no hay cultivo de salmón y la otra donde ha habido una presencia de granjas de salmón desde los años 80’s. Se utilizaron varios enfoques metodológicos,

incluidos la actividad enzimática de la β-glucosidasa y la MDH, técnicas fingerprinting (BIOLOG® EcoPlates para bacterias y la microplaca BIOLOG FF para hongos marinos) y la estimación de la respiración microbiana aeróbica.

2. HIPÓTESIS

H1: El incremento de la concentración de Azametifos provoca la disminución de la actividad β-glucosidasa en microorganismos provenientes de la columna de agua

H2: El incremento de la concentración de Azametifos provoca la disminución de la actividad malato dehidrogenasa en microorganismos provenientes de la columna de agua

H3: El incremento de las concentraciones de Azametifos disminuye la utilización de sustratos de carbono por parte de comunidades microbianas (bacterias y hongos) provenientes de la columna de agua

3. OBJETIVOS

3.1 Objetivo general

Determinar el impacto del pesticida Azametifos sobre el metabolismo de comunidades microbianas marinas a través de la respuesta enzimática de la β-glucosidasa, Malato dehidrogenasa y cambios fingerprintings metabólicos.

3.2 Objetivos específicos

3.2.1. Determinar el impacto de diferentes concentraciones de Azametifos sobre las actividades β-glucosidasa y malato dehidrogenasa en comunidades microbianas de la columna de agua.

3.2.2. Evaluar cambios por diferentes concentraciones de Azametifos en los perfiles metabólicos de comunidades microbianas (bacterias y hongos) provenientes de columna de agua.

4. MATERIALES Y MÉTODOS

4.1 Áreas de estudio

Se colectaron muestras de agua de mar de tres zonas costeras: (i) Bahía de Coliumo en la Región del Bío-Bío de Chile (Sitio 1; Lat. -36.602ºS, Long. -73.371ºW), donde no hay cultivos de salmón, y (ii) dos sitios en canales de la Isla de Chiloé en la X Región (Sitio 2: Lat. -42,556ºS, Long. -73,566ºW, y sitio 3: Lat. -42.602ºS, Long. -73.371ºW), donde ha habido una fuerte presencia de salmonicultura desde la década de los 80's (Figura 1). El agua de mar se colecto con botellas Niskin de 20 L. Se tomaron muestras a 5 m de profundidad en el sitio 1 en diciembre de 2016 y a 10 m de profundidad en los sitios 2 y 3 en enero de 2017. Los parámetros físico-químicos de la columna de agua (salinidad y temperatura) se midieron en los tres sitios con una Sonda CTD (Minos X, AML Oceanographic).

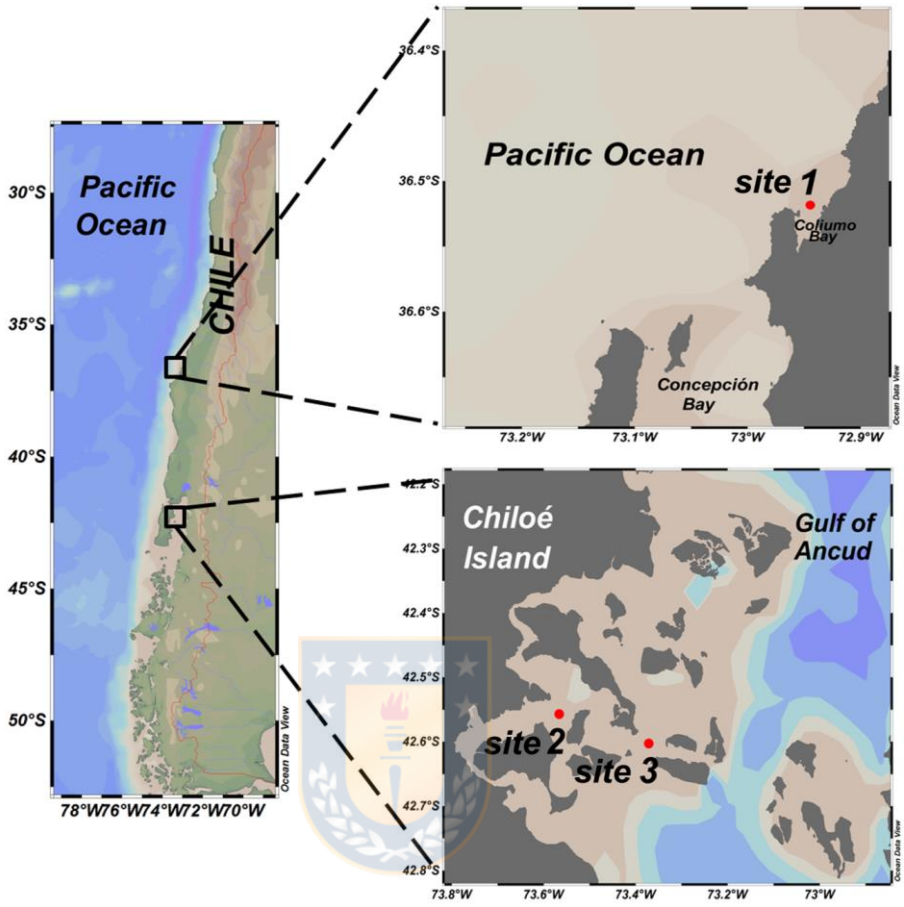

Figura 1. Zonas de muestreo. La zona 1 indica la zona muestreada ubicada en la Bahía de Coliumo, VIII región, Chile y las zonas 2 y 3 indican las zonas muestreadas en la Isla Grande de Chiloé, X región de Los Lagos, Chile.

4.2 Diseño experimental

Se realizaron estudios de microcosmos en los tres sitios. Los microcosmos consistieron en bidones de polietileno de alta densidad rellenos con 20 litros de agua de mar previamente filtrada en un tamiz de 100 µm más el pesticida Azametifos en concentraciones de: (1) 2 µg L^{-1}, (2) 10 µg L^{-1}, (3) 100 µg L^{-1} y (4) 1000 µg L^{-1}, así como un control sin Azametifos (CTR). Se utiliza una concentración de Azametifos de 100 µg

L^{-1} en tratamientos contra *Caligus* de acuerdo con los estándares regulatorios chilenos. Los experimentos se realizaron por triplicado para los sitios 1 y 3 (con una réplica para el sitio 2), y se incubaron en oscuridad en una cámara fría a ~12.5 ° C durante 5 días.

Se recolectaron muestras para análisis de nutrientes, clorofila-a y abundancia de bacterioplancton en (1) el control ambiental (CTRA) antes del experimento, (2) en la Fracción total (FT) en el momento 0 del experimento, y (3) el FT de todos los microcosmos al final del experimento (día 5). Para determinar el uso de sustratos de carbono en los principales grupos heterotróficos de microbios, también se recolectaron muestras de las fracciones F1 (25-100 µm para hongos) y F4 (0.22-3 µm para bacterioplancton).

Se analizó el efecto de Azametifos sobre la actividad enzimática, recolectando 500 mL para analizar MDH y 500 mL para determinar la concentración de Adenosín Trifosfato (ATP). Para determinar la actividad extracelular de la β-glucosidasa (β-glu) recolectamos 5 mL de cada fracción (FT, F1: 25-100 µm, F2: 5-25 µm, F3: 3-5 µm y F4: 0.22-3 µm) cada 24 horas durante los 5 días del experimento.

4.3 Determinación de concentración de Azametifos en microcosmos

Se colectaron muestras desde los sitios de muestreo (CTRA) para descartar la presencia previa de Azametifos. Se colectaron 500 mL de agua en botellas de vidrio para evaluar la degradación de Azametifos por microorganismos después de cinco días. Estas muestras se congelaron a -20 °C y se enviaron inmediatamente a la empresa SGS Chile (Puerto Varas, Chile) para su análisis mediante cromatografía líquida de alta resolución (HPLC) con un detector de fluorescencia.

El efecto de la hidrólisis sobre las concentraciones de Azametifos utilizadas se estimó a partir de la información de la literatura (SEPA, 2008). Se asumió una degradación lineal en base a los datos reportados de degradación a las 3 h (1%) y después de 3 días (21%), y

una vida media = 8.9 días, con lo cual se estimó un valor porcentual de hidrólisis de 28,1% para el día 5.

4.4 Nutrientes

Los macronutrientes PO_4, $Si(OH)_4$, NO_2, NO_3 se determinaron filtrando muestras de agua a través de filtros Whatman® GF/F de 0,7 µm. Los análisis se realizaron utilizando un autoanalizador Technicon® II en el Laboratorio de Biogeoquímica del Departamento de Oceanografía de la Universidad de Concepción. PO_4, NO_2 y NO_3 se midieron de acuerdo con la metodología propuesta por Strickland y Parsons (1972) y $Si(OH)_4$ se determinó de acuerdo con Aminot y Kerouel (2007)

4.5 Clorofila-*a*

La concentración de clorofila-*a* se determinó según Holm-Hansen *et al*. (1965), a partir del filtrado de 0,3 L de agua de mar, utilizando filtros Whatman® GF/F 0.7 µm y preservados a -20 ºC hasta su posterior análisis. La extracción fue realizada con acetona 90% y su concentración medida por fluorescencia en un fluorómetro (Trilogy, Turner Designs).

4.6 Abundancia bacteriana

Para estimar las abundancias bacterianas (cel mL^{-1}) se tomaron muestras de 50 mL de agua en tubos Falcon, se fijaron con formalina (1% concentración final) y fueron almacenadas en oscuridad a 4 °C. Las muestras fueron teñidas con 4'6-diamidino-2-phenylindole (DAPI, 5 µg mL^{-1} concentración final) sobre filtros de policarbonato negro (0,2 µm) y almacenadas en oscuridad a -20 °C (Porter y Feig 1980). El conteo bacteriano

se realizó utilizando un microscopio de epifluorescencia (Axioscope 2 plus, Zeiss) realizando un conteo de 10 campos por muestra.

4.7 Respiración Aeróbica

El consumo de oxígeno fue determinado a través de un respirómetro (FIBOX 3, PreSens) con sensores Optode. Se midió el consumo de oxígeno diario en los microcosmos en la fracción total (FT). Las tasas de respiración fueron medidas en los microcosmos en las concentraciones 0, 2, 10, 100 y 1000 µg L^{-1} en los sitios 1 y 3, y a las concentraciones 0 y 100 µg L^{-1} en el sitio 2.

4.8 Determinación de Adenosín Trifosfato (ATP)

El ATP es un indicador potencial de biomasa microbiana viable (Karl, 1993). El ATP se cuantificó en un ensayo de bioluminiscencia (Holm-Hansen y Booth, 1966; Bulleid, 1978; Karl, 1993). La primera etapa consistió en una extracción de ATP de los filtros mediante un baño de ebullición con buffer orgánico (Tris buffer 20 mM, pH 7,7). La segunda etapa consistió en la cuantificación del ATP mediante un ensayo de bioluminiscencia usando un luminómetro (GloMax, Promega).

4.9 Efecto de Azametifos en las actividades enzimáticas del plancton microbiano

4.9.1 Actividad enzimática β-glucosidasa

La actividad enzimática de la β-glucosidasa (EC 3.2. 1.3) se analizó mediante ensayos fluorométricos en microplacas basadas en metilumbeliferona con MUB-β-D-glucopiranosido, utilizando la metodología propuesta por Jackson *et al*. (2013). Las lecturas de fluorescencia se realizaron con un lector de microplacas Multi-Modal Synergy 2 (Biotek).

4.9.2 Actividad enzimática Malato Dehidrogenasa (MDH)

La actividad de MDH se evaluó utilizando una versión modificada de la metodología propuesta por Schiedek (1997). Los medios de extracción (tampón de homogeneización) consistieron en tampón fosfato 200 mM (K_2HPO_4), pH 7,9, ditiotreitol (DTT) 1 mM, polivinilpirrolidona (PVP) al 0.3% (p/v), EDTA 5 mM, 0,1% (v/v) Triton X-100 y 3% (p/v) de albúmina de suero bovino (BSA). Las muestras se homogeneizaron mediante el uso de un Ultra-Turrax durante 15 segundos a la velocidad máxima con giros cortos en un baño de hielo. La temperatura se mantuvo a 4 °C durante el procedimiento de homogeneización para evitar pérdidas de actividad enzimática. Las muestras homogeneizadas se centrifugaron a 5000 rpm durante 5 minutos a 4 °C, y los sobrenadantes se usaron para ensayos enzimáticos.

Se analizó la actividad de MDH, ya que cataliza la formación de malato a partir de oxaloacetato, utilizando un procedimiento general modificado de Childress y Somero (1979) y Vetter *et al*. (1994). El medio del ensayo contenía 80 mM de tampón K_2HPO_4 pH 7.9 a 20 °C, 0.1 mM NADH, 150 µM $MgCl_2$ $6H_2O$ y 0.2 mM moxaloacetato. La absorción se midió a 340 nm después de la adición del sobrenadante. Las medidas de actividad de MDH se corrigieron para la oxidación inespecífica de NADH.

El ATP se usa como una variable proxy de la biomasa de microplancton, usamos el término "actividad específica" como unidades de actividad “MDH/unidades de ATP” (González y Quiñones, 2009).

4.10 Efecto de Azametifos en perfiles metabólicos de comunidades microbianas

4.10.1 Efecto de Azametifos en perfiles metabólicos de comunidades bacterianas

El sistema EcoplateTM (Biolog®) se utilizó para caracterizar y evaluar los cambios en los perfiles metabólicos de las comunidades de bacterioplancton en las columnas de agua de

los sitios 2 y 3. Las Ecoplacas están compuestas por 96 pocillos que contienen 31 fuentes de carbono diferentes y un control sin sustrato; por triplicado (Frąc, 2012). Para obtener solo la fracción de bacterioplancton, se concentraron 500 ml de las muestras en 50 ml de la fracción <3 µm en un filtro de 0,22 µm. Se inocularon 120 µl de muestra en cada pocillo. La formación del color púrpura ocurre cuando los microbios pueden utilizar la fuente de carbono y comenzar a respirar. La respiración de las células en la comunidad reduce un colorante de tetrazolio que se incluye con la fuente de carbono. Las microplacas se incubaron a 26 °C y se midió la densidad óptica (OD) cada 24 horas (λ = 590 nm) con un lector de microplacas (ELx800 Epoch^{TM}, BIO TEK®).

4.10.2 Efecto de Azametifos en perfiles metabólicos de comunidades de hongos marinos

El sistema FF Microplate (Biolog®) se utilizó para caracterizar y evaluar los cambios en el perfil metabólico de las comunidades de hongos en la columna de agua (e.g. Shengnan *et al*., 2011; Fuentes y Quiñones, 2016) de los sitios 2 y 3.

Biolog FF MicroPlates contiene 95 pocillos con diferentes compuestos que contienen carbono y 1 pocillo con agua como control. Se usó violeta de yodonitrotetrazolio (INT) como colorante redox para medir colorimétricamente la actividad mitocondrial (valor redox) resultante de la oxidación de fuentes de carbono metabolizables. Todos los pocillos son incoloros cuando se inoculan por primera vez. La oxidación del succinato a fumarato en el ciclo del ácido cítrico, mediado por la succinato deshidrogenasa y el FAD, reduce el INT al colorante violeta de formazan con una absorción máxima a 490 nm (Kubicek *et al*., 2003; Tanzer *et al*., 2003; Bochner, 2009) La reducción del tinte de tetrazolio debido al aumento de la respiración celular vuelve el pocillo morado (Tanzer *et al*., 2003). La reducción de INT y la producción de formazan coloreado es irreversible, y la acumulación de formazan, medida espectrofotométricamente, refleja cuantitativamente la oxidación del sustrato de prueba. La lectura de 750 nm mide la turbidez, lo que refleja la producción de

micelios del sustrato de prueba. Debido a que el espectro de absorbancia del micelio hialino está esencialmente nivelado en el rango de 490 a 750 nm, se obtiene un valor redox corregido (CRV) para la producción de formazán restando la lectura de 750 nm de la lectura de 490 nm (490–750 nm) (Kubicek *et al*., 2003). El CRV de cada pozo se corrigió restando el valor de control (agua).

Para inocular las microplacas FF se concentraron 500 mL en 50 mL, de la fracción de 25-100 µm, fracción en la cual se ha encontrado la mayor abundancia de hongos marinos (Gutiérrez *et al*., 2011), y se inocularon 120 µl en los pocillos. Las microplacas se incubaron a 26 °C y la densidad óptica (λ = 490 y 750 nm) de cada pocillo se midió cada 24 horas con un lector de microplacas (ELx800 Epoch^{TM} de BIO TEK®).

4.10.3 Análisis estadístico de los perfiles metabólicos.

El desarrollo promedio del color de los pocillos (AWCD) de todos los sustratos expresa el uso general del sustrato de carbono por parte de la comunidad microbiana, que se calcula con la siguiente ecuación: AWCD = Σ (C - R) / N, donde C es la densidad óptica (DO) en cada fuente de carbono (pocillo), R es DO del control y N es el número de sustratos de la placa. Los niveles de DO del sustrato se ajustaron restando la DO del control y las lecturas negativas. El AWCD de todos los tratamientos se comparó usando una prueba de Kruskal-Wallis y análisis de comparación múltiple (z' value) usando el software Statistica v.13.

Los sustratos se dividieron en seis categorías (polímeros, aminoácidos, carbohidratos, aminas, ácidos carboxílicos y misceláneos), de acuerdo con Buyer y Drinkwater (1997). Los valores corregidos de absorbancia de los sustratos se expresaron como un porcentaje de la absorbancia total de la placa para un tratamiento particular (Frąc *et al*., 2012). El AWCD y el OD medio de los seis tipos de sustrato se compararon con una prueba de Kruskal-Wallis y los diferentes tratamientos con un análisis de comparación múltiple (z score) con Statistica v.13. Los sustratos utilizados con mayor frecuencia, en los dos sitios

estudiados, en las microplacas Ecoplates y FF fueron analizado en la colección metabólica KEGG (http://www.genome.jp/kegg/pathway.html).

4.11 Análisis estadísticos

Todos los análisis estadísticos se realizaron con el software Statistica v.13. La normalidad de la distribución de todos los datos se verificó mediante las pruebas de Kolmogorov-Smirnov.

Se usó un análisis de varianza unidireccional (ANOVA) y una prueba post hoc Tukey HSD para evaluar las diferencias entre los tratamientos en cuanto a nutrientes y a la abundancia de bacterioplancton. Los resultados de las mediciones de respiración en los diferentes tratamientos se evaluaron con un ANOVA de 2 vías y una prueba post hoc Tukey HSD.

Para el análisis de β-glucosidasa y MDH/ATP, primero se realizó una transformación de raíz cuarta a los datos y luego se analizaron con un ANOVA factorial (factores: día, tratamiento, día*tratamiento) y pruebas Tukey HSD. Para la utilización del sustrato, se comparó la AWCD y la DO medias de las seis categorías de sustrato mediante la prueba de Kruskal-Wallis, y se compararon los diferentes tratamientos con un análisis de comparaciones múltiples (z' value). Los datos para ambas microplacas, en cada sitio, se ordenaron mediante un análisis de agrupamiento de unión.

5. RESULTADOS

5.1 Efecto de Azametifos sobre la actividad enzimatica y huellas digitales metabolicas de comunidades microbianas marinas de la columna de agua

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Resumen

La salmonicultura en Chile ha experimentado un significativo crecimiento a partir de la década de los noventa. Sin embargo, la susceptibilidad de los centros de cultivo a los brotes de *Caligus rogercresseyi*, ha dado lugar a importantes pérdidas económicas. Los pesticidas, entre ellos Azametifos, se utilizan para mitigar el impacto de este piojo de mar. Azametifos puede afectar a especies "no objetivo" que viven cerca de granjas de salmón como el plancton microbiano, aunque hay poca información al respecto. El objetivo de esta investigación fue determinar el efecto de diferentes concentraciones de Azametifos, sobre el metabolismo de comunidades microbianas marinas (<100 µm) en dos zonas contrastantes, una sin presencia de salmonicultura y la otra con un alto nivel de actividad acuícola. Se utilizaron varios enfoques metodológicos, incluidas las mediciones de la actividad enzimática de la β-glucosidasa y la Malato Deshidrogenasa (MDH), técnicas fingerprints (EcoPlates para bacterias y la microplaca FF para hongos marinos) y la estimación de la respiración aeróbica microbiana. Se observó una mayor degradación, por microorganismos marinos, del pesticida Azametifos en la zona donde hay cultivos de salmones (~ 72,4%) en comparación con un área no utilizada para la producción acuícola

(~ 26,1%). La respuesta de la actividad microbiana de la β-glucosidasa a Azametifos es muy variable, pero existe una tendencia a actividades más altas en las fracciones de menor tamaño (5-25 μm y 0.22-3 μm) cuando se exponen a altas concentraciones de Azametifos. Además, se detectó una respuesta muy variable en la actividad microbiana de MDH. Los sustratos de carbono más utilizados por bacterioplancton y hongos marinos son los relacionados con el metabolismo de los carbohidratos. También se encontró un mayor uso de ácidos carboxílicos y aminoácidos en los tratamientos con concentraciones más altas de azametifos. La actividad enzimática, junto con las huellas metabólicas, podrían usarse como un indicador de perturbaciones en comunidades microbianas marinas

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Effect of Azamethiphos on enzymatic activity and metabolic fingerprints of marine microbial communities from the water column

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ABSTRACT

Salmon production in Chile has increased significantly since the 1990s. However, there have been significant economic losses owing to outbreaks of *Caligus rogercresseyi*. Pesticides, among them Azamethiphos, are used to mitigate the impact of this sea louse. Azamethiphos can affect non-target species living near salmon farms such as microbial plankton, although there is little information about this. The general objective of this study was to determine the effect of different concentrations of the pesticide Azamethiphos on the metabolism of microbial plankton communities (< 100 μm) in two contrasting zones, one without salmon farming and the other with a high level of aquaculture activity. Several methodological approaches were used, including the enzymatic activity of β-glucosidase and malate dehydrogenase (MDH), fingerprinting techniques (EcoPlates for bacteria and FF microplate for marine fungi) and the estimation of microbial aerobic respiration. We found greater degradation of the pesticide Azamethiphos in the salmon culture zone (~72.4%) than in an area not previously used in salmon production (~26.1%). The response of microbial β-glucosidase activity to Azamethiphos is highly variable, but there is a tendency toward more activity in the smaller size fractions (5–25 μm and 0.22–3 μm) when exposed to high Azamethiphos concentrations. We also detected a highly variable response in microbial MDH activity. The most widely used carbon substrates by bacterioplankton and marine fungi are those related to carbohydrate metabolism. We also found increased use of carboxylic acids and amino acids in the treatments with higher concentrations of Azamethiphos. Enzyme activity, together with metabolic fingerprints, could be used as an indicator of perturbations in marine microbial communities.

1. Introduction

Salmon farming has experienced significant growth in Chile since the 1990s (Ibieta et al., 2011; Avendaño-Herrera, 2018). The country is today the second most important salmon producer worldwide after Norway (FAO, 2018). However, the outbreak of diseases owing to ectoparasites has been responsible for significant economic losses related to the reduced quality of the final product, the slow growth rates of affected fish, increased susceptibility to other diseases and the costs of treatments in response (Johnson et al., 2004; Costello, 2006; Torrissen et al., 2013; Quiñones et al., 2019).

The sea louse (*Caligus rogercresseyi*) has the most significant economic impact of any parasitic pest on the Chilean salmon industry (Bravo et al., 2015). Salmon producers have employed pesticides to reduce this impact. Seven pesticides have been authorized to date by the Chilean Agriculture and Livestock Service (www.sag.gob.cl): Emamectin benzoate 0.2% (chloride channel activator); Deltramethrin 1% and Cypermethrin 5% (sodium channel modulator); Diflubenzuron 80%, Lufenuron 10% and Hexaflumuron 100 mg mL^{-1} (chitin synthesis inhibitor); and Azamethiphos 50% (acetyl cholinesterase inhibitor). Azamethiphos is a water-soluble organophosphate (1.1 g L^{-1}) that remains in the aqueous phase in the environment (Burridge, 2013); It decomposes mainly by hydrolysis in water, with a half-life of 8.9 days (SEPA, 2008). In addition to pesticides, hydrogen peroxide has also been used in Chile, especially during the ISA crisis in 2007–2010 (Bravo et al., 2010). Fresh water baths have recently been introduced in Chile as a tool to combat Caligidiosis, although its use is still at the experimental stage in a few salmon companies. However, hydrogen peroxide and fresh water baths are widely used in Norway and other countries, especially because they are more ecologically friendly alternatives to combat sea lice than pesticides (Hjeltnes et al., 2018).

Pesticide use has been identified as a significant environmental problem (Nash, 2003; Gebauer et al., 2017; Quiñones et al., 2019), given that pesticides and/or their subsequent intermediates can affect

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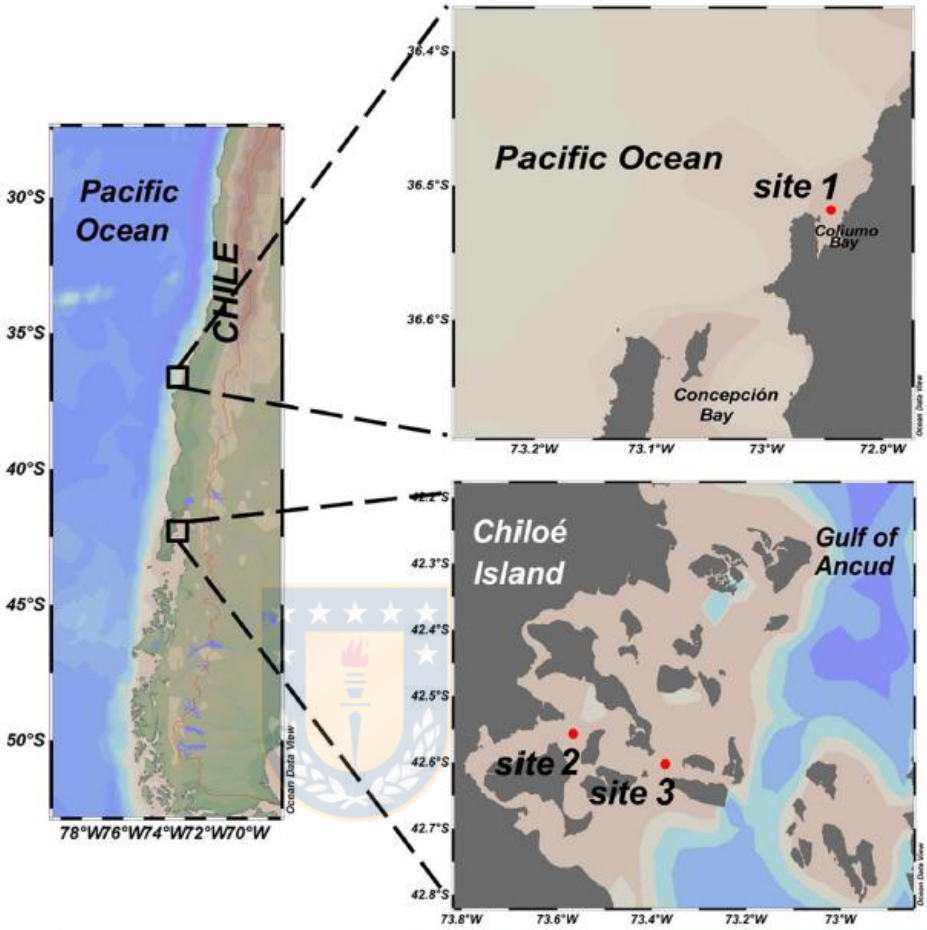

Fig. 1. Sampling sites: Point 1 is the sampled area in Coliumo Bay in Chile's VIII Region. Points 2 and 3 are the sampled areas off Chiloé Island in the X Region of Chile.

non-target species (Rohr et al., 2006; Urbina et al., 2018). In particular, treatments against *Caligus rogercresseyi* lack specificity and can affect autochthonous organisms like fish and aquatic invertebrates, and alter population structures in the immediate surroundings where treatments are applied (Johnson et al., 2004; Burridge et al., 2010, 2014; Ernst et al., 2014).

Marine microorganisms are non-target species that are also affected by pesticides. The majority of studies on the effects of toxic substances on microorganisms have focused on microbial degradation (DeLorenzo et al., 2001). Due to pesticide use in farming and livestock, there have been many more studies of the ecotoxicological effects of pesticides on soil microorganisms than on marine or estuary microbial populations (DeLorenzo et al., 2001; Muturi et al., 2017).

Rain-Franco et al. (2018) found that there is a significant effect on marine microbial communities when a pesticide is applied, whereas combined treatments did not show a significant impact on primary photoautotrophic production (Rain-Franco et al., 2018). Emamectin benzoate decreases photoautotrophic and chemoautotrophic carbon fixation by 60 to 90%. It has also been found that adding Azamethiphos increases the rate of in situ primary production in northern Chilean Patagonia (Rain-Franco et al., 2018).

One approach to studying the effect of compounds like Azamethiphos on marine microorganisms is to identify alterations in their metabolic response. Pesticides that enter the soil of land environments can alter local metabolism and enzymatic activity of microorganisms (Engelen et al., 1998; Liu et al., 2008; Hussain et al., 2009), with negative effects on the enzymatic activity of hydrolases, oxydoreductases and dehydrogenases of the bacterial communities inhabiting agricultural soils (Perucci and Scarponi, 1994; Ismail et al., 1998; Malkomes and Dietze, 1998; Monkiedje and Spiteller, 2002; Monkiedje et al., 2002; Menon et al., 2005; Cáceres et al., 2009).

The quantification of the extracellular enzymatic activity of soil microorganisms such as β- glucosidase is used to assess functional diversity, environmental quality and anthropogenic effects on ecosystem health (Marx et al., 2005; Ai et al., 2012). The activity of dehydrogenase enzymes is used as a metabolic index of fertilization (Skujins, 1973; Parinkina et al., 1994; Alef, 1995; Salazar et al., 2011). The activity of the intracellular enzyme malate dehydrogenase (MDH) has been proposed as an indicator of microbial activity, given that it reflects the global oxidative metabolic reaction of the microbial community (Martínez et al., 2002). Increased MDH activity in soils indicates increased remineralization of organic matter (Martínez et al., 2002).

Other potential tools for monitoring changes in functional microbial diversity in soils and estuarine sediments subject to some type of perturbation are the BIOLOG® EcoPlate ™ system (e.g. Campbell et al., 1997; Staddon et al., 1997; Classen et al., 2003; Calbrix et al., 2005;

Echavarri-Bravo et al., 2015) proposed for bacteria by Insam (1997), and the BIOLOG® FF microplate system for fungi (e.g. Dobranic and Zak, 1999). These techniques have been used to study community level physiological profiles to understand the functioning of microbiota that participate in the carbon cycle (Schutter and Dick, 2001) and to assess changes in functional microbial diversity produced by adding antibiotics to soil. The loss of capacity of microbial communities to maintain functions like catabolic uniformity or uniform substrate use are indicators of decline in soil health (Chapman et al., 2007).

The main objective of the present study is to determine the effects of the pesticide Azamethiphos on the metabolism of microbial plankton communities in two contrasting areas, one where there is no salmon farming and the other where there has been a significant presence of salmon farms since the 1980s. Several methodological approaches were used, including the enzymatic activity of β-glucosidase and MDH, fingerprinting techniques (BIOLOG® EcoPlates for bacteria and BIOLOG FF microplate for marine fungi) and the estimation of microbial aerobic respiration.

2. Methodology

2.1. Study areas

Seawater samples were collected from three coastal areas, Coliumo Bay in the Bío-Bío Region of Chile (Site 1; Latitude 36.602°S, Longitude 73.371°W), where there is no salmon farming, and two sites in Chiloé Island in the X Region (Site 2: Latitude 42,556°S, Longitude 73,566°W, and site 3: Latitude 42.602°S, Longitude 73.371°W), where there has been a strong presence of fish farming since the 1980s (Fig. 1). Seawater was collected with 20-L Niskin bottles. Samples were taken at 5 m depth at site 1 in December 2016 and at 10 m depth at sites 2 and 3 in January 2017. The physical-chemical parameters of the water column (salinity, and temperature) were measured at the three sites with a CTD probe (Minos X, AML Oceanographic).

2.2. Experimental design

Microcosm studies were conducted with microbial communities collected from the three sites. The microcosms consisted of high-density polyethylene carboys filled with 20 L of seawater pre-filtered in a 100-µm sieve, plus the pesticide Azamethiphos in concentrations of: (i) 2 µg L^{-1}, (ii) 10 µg L^{-1}, iii) 100 µg L^{-1}, and (iv) 1000 µg L^{-1}, as well as a control without Azamethiphos (CTR). An Azamethiphos concentration of 100 µg L^{-1} is used in treatments against *Caligus* in accordance with Chilean regulatory standards. Nevertheless, taking into consideration the scarce knowledge on the ecotoxicological impact of Azamethiphos on natural marine microbial communities, we decided to include a much higher concentration (i.e. 1000 µg L^{-1}) in the experimental design than what can be found in the environment after an immersion treatment in order to detect a possible effective or lethal concentration in these microbial communities. The experiments were conducted in triplicate for sites 1 and 3 (with one replicate for site 2), and incubated in darkness in a cold chamber at ~12.5 °C for 5 days.

We collected samples for nutrients, chlorophyll-*a* and bacterioplankton abundance in: (i) the environmental control (CTRA) before the experiment, (ii) in the total fraction (TF) at time 0 of the experiment, and (iii) the TF of all the microcosms at the end of the experiment (day 5). We also collected samples of the fractions F1 (25–100 µm for fungi) and F4 (0.22–3 µm for bacterioplankton) to determine the use of carbon substrates in the main heterotrophic groups of microbes.

We collected 500 mL to analyze MDH and 500 mL to determine microbial biomass (adenosine triphosphate concentration) to evaluate the effect of Azamethiphos on enzyme activity. Five mL of each fraction (TF, F1: 25–100 µm, F2: 5–25 µm, F3: 3–5 µm, and F4: 0.22–3 µm) was collected every 24 h during the 5 days of the experiment to determine extracellular activity of β-glucosidase (β-glu).

2.3. . Determining Azamethiphos concentration

Samples were collected at the sampling sites (CTRA) to rule out the prior presence of Azamethiphos. 500 mL of water was collected in glass bottles to assess the degradation of Azamethiphos by microorganisms after five days. These samples were frozen at −20 °C and immediately sent to the company SGS Chile (Puerto Varas, Chile) for analysis using high performance liquid chromatography (HPLC) with a fluorescence detector.

The effect of hydrolysis on the Azamethiphos concentrations used in the experiments was estimated based on the Scottish Environmental Protection Agency (SEPA, 2008). Linear hydrolytic decomposition of Azamethiphos was assumed based on the results reported by SEPA (2008), which found 1% degradation at 3 h and 21% at 3 days, and a half-life of 8.9 days. Therefore, in the case of our experiments, hydrolytic decomposition of Azamethiphos of 28.1% was estimated at 5 days of experimentation.

2.4. Nutrients

The macronutrients PO_4, $Si(OH)_4$, NO_2 and NO_3 were determined by passing water samples through 0.7 µm GF/F filters. The analyses were performed using a Technicon® autoanalyzer II at the Biogeochemistry Laboratory of the Oceanography Department of the Universidad de Concepción. PO_4, NO_2, and NO_3 were measured according to Strickland and Parsons (1972) and $Si(OH)_4$ was determined according to Technicon (1977).

2.5. Chlorophyll-a

Chlorophyll-*a* concentration was determined according to Holm-Hansen et al. (1965) from the extracts of 0.3 L of seawater passed through 0.7-µm GF/F filters. The extraction was conducted with acetone at 90%, and the chlorophyll-*a* concentration was measured by fluorescence with a fluorometer (Trilogy, Turner Designs).

2.6. Bacterioplankton abundance

To estimate bacterioplankton abundance (cell mL^{-1}), 50 mL of water stored in Falcon tubes was fixed with formalin (1% final concentration) and subsequently stored in the dark at 4 °C. The samples (2 mL) were stained in triplicate with 4′6-diamidino-2-phenylindole (DAPI, 5 µg mL^{-1} final concentration) over black polycarbonate filters (0.2 µm), in accordance with Porter and Feig (1980). Bacteria were counted with an epifluorescence microscope (Axioscope 2 plus, Zeiss).

2.7. Aerobic respiration

Oxygen consumption in the microcosms was determined with a fiber optic oxygen meter (FIBOX 3, PreSens) with optode sensors. Oxygen consumption in the microcosms in the TF was measured daily. Respiration rates were measured with Azamethiphos concentrations of 0, 2, 10, 100 and 1000 µg L^{-1} for Sites 1 and 3, and at concentrations of 0 and 100 µg L^{-1} for Site 2.

2.8. Determination of adenosine triphosphate (ATP)

ATP concentration is used as an indicator of viable microbial biomass (Karl, 1993). ATP was quantified by a bioluminescence assay (Holm-Hansen and Booth, 1966; Bulleid, 1978; Karl, 1993) with a luminometer (GloMax, Promega).

2.9. Effect of Azamethiphos on the enzymatic activity of microbial plankton communities

2.9.1. Enzymatic activity of β-glucosidase

The enzymatic activity of β-glucosidase (EC 3.2. 1.3) was analyzed in fluorometric assays in microplates based on methylumbelliferone with MUB-β-D-glucopyranoside, using the methodology proposed by Jackson et al. (2013). The fluorescence readings were made with a Synergy™ 2 Multi-Mode Microplate Reader (Biotek).

2.9.2. Malate dehydrogenase (MDH) enzymatic activity

MDH activity was assessed using a modified version of the methodology proposed by Schiedek (1997). The extraction media (homogenization buffer) consisted of 200 mM phosphate buffer (K_2HPO_4), pH 7.9, 1 mM dithiothreitol (DTT), 0.3% (*w/v*) polyvinylpyrrolidone (PVP), 5 mM EDTA, 0.1% (*v/v*) Triton X-100, and 3% (w/v) bovine serum albumin (BSA). Samples were homogenized in an Ultra-Turrax for 15 s at maximum speed with short spins on an ice bath. Temperature was kept at 4 °C during the homogenization procedure to avoid enzymatic activity losses. The homogenized samples were centrifuged at 5000 rpm for 5 min at 4 °C, and the supernatants were used for enzyme assays.

The activity of MDH was assayed as it catalyzed the formation of malate from oxaloacetate, using a general procedure modified from Childress and Somero (1979) and Vetter et al. (1994). The assay medium contained 80 mM K_2HPO_4 buffer pH 7.9 at 20 °C, 0.1 mM NADH, 150 µM $MgCl_2$ $6H_2O$ and 0.2 mM oxaloacetate. Absorption was monitored at 340 nm following the addition of the supernatant. MDH activity measurements were corrected for nonspecific NADH oxidation.

Since ATP is used as a proxy variable of microplankton biomass, we used the term "specific activity" as units of "MDH activity/ATP units" (González and Quiñones, 2009).

2.10. Effect of Azamethiphos on metabolic profiles of microbial communities

2.10.1. Effect of Azamethiphos on the metabolic profiles of bacteria

The Ecoplate™ (Biolog®) system was used to measure and assess changes in the metabolic profiles of bacterioplankton communities in the water columns of sites 2 and 3. The Ecoplates were composed of 96 wells containing 31 different carbon sources and a control without a substrate; all in triplicate (Frąc et al., 2012). To obtain just the bacterioplankton fraction, 500 mL of the samples were concentrated into 50 mL of the < 3 µm fraction in a 0.22 µm filter. 120 µL of the sample was inoculated in each well. The sample turns purple when the microbes use the carbon source and begin to respire. The respiration of the cells in the community reduces a tetrazolium dye included in the carbon source. The microplates were incubated at 26 °C, and optical density (OD) was measured every 24 h (λ = 590 nm) with a microplate reader (ELx800 Epoch™, BIOTEK®).

2.10.2. Effect of Azamethiphos on the metabolic profiles of marine fungi

The FF Microplate (Biolog®) system was used to characterize and assess changes in the metabolic profile of fungal communities in the water column (e.g. Shengnan et al., 2011; Fuentes and Quiñones, 2016) of sites 2 and 3.

The Biolog FF MicroPlates test panel contained 95 wells with different carbon-containing compounds and 1 well with water as a control. Iodonitrotetrazolium violet (INT) was used as a redox dye to colorimetrically measure the mitochondrial activity (redox value) resulting from oxidation of metabolizable carbon sources. All the wells were colorless when first inoculated. The oxidation of succinate to fumarate in the citric acid cycle, mediated by succinate dehydrogenase and FAD, reduces INT to a violet formazan dye with peak absorbance at 490 nm (Kubicek et al., 2003; Tanzer et al., 2003). Reduction of the tetrazolium dye due to increased cell respiration turns the well purple (Tanzer et al., 2003). The reduction of INT and production of colored formazan is irreversible, and the accumulation of formazan, measured spectrophotometrically, reflects quantitatively the oxidation of the test substrate. The 750 nm reading measures turbidity, reflecting mycelial production of the tested substrate. Because the absorbance spectrum of hyaline mycelium is essentially level in the range of 490 to 750 nm, a corrected redox value (CRV) for the production of formazan is obtained by subtracting the 750 nm reading from the 490 nm reading (490–750 nm) (Kubicek et al., 2003). The CRV of each well was corrected by subtracting the control value (water).

To inoculate the FF microplates, 500 mL of the 25–100 µm fraction was concentrated to 50 mL, and 120 µL were inoculated in the wells. The microplates were incubated at 26 °C and the optical density (λ = 490 and 750 nm) of each well was measured every 24 h with a microplate reader (ELx800 Epoch™ de Bio Te®). The 25–100 µm fraction has been reported as the size range in which the highest abundance of marine fungi has been found off the Chilean coast (Gutiérrez et al., 2011).

2.10.3. Statistical analysis of metabolic profiles

Average well color development (AWCD) of all the substrates expresses the general use of the carbon substrate by the microbial community, which is calculated with the following equation: AWCD = Σ (C - R) / N, where C is the optical density (OD) of the carbon source (well), R is the OD of the control and N is the number plate substrates. The substrate OD levels were adjusted by subtracting the control well OD and negative readings. The AWCD of all the treatments was compared using a Kruskal-Wallis test and multiple comparison analyses (z values) using Statistica v.13 software.

The substrates were divided into six categories (polymers, amino acids, carbohydrates, amines, carboxylic acids and miscellaneous), in accordance with Buyer and Drinkwater (1997). The corrected absorbance values of the substrates were expressed as a percentage of total absorbance of the plate for a particular treatment (Frąc et al., 2012). The AWCD and mean OD of the six types of substrate were compared with a Kruskal-Wallis test and the different treatments with a multiple comparison analysis (z score) with Statistica v. 13. The substrates used most often in the Ecoplates and FF microplates were analyzed with the metabolic KEGG collection (http://www.genome.jp/kegg/pathway.html) from the two sites.

2.11. Statistical analysis

All statistical analyses were performed using the Statistica v.13 software. The normality of the distribution of all data was verified using Kolmogorov-Smimov tests.

We used a one-way analysis of variance (ANOVA) and the Tukey HSD post-hoc test to evaluate the differences between treatments in nutrients, and bacterioplankton abundance. The respiration results were evaluated with a 2-way ANOVA and a Tukey HSD post-hoc test.

For the analysis of β-glucosidase and MDH/ATP, the data were first 4th root transformed and then analyzed with a factorial ANOVA (factors: Day, Treatment, Day*Treatment) and Tukey HSD tests. For substrate utilization, the mean AWCD and OD of the six substrate categories were compared by the Kruskal-Wallis test, and the different treatments were compared with an analysis of multiple comparisons (z' value). The data for both microplates in each site were ordered using a joining cluster analysis.

3. Results

3.1. Oceanographic variables in the study zones

The oceanographic conditions at site 1 in Coliumo Bay showed values expected for the studied season. At 5 m deep, temperature was 13.5 °C and salinity was 34.5, while at a greater depth the mean

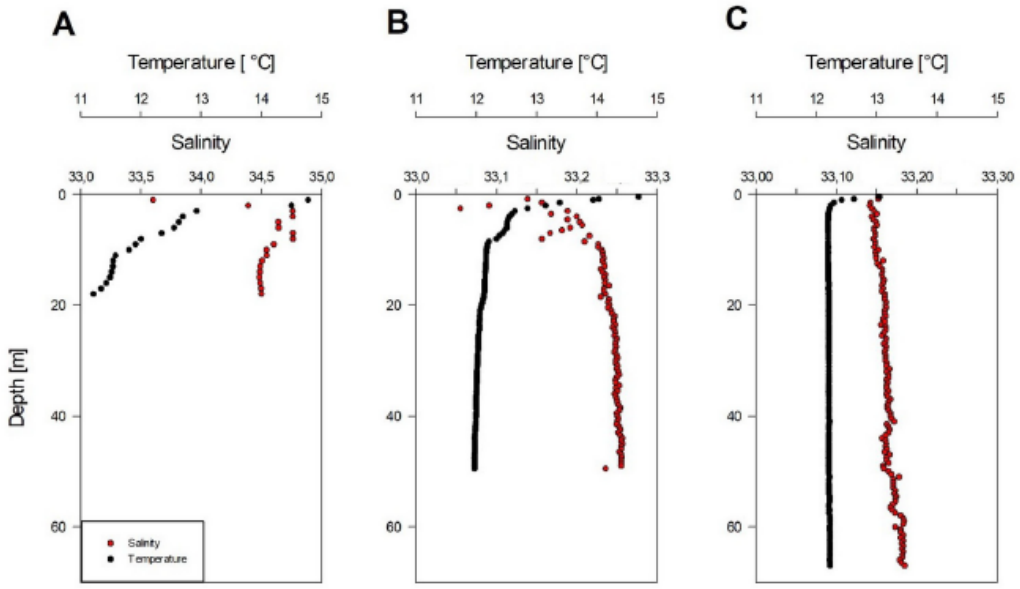

Fig. 2. Oceanographic temperature and salinity profiles of the water column at (A) site 1, (B) site 2, and (C) site 3.

temperature went down to 11.7 °C, while the salinity was constant up to 18 m down (Fig. 2A). At sites 2 and 3 (Achao, Chiloé), the mean temperature in the first 10 m was 12.3 °C and salinity was 33.2 and 33,15 respectively. The latter remained constant at greater depths (Fig. 2B and C).

3.2. Degradation of Azamethiphos in the microcosms

Azamethiphos was not found in the CTRA of the three study sites (Fig. 3). Azamethiphos was more degraded in the sites 2 and 3 treatments, with levels below the detection limit ($< 1.4\,\mu g\,L^{-1}$) in the 2 and 10 $\mu g\,L^{-1}$ treatments. The average level of degradation for site 2 was 70.0% (84.0% and 56% for the 100 and 1000 $\mu g\,L^{-1}$ treatments, respectively), while the average level of degradation for site 3 was 74.7% (77.0% and 72.4% for the 100 and 1000 $\mu g\,L^{-1}$ treatments, respectively). In contrast, the average level of Azamethiphos degradation for site 1 was 26.1% (21.0%, 25.9%, and 31.5% for the 10, 100 and 1000 $\mu g\,L^{-1}$ treatments respectively) at 5 days, which was close to the estimated 28% degradation at 5 days due to hydrolysis (Fig. 3).

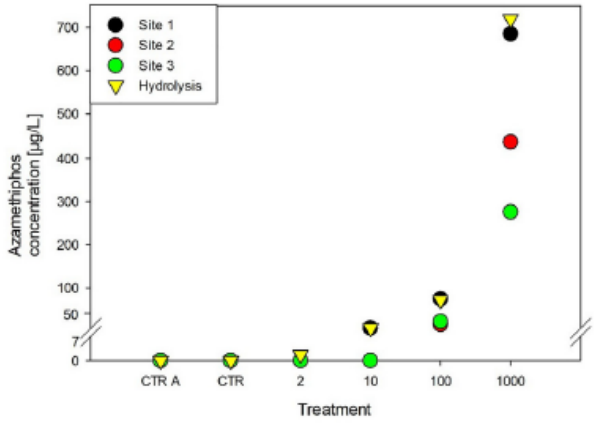

Fig. 3. Initial Azamethiphos concentrations at the sampled sites (CTRA: Environmental control), and the decrease in Azamethiphos concentrations in the controls, and treatments with 2, 10, 100 and 1000 $\mu g\,L^{-1}$ at sites 1, 2 and 3 measured at day 5 of the experiment. The hydrolytic decomposition of Azamethiphos was calculated based on SEPA (2008). See Section 2.3.

3.3. Nutrients in microcosms with Azamethiphos

Higher environmental concentrations of NO_3, PO_4 and $Si(OH)_4$ were found at site 1, while NO_2 was higher at sites 2 and 3.

All nutrients had increased by day 5 in the CTR of site 1 compared to its CTRA (Table 1). NO_3, PO_4 and $Si(OH)_4$ in treatments 2 and 10 $\mu g\,L^{-1}$ of site 1 remained near the values of the CTR, while in the 100 and 1000 $\mu g\,L^{-1}$ treatments, the values were closer to those of the initial CTRA. In treatments 10 and 1000 $\mu g\,L^{-1}$, NO_2 increased similarly to that of the CTR, and in the concentrations 2 and 1000 $\mu g\,L^{-1}$ the NO_2 concentration was close to the initial level of the CTRA (Table 1).

Table 1
Nutrient concentrations [$\mu mol\,L^{-1}$] NO_2, NO_3, PO_4 and $Si(OH)_4$ at time 0 (CTRA: environmental control) and after 5 days in different treatments with Azamethiphos (CTR, 2, 10, 100 and 1000 $\mu g\,L^{-1}$) at sites 1, 2 and 3.

Site	Treatment	NO_2	NO_3	PO_4	$Si(OH)_4$
Site 1	CTRA	0.37	18.52	1.31	12.12
	CTR	0.52	27.70	1.86	16.72
	2	0.30	27.21	1.70	14.92
	10	0.54	28.20	1.79	15.45
	100	0.50	24.71	1.53	13.30
	1000	0.33	17.52	1.18	10.83
Site 2	CTRA	0.78	14.98	0.94	7.42
	CTR	0.27	13.48	0.95	7.77
	2	0.19	12.77	0.84	6.82
	10	0.09	9.89	0.72	6.24
	100	0.16	15.58	1.21	10.07
	1000	0.58	13.43	0.79	6.26
Site 3	CTRA	0.79	16.21	1.06	8.44
	CTR	0.16	17.57	1.21	9.82
	2	0.18	16.71	1.20	9.96
	10	0.17	17.52	1.10	8.92
	100	0.15	16.24	1.14	9.73
	1000	0.47	18	1.15	9.51

CTR: experimental control.

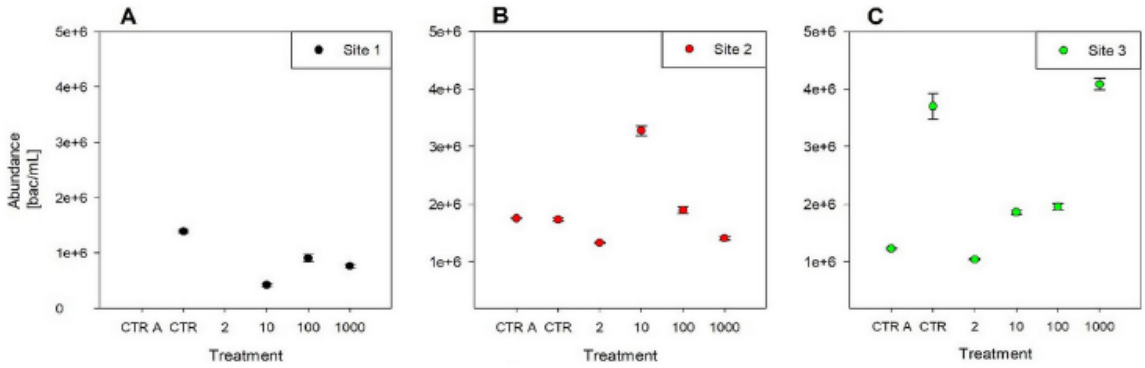

Fig. 4. Bacterioplankton abundance before (CTRA: Environmental control) and after 5 days of exposure to four concentrations of Azamethiphos (CTR, 2, 10, 100 and 1000 µg L^{-1}), at (A) site 1, (B) site 2 and (C) site 3.

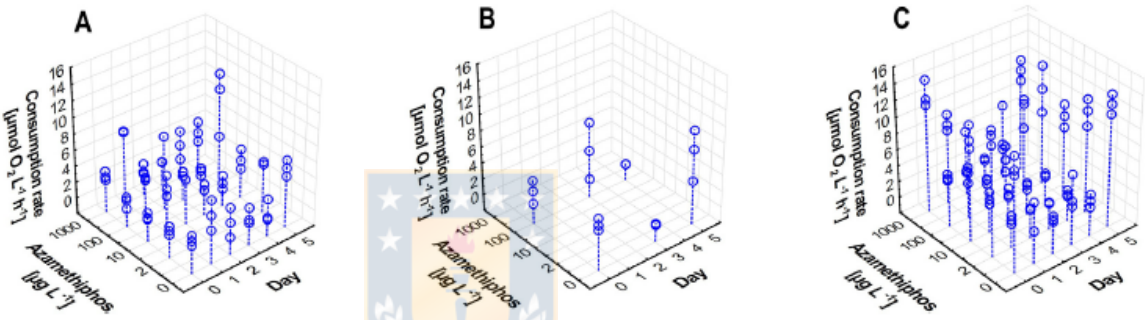

Fig. 5. Oxygen consumption of the total fraction (i.e. < 100 µm) in the microcosm experiments carried out with microbial communities from the three sampling sites over 5 days. (A) Site 1 at Azamethiphos concentrations of CTR, 2, 10, 100 and 1000 µg L^{-1}; (B) Site 2 at the concentrations of CTR and 100 µg L^{-1} Azamethiphos and (C) Site 3 at the concentrations of CTR, 2, 10, 100 and 1000 µg L^{-1} Azamethiphos.

Comparing the CTRA to the microcosm CTR in sites 2 and 3, NO_2 decreased, while NO_3, PO_4 and $Si(OH)_4$ remained constant (Table 1).

After 5 days, the nutrient levels in the 2 and 1000 µg L^{-1} treatments at site 2 were similar to those of the CTR, except for the NO_2 in treatment 1000 µg L^{-1}, which increased to 0.58 µmol L^{-1}. In treatment 10 µg L^{-1}, there was a slight decrease in the nutrients NO_2 NO_3, PO_4 and $Si(OH)_4$ to 0.09, 9.89, 0.72 and 6.24 µmol L^{-1}, respectively. There was an increase of NO_3, PO_4 and $Si(OH)_4$ in treatment 100 µg L^{-1} (Table 1).

All treatments with Azamethiphos in site 3 maintained similar values to those of the CTR for NO_2, NO_3, PO_4 and $Si(OH)_4$ after 5 days, with means of 0.23, 17, 1.1 and 9.4 µmol L^{-1}, respectively.

3.4. Abundance of bacterioplankton in microcosms with Azamethiphos

In the treatments with Azamethiphos, bacterioplankton abundance was significantly different from the control in the three sites. In site 1, control bacterioplankton abundance was 1.39E+06 bac. mL^{-1}, while the mean of treatments 10, 100 and 1000 µg L^{-1} was ~6.94E+05 bac. mL^{-1} (Fig. 4; Supplementary Table 1).

Bacterioplankton abundance in the site 2 CTR remained constant after 5 days, ~1.75E+06 bac. mL^{-1}. The mean abundance of treatments 2, 100 and 1000 µg L^{-1} was ~1.5E+06 bac. mL^{-1}, while in treatment 10 µg L^{-1}there was an increase to 3.28E+06 bac. mL^{-1} (Fig. 4B; Supplementary Table 1). In site 3 there was an increase in abundance from day 0 to 5 from 1.2E+06 to 3.70E+06 bac. mL^{-1}, and in treatment 1000 µg L^{-1}, abundance was 4.09E+06 bac. mL^{-1}, while the mean of treatments 2, 10 and 100 µg L^{-1} was ~1.62E+06 bac. mL^{-1}, near the bacterioplankton abundance in CTRA (Fig. 4C; Supplementary Table 1).

3.5. Chlorophyll-a in microcosms with Azamethiphos

Environmental chlorophyll-*a* concentrations were much lower in site 1 than in the southern sites. Chlorophyll-*a* decreased in the site 1 CTR from 0.49 mg m^{-3} at day 1 to 0.2 mg m^{-3}at day 5. The concentrations in treatments with 2, 10, 100 and 1000 µg L^{-1} were similar to that of CTR (~0.15 mg m^{-3}). Chlorophyll-*a* levels in the CTR of sites 2 and 3 remained constant from day 1 to day 5 (~2 and 0.9 mg m^{-3}), while the levels in treatments 2, 10, 100 and 1000 µg L^{-1} remained near those of the respective CTR.

3.6. Microbial respiration in microcosms with Azamethiphos

All the experiments began under aerobic conditions, with oxygen concentrations over 200 µmol O_2 L^{-1} (4.4 mL L^{-1}). The highest respiration rates were measured in site 3 (ca. 14 µmol O_2 L^{-1} h^{-1}), although with highly variable responses between treatments and over time. The mean oxygen consumption of the TF (< 100 µm) in the microcosms with different treatments with Azamethiphos in sites 1, 2 and 3 were 2.67, 2.8 and 5.4 µmol O_2 L^{-1} h^{-1}, respectively (Fig. 5). The 2-way ANOVA found a significant difference in site 3 among treatments ($F_{5,60}$) =144.61, p = .000; Supplementary Table 2), and the Tukey post-hoc test indicated that treatment 10 µg L^{-1} (p = .010) accounted for the difference with the CTR (Supplementary Tables 3).

There were differences between experimental days in sites 1 ($F_{5,48}$ = 35.44, p = .000) and 3 ($F_{5,60}$ = 144.61, p = .000) (Supplementary Tables 4). Oxygen consumption at site 1 increased from 2 to 3.7 µmol O_2 L^{-1} h^{-1} by day 5, while it decreased at site 3 from 11.6 O_2 L^{-1} h^{-1} at day 2 to ~2.6 µmol O_2 L^{-1} h^{-1} at day 4, and increased to ~10.6 µmol O_2 L^{-1} h^{-1} by day 5 (Fig. 5).

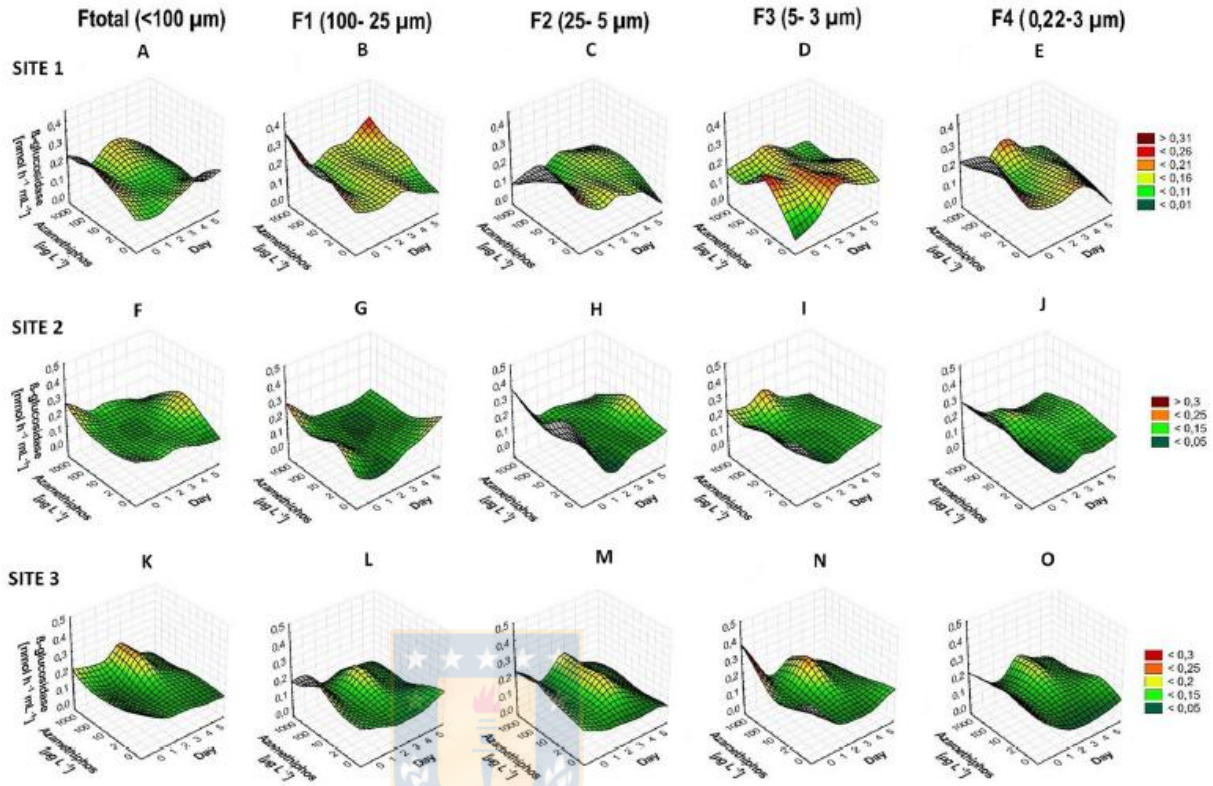

Fig. 6. β-glucosidase microbial enzyme activity over the 5 days of the experiments at 3 concentrations of Azamethiphos (10, 100 and 1000 µg L^{-1}) in addition to CTR. β-glucosidase activity of the microbial communities from site 1 (A) Total Fraction (TF), (B) Fraction 100–25 µm (F1), (C) Fraction 25–5 µm (F2), (D) 5–3 µm (F3), (E) Fraction 0.22–3 µm (F4); site 2 (F) TF, (G) F1, (H) F2, (I) F3, (J) F4; and site 3 (K) TF, (L) F1, (M) F2, (N) F3, (O) F4.

3.7. Enzyme activity of β-glucosidase in microcosms with Azamethiphos

The mean enzyme activity of β-glucosidase of the microbial community from sites 1, 2 and 3 was 0.142 (± 0.072), 0.114 (± 0.059), 0.126 (± 0.074) nmol h^{-1} mL^{-1}, respectively. The response of microbial β- glucosidase activity to Azamethiphos is highly variable, but there is a tendency for activity to increase in the smaller-size fractions [F3 (3–5 µm) and F4 (0.22–3 µm)] when exposed to high Azamethiphos concentrations. In site 1, only fraction F3 showed a significant difference in activity among treatments in the concentration of 10 µg L^{-1} (Fig. 6D; F:3.275, p:0.029). There were significant differences in the β-glucosidase activity of fractions F1, F2, F3 and F4 among the days (Figs. 6B-E; Supplementary Table 5). No significant differences were found for the TF between days or treatments, but the tendencies were the same as in the other fractions (Fig. 6A; Supplementary Tables 5, 6 and 7).

In site 2, there were significant differences among days in fractions F1, F2, F3 and F4, due to an increase in activity over time (days), related to greater concentrations of Azamethiphos (Figs. 6G-J; Supplementary Tables 6 and 7). There were also significant differences in treatments 100 µg L^{-1} (F2 and F4) and 1000 µg L^{-1} (F2), which increased compared to their respective CTR (Supplementary Table 7). There were significant differences in site 3 among days in all fractions (Figs. 6K-O; Supplementary Tables 5 and 6), due to an increase in the β-glucosidase activity on day 4 in the treatments with higher concentrations of Azamethiphos (notably greater at 100 µg L^{-1}).

3.8. Malate dehydrogenase activity in microcosms with Azamethiphos

Mean specific enzyme activity measured as MDH/ATP in sites 1, 2 and 3 was 1.673 (± 1.25), 1.428 (± 1.14) and 2.082 (± 1.55) pmol NADH min^{-1} pg ATP^{-1}, respectively (Fig. 7). In the TF of site 1 there was a significant decrease in MDH activity in treatment 10 µg L^{-1} compared to the CTR (Supplementary Table 8 and 9), and differences among days (Fig. 7A; Supplementary Table 10), while there was a significant increase in specific activity in the treatment with 1000 µg L^{-1} in site 3 (Fig. 7K; Supplementary Tables 8, 9 and 10).

MDH activity in site 1 increases in F1, F2 and F4 at higher concentrations. In F4 (on day 4) there was a peak in MDH activity in the treatment with 1000 µg L^{-1} of Azamethiphos (Figs. 7B-E).

There was an increase in MDH activity at site 2 in the F1, F2, F3 and F4 fractions at higher Azamethiphos concentrations (Figs. 7G-J). The activity increased more rapidly in the smallest fractions.

MDH activity was more heterogeneous in site 3. There were two peaks in fraction F1 on day 2 with the treatments with the highest level of Azamethiphos, and on day 5 in the treatment with 10 µg L^{-1}. Fraction F2 peaked on day 5 in treatment 1000 µg L^{-1} (Figs. 7L-O).

3.9. Utilization of carbon substrates by bacterioplankton from sites 2 and 3 in microcosms with Azamethiphos

The Kruskal-Wallis test ($H_{1,186} = 2.81$, $p = .093$) indicated that there were no significant differences initially between sites 2 and 3 in substrate use by bacterioplankton. Significant differences were found in site 2 in the treatments with 2, 10 and 1000 µg L^{-1} of Azamethiphos (Fig. 8B; Supplementary Table 11). This was due to a generally low

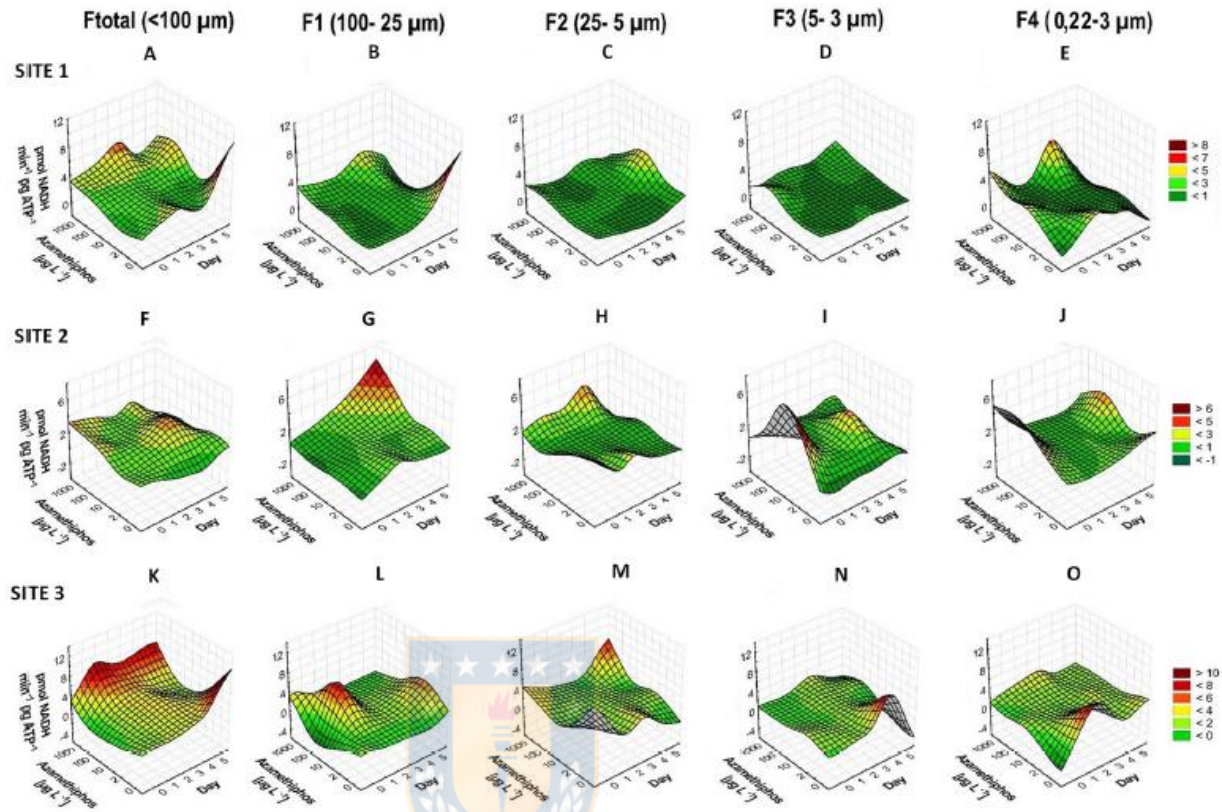

Fig. 7. MDH/ATP microbial enzyme activity over the 5 days of the experiment at 3 concentrations of Azamethiphos (10, 100 and 1000 µg L^{-1}) in addition to CTR. MDH/ATP activity of the microbial communities from site 1 (A) Total Fraction (TF), (B) Fraction 100–25 µm (F1), (C) Fraction 25–5 µm (F2), (D) 5–3 µm (F3), (E) Fraction 0.22–3 µm (F4); site 2 (F) TF, (G) F1, (H) F2, (I) F3, (J) F4; and site 3 (K) TF, (L) F1, (M) F2, (N) F3, (O) F4.

level of microbial activity in the treatments with 2 and 10 µg L^{-1} (AWCD = 0.059 and 0.067) and increased substrate use in treatment 1000 µg L^{-1} (AWCD = 0.577) compared to the CTR (Fig. 8A).

In site 3, multiple comparisons of color development between the CTR and Azamethiphos treatments indicated significant differences in treatments 100 and 1000 µg L^{-1} (Fig. 8B; Supplementary Tables 11 and 12). This was produced by greater substrate use in treatment 100 µg L^{-1} and less use in treatment 1000 µg L^{-1} (Supplementary Table 11). However, no significant differences were found when the substrates were grouped (Supplementary Table 12).

The difference in treatment 2 µg L^{-1} was produced by decreased carbohydrate use ($z' = 3.139$, $p = .025$), and in treatment 10 µg L^{-1} by decreased carboxylic acid use. Although all substrate use was greater than the CTR levels in the treatment with 1000 µg L^{-1}, only carbohydrate and polymer use were significantly higher (Supplementary Table 12; Supplementary Fig. 1B).

The routes used by bacterioplankton at both sites were mainly carbohydrate metabolism (Figs. 9A and B). In the CTR of site 2, bacterioplankton used substrates of the miscellaneous group, the metabolic routes of glycolysis, the Krebs cycle (glucose-1-phosphate, DL-α-glycerol phosphate, methyl ester of pyruvic acid), carbohydrates of glycolysis metabolism (i-erythritol, D-cellobiose) and amino acids (glycyl-L-glutamic acid, L-threonine). Substrate use in the treatment with 1000 µg L^{-1} was less than in the CTR, and the most used substrates were carboxylic acids (γ-hydroxybutyric acid, α-ketobutyric acid, 2-hydroxybenzoic acid) (Fig. 8A).

The substrates used by bacterioplankton in the site 3 CTR were also from the carbohydrate group (β-methyl-D-glucoside, i-erythritol, D-xylose, α-D-lactose), suggesting the use of metabolic pathway such as glycolysis, and the carboxylic acid group (D-malic acid, γ-hydroxybutyric acid). This tendency was maintained in all treatments with Azamethiphos; the substrates most used were also from the carbohydrate group (*N*-acetyl-D-glucosamine, β-methyl-D-glucoside, i-erythritol), but at higher pesticide concentrations (100 and 1000 µg L^{-1}). Amino acids (L-arginine, L-asparagine) and a carboxylic acid (D-malic acid) were also used.

3.10. Use of carbon substrates by marine fungi (25–100 µm fraction) from sites 2 and 3 exposed to Azamethiphos

The Kruskal-Wallis test ($H_{1,380} = 77.92$, $p = .000$) indicated that there were initially significant differences between sites 2 and 3 in the use of substrates by marine fungi. There was a significant difference in site 2 between CTR and CTRA ($z' = 4.65$, $p = .000$) and between CTR and treatment 2 ($z' = 3.52$, $p = .006$) (Fig. 9A; Supplementary Table 12). This appears to be due to lower overall activity of the fungal community in CTRA (AWCD = 0.059) and in treatment 2 (AWCD = 0.086) compared to CTR (AWCD = 0.166). No significant differences were observed between the utilization of substrate groups, which indicates that the differences were due to different overall use (i.e. the entire microplate) rather than to the use of a specific group of carbon substrates (Supplementary Table 12; Supplementary Fig. 1C).

There was less microbial activity in the site 3 treatments with 2 µg L^{-1} (AWCD = 0.135) and 100 µg L^{-1} (AWCD = 0.114) than in the CTR (AWCD = 0.210) (Fig. 9B, Supplementary Table 12). However, comparing carbon substrate use, the only difference found was in the

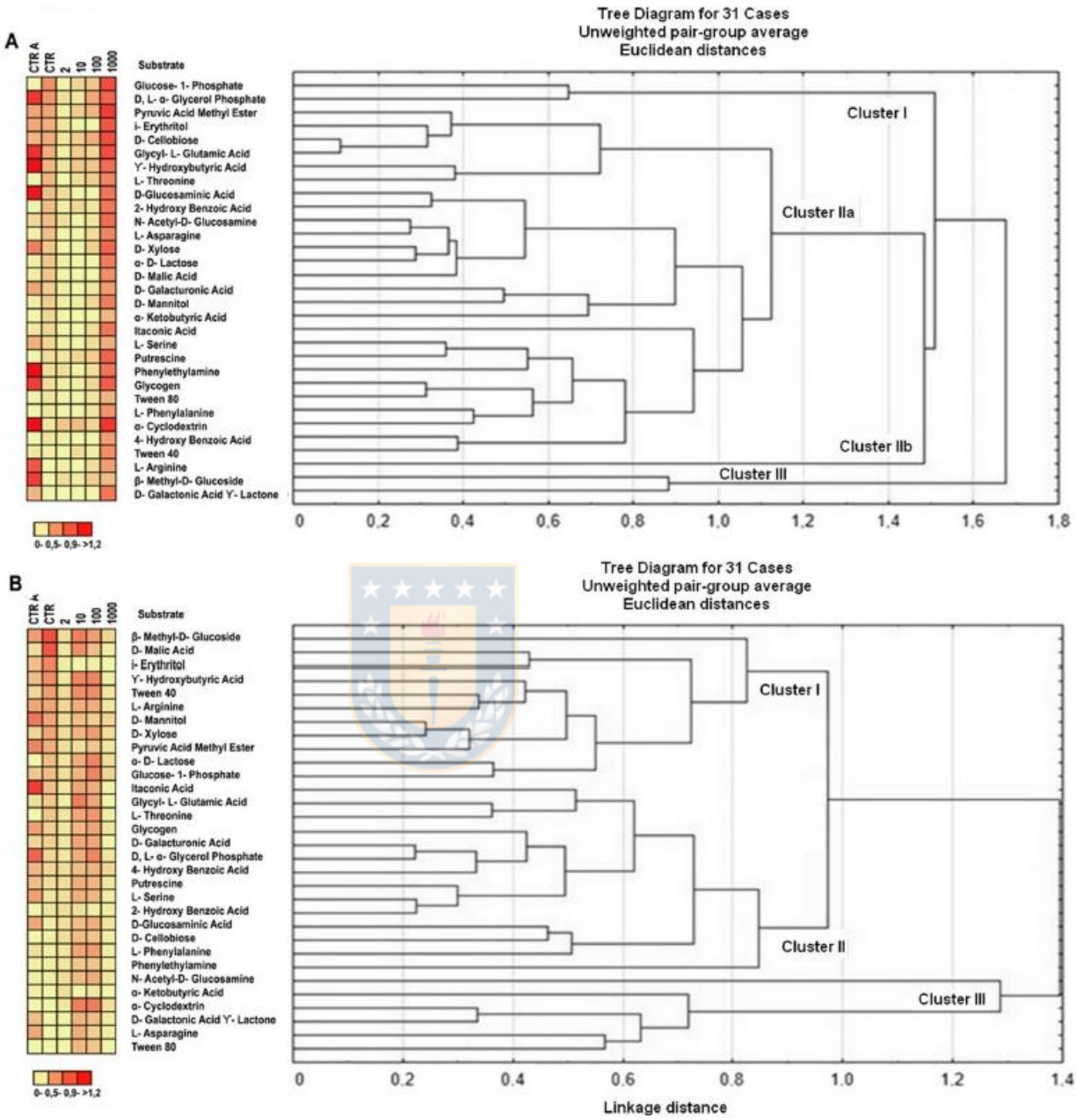

Fig. 8. Use of 31 substrates by bacterioplankton from (A) site 2 and (B) site 3 in CTRA and at day 5 of the experiment at different concentrations of Azamethiphos (CTR, 2, 10, 100 and 1000 μg L^{-1}), ordered based on the cluster analysis applied to the substrate use of the control from each area.

use of carboxylic acids in the treatment with 100 μg L^{-1} ($z' = 3.030$, $p = .036$). No significant differences were found for the groups in the treatment with 2 μg L^{-1}, indicating different overall use of carbon substrates (Supplementary Table 12; Supplementary Fig. 1D).

Substrate use in the CTRs was heterogeneous, while the use of carbohydrates and amino acids in the Azamethiphos treatments was notable (Figs. 9C and Supplementary Fig. 1D). The metabolic pathways used preferentially in the site 2 CTR were those of fructose and mannose, and the amino acids (L-glutamic acid, proline, serine, tyrosine). In all the treatments with the pesticide, the fungi used compounds that participate in the metabolism of starch and the carbohydrates trehalose, mannitol, sucrose and dextrose. One of the main substrates used in all treatments with the pesticide, and especially the treatment with 1000 μg L^{-1}, was α-keto-glutaric acid, which has been identified in the metabolic degradation of xenobotic compounds.

According to our analysis, the metabolic routes most likely used by fungi in the site 3 CTR were pentose-phosphate (D-gluconic acid), carbohydrates (D-galactose, D-fructose) and the Krebs cycle (L-malic acid).

One of the substrates most used in the CTRA and CTR was α-keto-glutaric acid, which is implicated in xenobiotic metabolism. There was also use of D-glucuronic acid in treatments with 2 and 100 μg L^{-1}. This

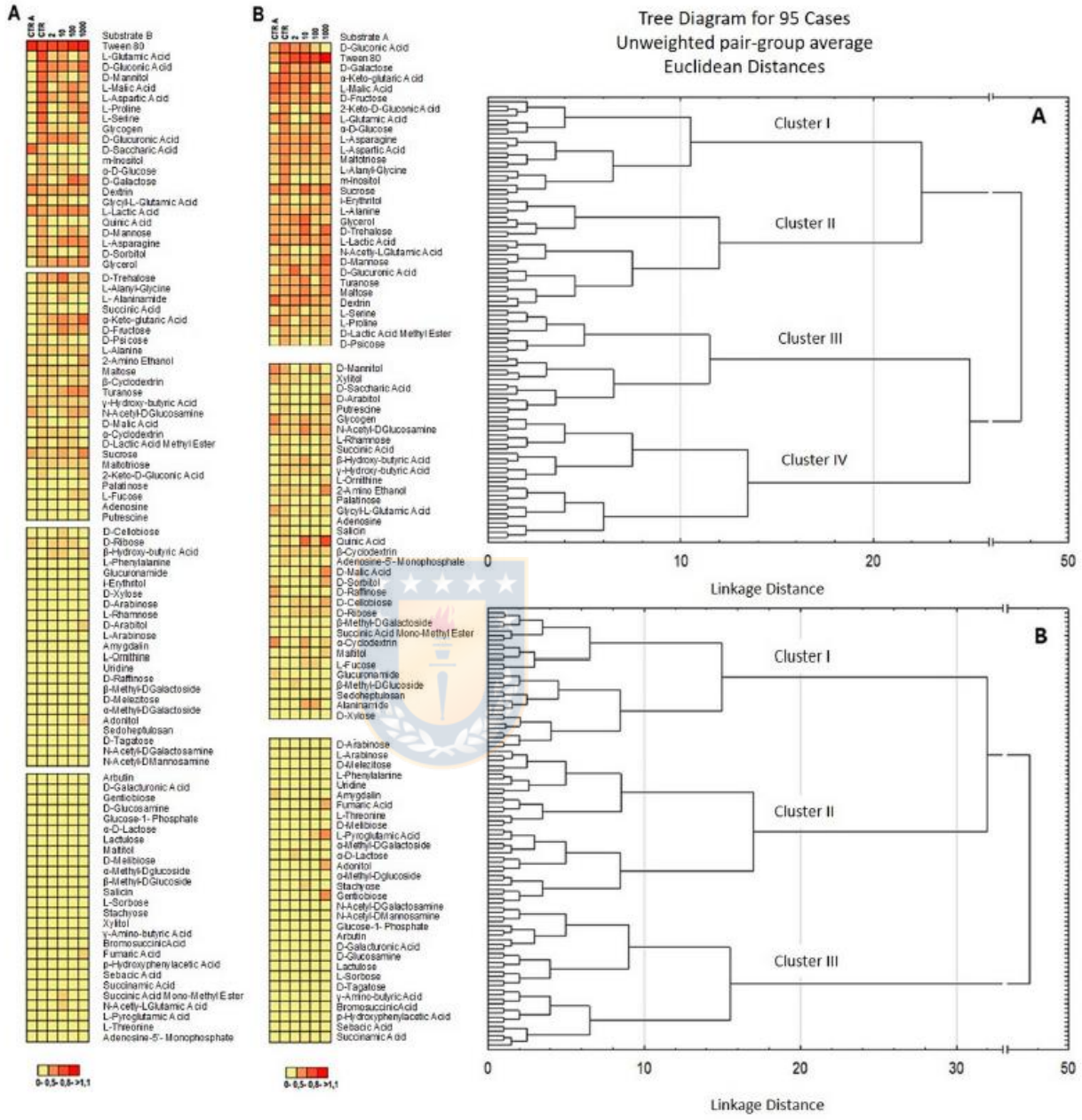

Fig. 9. Use of 95 carbon substrates by marine fungi from (A) site 2 and (B) site 3 in CTRA and at day 5 of the experiment at different concentrations of Azamethiphos (CTR, 2, 10, 100 and 1000 μg L^{-1}), ordered based on the response of the control at day 5.

substrate has also been implicated in xenobiotic metabolism.

4. Discussion

As pesticide use increases, the impact of residues on aquatic ecosystems is also likely to increase. It is particularly important to understand the effect of pesticides on microbial communities given their role in these ecosystems (Verma et al., 2014; Staley et al., 2015). This study involved experiments with microcosms of microbial communities from two areas, one without fish farming (Coliumo Bay, site 1) and the other two with intensive levels of salmon farming (Achao, Chiloé, sites 2 and 3), with the aim of studying the metabolic response of aquatic microorganisms (< 100 μm) to five days of exposure to the pesticide Azamethiphos under controlled conditions.

4.1. Environmental conditions

The environmental conditions at site 1 in Coliumo Bay (southern central Chile) and at sites 2 and 3 in Achao (Chiloé) were within the expected ranges for the spring season when the sampling took place

(Schneider et al., 2017; Narváez et al., 2019). Differences in surface temperature among sites 1, 2 and 3 were up to 2 °C (Fig. 2). These differences were even less at depths between 5 and 10 m (< 1.2 °C). Differences in salinity among sites 1, 2 and 3 were minor (~1.4) at 10 m deep. The differences observed in salinity and temperature are not relevant considering the natural environmental variability in the studied areas, even at a daily time scale.

Nutrient concentrations in the surface waters of the coastal region of northern Patagonia (Chiloé) are lower (Silva et al., 1997; Iriarte et al., 2007; Jacob et al., 2014; Martínez et al., 2015) than those in southern central Chile (Quiñones et al., 2010; Morales and Anabalón, 2012). The nutrient ratios indicate silicate deficiency (Si:N < 1; and Si:P < 10; Souchu et al., 2010) in the three studied sites, which could explain why chlorophyll-*a* concentrations were not higher in spring-summer (Martínez et al., 2015; Anabalón et al., 2016).

4.2. Azamethiphos degradation

High concentrations of Azamethiphos in the microcosms at 5 days were found in all Coliumo Bay treatments due to low degradation. The ~26.1% Azamethiphos degradation measured in Coliumo experiment was close to the ~28% expected by hydrolysis at 5 days (SEPA, 2008) (Fig. 3). Degradation was much greater at sites 2 and 3 in the Achao area, ~72.4% in all the treatments (Fig. 3). Most organophosphates (including Azamethiphos) are degraded by microorganisms as a source of carbon and/or phosphorous (Kumar et al., 2018; Serasinghe and Manage, 2019). This is accomplished by hydrolysis of alkyl and aryl PO bonds with the help of enzymes such as hydrolases (Cui et al., 2001; Kapoor and Rajagopal, 2011; Gao et al., 2012, Lu et al., 2013), phosphotriesterase and phosphatase (Ortiz-Hernández et al., 2001; Bhadbhade et al., 2002; Sogorb and Vilanova, 2002; Iyer et al., 2013). Azamethiphos degradation occurred in Chiloé sites even though there was no nutrient deficiency or N:P deviations from Redfield stoichiometry during the study.

We suggest that the microbial community is responsible for non-specific Azamethiphos degradation by released enzymes, which depends on: (i) differences in the microbial composition because the two studied areas are oceanographically distinct (e.g. DeLorenzo et al., 2001); and (ii) the possible adaptation of microbial communities to exposure to organophosphate pesticides, given that the studied sites in Achao have been exposed to decades of salmon farming activity. Immersion treatments with organophosphates (metrifonate and dichlorvos) were applied in Chile from 1981 to 2001 (Bravo et al., 2014; Urbina et al., 2018), and subsequently the organophosphate Azamethiphos entered the Chilean market in 2013 (Helgesen et al., 2014). Several authors have suggested the adaptation of microbial communities in areas exposed to organophosphate pesticides, which would be more efficient in degrading them (Racke and Coats, 1988; Coppola, and Castillo, M.d.P., Monaci, E., Vischetti, C., 2007; Itoh et al., 2014). According to Itoh et al. (2014), after the application of organophosphate pesticides in soils, changes were observed in enzymatic activity and biodiversity of resident microbial communities.

Studies have shown adverse impacts of pesticides on microbial biomass and soil respiration (Pampulha and Oliveira, 2006; Zhou et al., 2006). Here we only observed a significant increase in oxygen consumption in the site 3 treatment with 10 $\mu g\,L^{-1}$ (Fig. 5). Although there is no information about the effect of organophosphate pesticides on marine microbial respiration, it has been suggested that most organophosphate insecticides do not adversely affect microbial respiration (Jail et al., 2014). A possible explanation for increased respiration is that microorganisms oxidize insecticides (Tu, 1970; Jones and Ananyeva, 2001; Eisenhauer et al., 2009; Jail et al., 2014).

4.3. Enzymatic activity

We detected differences in β-glucosidase activity, with increased activity mainly in the smaller size fractions F2 (5–25 μm; nanoplankton) and F4 (0.22–3 μm, picoplankton) (Fig. 6). In site 2, nanoplankton increased in the 100 and 1000 $\mu g\,L^{-1}$ treatments, and picoplankton in the 100 $\mu g\,L^{-1}$ treatment, while in site 3, nanoplankton increased in the 10 $\mu g\,L^{-1}$ treatment and picoplankton increased in all the treatments. The F2 fraction includes a wide variety of organisms like fungi, dinoflagellates, flagellates and ciliates, which dominate the study zone mainly during periods of lower productivity (Alves-de-Souza et al., 2008; Montero et al., 2011, 2017). Together with the picoplankton fraction (bacteria, archaea), fungi may benefit from phosphorous inputs. Working in the Aysén Fjord, Czypionka et al. (2011) highlighted the complexity of the effects of variation in nutrients on nanoplankton; changes in phosphorous concentrations could change the trophic state of nanoflagellates. Studies have shown that several bacteria (Itoh et al., 2014) and fungi (Pandey and Singh, 2004) groups increase their metabolic rates in the soil when exposed to pesticides.

Specific (MDH/ATP) enzymatic activity decreased in the < 100 μm fraction in the 10 $\mu g\,L^{-1}$ site 1 treatment and increased in the 1000 $\mu g\,L^{-1}$ treatment, while enzymatic activity in the other treatments was near that of the CTR (Fig. 7). Increased MDH activity indicates increased remineralization of organic matter (Martínez et al., 2002), and is an indicator of microbial activity in microplankton communities (Quiñones et al., 2006; González and Quiñones (2009). MDH/ATP activity levels for sites 1, 2 and 3 were 1.673, 1.428 and 2.082 pmol NADH min^{-1} pg ATP^{-1}, respectively (Fig. 7), which were higher than those found by González and Quiñones (2009). This could be because microbial biomass (i.e. ATP concentration) in the three sites (TF means: Coliumo = 0.021; Achao-site 2 = 0.022; Achao-site 3 = 0.023 μg ATP L^{-1}) were lower than the 0.2 μg ATP L^{-1} in southern central Chile, and the 0.05 μg ATP L^{-1} in northern Chile reported by González and Quiñones (2009), showing that there was less microbial biomass in our study.

Our results indicate that Azamethiphos could cause an increase of extracellular enzyme activity, and thus increases organic matter remineralization. Intracellular activity in marine microorganisms is difficult to evaluate because of the variability of observed responses. Previous studies have shown that pesticides can perturb local metabolism and/or enzyme activity in soils (Topp et al., 1997; Engelen et al., 1998; Liu et al., 2008; Gill and Garg, 2014), indicating the negative impact of pesticides on the activity of hydrolases, oxidoreductases and dehydrogenases (Schuster and Schroeder, 1990; Tu, 1992; Perucci and Scarponi, 1994; Ismail et al., 1998; Monkiedje and Spiteller, 2002; Menon et al., 2005). There is also evidence that enzyme activity and ATP content increase with some pesticides (Shukla, 1997; Megharaj et al., 1999).

4.4. Substrate utilization

The substrates included in the EcoPlates and FF microplate wells represent those found in environmental samples (Insam, 1997; Prakash-Singh, 2009; Atanasova and Druzhinina, 2010). We found different responses in sites 2 and 3 in the utilization of carbon substrates by bacterioplankton (Ecoplate) with Azamethiphos (Fig. 8). There was a decrease in the utilization of carbon substrates by marine fungi (FFmicroplate) from sites 2 and 3, exposed to Azamethiphos (Fig. 9). Mishra and Pandey (1989) and Murage et al. (2007) suggested that the toxic effects of pesticides on microorganisms in the environment are influenced by the type of organic matter available, since the addition of carbon sources such as glucose, acetate and amino acids (glutamine, arginine, serine and tryptophan) increase the resistance of some fungal species to pesticide toxicity. This concurs with our results and with those of Fuentes and Quiñones (2016) that the carbon substrates most used by marine fungi are those with carbohydrate metabolism. We also found increased use of carboxylic acids and amino acids in the treatments with higher concentrations of Azamethiphos.

Previous soil studies using Biolog Ecoplate detected changes in the

functional diversity of bacteria and increased use of *N*-acetyl-D-glucosamine with the application of the antibiotic sulfamethoxazole (Liu et al., 2012) and the pesticides vinclozolin (fungicide) and λ- cyhalothrin (insecticide) (Lupwayi et al., 2009). This concurs with our results since *N*-acetyl-D-glucosamine was one of the four most utilized substrates in the 100 and 1000 μg L^{-1} site 2 treatments, and the most utilized in site 3 treatments with 2, 10 and 1000 μg L^{-1}. Lupwayi et al. (2009) suggested that the use of *N*-acetyl-D-glucosamine increases because bacteria can catabolize from the dead biomass of fungi and arthropods in treatments with pesticides, since *N*-acetyl-D-glucosamine forms part of the fungal cell wall (Cousin, 1995) and the skeletons of insects (Togawa et al., 2004).

In the treatment with higher concentration of Azamethiphos in site 2, fungi used the substrate α-ketoglutaric acid (carboxylic acid), a component of the Krebs cycle that has been identified in xenobiotic biodegradation pathways (Keggs metabolic pathways; www.genome.jp). α-ketoglutaric acid was utilized by fungi in all the site 3 treatments with Azamethiphos, which suggests previous perturbation by a different xenobiotic, since no Azamethiphos was found in the water before the study. Several pesticides have been detected near salmon farms in southern Chile (Tucca et al., 2018; Quiñones et al., 2019). *We suggest* α-ketoglutaric acid use increases at higher concentrations of Azamethiphos because the pesticide is a xenobiotic that contains phosphorous in its structure. Phosphate-solubilizing microorganisms use α-ketoglutaric acid to convert insoluble forms of phosphate to soluble ones (Mardad et al., 2013). The main mechanism for mineral phosphate solubilization is thought to be the liberation of microbial metabolites, such as organic acids (Cunningham and Kuiack, 1992; Singh and Amberger, 1997; Gadd, 1999; Whitelaw, 2000; Lin et al., 2006) like α-keto-glutaric, whose hydroxyl and carboxyl groups chelate the cations bonded to phosphate, converting them into soluble forms (Kpomblekou and Tabatabai, 1994; Chen et al., 2006).

Although responses of the microbial communities were found in the treatment with 1000 μg L^{-1}, this concentration is 10 times greater than the reference concentration used in Azamethiphos treatments in salmon farming. However, it is an indicator of what would happen if natural communities were exposed to much greater concentrations of the pesticide.

5. Conclusion

Given the key role of microbial activity in the marine environment, it is important to be able to evaluate the environmental effects on these communities of the pesticides used in Chilean salmon farming. This study found greater microbial degradation capacity of the pesticide Azamethiphos in the area with salmon farming (~72.4%) compared to an unexposed area (~26.1%).

Our results indicate that Azamethiphos increases extracellular β-glucosidase activity in the size fractions 5–25 μm and 0.22–3 μm, which in turn suggests an increase in the remineralization of organic matter. Azamethiphos exposure produces high variability in intra-cellular MDH activity in marine microorganisms.

The assimilation of carbon substrates in the marine fungi size-fraction was lower in the treatments with ≤100 μg L^{-1} Azamethiphos. There were highly variable responses in substrate use by bacterioplankton. Increased MDH activity at site 3 was related to utilization of carbohydrate, carboxylic acids and amino acids, which may help to decrease the toxic effects of pesticides.

The carbon substrates most used by bacterioplankton and marine fungi were those related to carbohydrate metabolism. Increased use of carboxylic acids and amino acids was found in the treatments with higher concentrations of Azamethiphos.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Table 1. One-way ANOVA and Tukey HSD post-hoc test for bacterial abundance in treatments with Azamethiphos (CTR, 2, 10,100 and 1000 µg L^{-1}) at day 5, at sites 1, 2 and 3, with $p < 0.05$ of significance in bold letters. CTR = control without Azamethiphos.

		Tukey HSD (*p*)
Site	Treatment	CTR
Site 1	ANOVA: F (3, N=12) =246.751, p=0.000	
	10	**0.000**
	100	**0.000**
	1000	**0.000**
Site 2	ANOVA: F (4, N=15) =788.213, p=0.000	
	2	**0.000**
	10	**0.000**
	100	**0.014**
	1000	**0.000**
Site 3	ANOVA: F (4, N=15) =391.694, p=0.000	
	2	**0.000**
	10	**0.000**
	100	**0.000**
	1000	**0.000**

Supplementary Table 2. Two-way ANOVA statistical analysis for oxygen consumption between the different concentrations of Azamethiphos and the days of experimentation for sites 1, 2 and 3, with $p < 0.05$ of significance in bold letters.

Site	Two ways ANOVA	
Site 1		
	Treatment:	F (3, N=72) = 0.165, p=0.919
	Day:	F (5, N=72) = 35.440, p=**0.000**
	Treatment*Day:	F (15, N=72) = 6.545, p=**0.000**
Site 2		
	Treatment:	F (1, N=18) =2.664, p=0.129
	Day:	F (2, N=18) =0.665, p=0.531
	Treatment*Day:	F (2, N=18) =9.553, p=**0.003**
Site 3		
	Treatment:	F (4, N=90) =5.105, p=0.001
	Day:	F (5, N=90) =144.605, p=**0.000**
	Treatment*Day:	F (20, N=90) =3.353, p=**0.000**

Supplementary Table 3. Tukey HSD post-hoc test for oxygen consumption rates between the control (CTR) and treatments with Azamethiphos for sites 1, 2 and 3, with p <0.05 of significance in bold letters.

	Tukey HSD (*p*)	
Site	Treatment	CTR
Site 1	ANOVA: F (3, N=72) =0.165, p=0.919	
	10	0.978
	100	0.938
	1000	0.999
Site 2	ANOVA: F (1, N=18) =2.664, p=0.129	
	100	0.128
Site 3	ANOVA: F (4, N=90) =5.105, p=0.001	
	2	0.093
	10	**0.010**
	100	0.997
	1000	0.567

Supplementary Table 4. Tukey HSD post-hoc test for oxygen consumption rates between different days, for sites 1, 2 and 3, with p <0.05 of significance in bold letters.

		Tukey HSD (*p*)					
Site	Day	0	1	2	3	4	5
Site 1	ANOVA: F (5, N=48) =35.440, p=0.000						
	0		**0.000**	**0.000**	0.999	0.778	**0.000**
	1	**0.000**		**0.001**	**0.000**	**0.000**	0.933
	2	**0.000**	**0.001**		**0.000**	**0.000**	**0.017**
	3	0.999	**0.000**	**0.000**		0.839	**0.000**
	4	0.778	**0.000**	**0.000**	0.839		**0.000**
	5	**0.000**	0.933	0.017	**0.000**	**0.000**	
Site 2	ANOVA: F (2, N=12) =0.665, p=0.531						
	0				0.799		0.869
	3	0.799					0.502
	5	0.869			0.502		
Site 3	ANOVA: F (5, N=60) =144.605, p=0.000						
	0		**0.000**	**0.000**	**0.000**	**0.000**	0.394
	1	**0.000**		0.989	0.999	0.182	**0.000**
	2	**0.000**	0.989		0.971	**0.045**	**0.000**
	3	**0.000**	0.999	0.971		0.245	**0.000**
	4	**0.000**	0.182	**0.045**	0.245		**0.000**
	5	0.394	**0.001**	**0.000**	**0.000**	**0.000**	

Supplementary Table 5. Factorial ANOVA performed for sites 1, 2, and 3 between the β-glucosidase activities of the different size fractions evaluated (TF, F1, F2, F3 and F4) and the different concentrations of Azamethiphos (CTR, 10 100 and 1000 µg L^{-1}) and the 5 days of experimentation, with $p < 0.05$ of significance in bold letters.

		ANOVA F(p)				
Site	Factor	Ftotal	F1	F2	F3	F4
Site 1	Day	2.107 (0.081)	**3.180 (0.015)**	**3.174 (0.015)**	**5.313 (0.001)**	**3.455 (0.010)**
	Treatment	1.195 (0.321)	0.640 (0.593)	1.885 (0.145)	**3.275 (0.029)**	0.674 (0.572)
	Day * Treatment	1.001 (0.469)	0.790 (0.681)	1.412 (0.180)	**1.889 (0.049)**	1.512 (0.138)
Site 2	Day	2.398 (0.051)	**4.081 (0.004)**	**24.291 (0.000)**	**8.217 (0.000)**	**17.402 (0.00)**
	Treatment	1.599 (0.202)	**3.072 (0.036)**	**8.659 (0.000)**	2.268 (0.092)	**3.558 (0.021)**
	Day*Treatment	**3.118 (0.001)**	**4.567 (0.000)**	**5.755 (0.000)**	**3.414 (0.001)**	**5.192 (0.000)**
Site 3	Day	**4.499 (0.002)**	**7.149 (0.000)**	**9.409 (0.000)**	**6.912 (0.000)**	**8.512 (0.000)**
	Treatment	1.575 (0.207)	2.416 (0.078)	**3.116 (0.035)**	2.186 (0.102)	**6.062 (0.001)**
	Day * Treatment	1.063 (0.413)	1.702 (0.083)	1.676 (0.089)	**2.026 (0.033)**	**2.312 (0.014)**

Supplementary Table 6. Tukey HSD post-hoc test for β-glucosidase activity between day 0 and the different days of experimentation in the different size fractions (TF, F1, F2, F3 and F4) at sites 1, 2 and 3, with $p < 0.05$ of significance in bold letters.

	Tukey HSD (*p*) Day 0			
Fraction	Day	Site 1	Site 2	Site 3
FTotal				
	1	0.070	0.034	0.995
	2	0.998	0.717	0.907
	3	0.903	0.130	**0.048**
	4	0.991	0.278	0.907
	5	0.533	0.750	1.000
F1				
	1	**0.023**	**0.014**	**0.001**
	2	0.971	**0.020**	0.112
	3	1.000	**0.008**	1.000
	4	0.201	0.075	**0.009**
	5	0.872	0.668	**0.009**
F2				
	1	**0.019**	**0.000**	**0.001**
	2	1.000	**0.000**	0.999
	3	0.763	**0.000**	0.852
	4	0.999	**0.000**	**0.013**
	5	0.583	**0.000**	0.092
F3				
	1	0.466	**0.000**	**0.005**
	2	0.061	**0.025**	0.518
	3	0.999	**0.002**	0.902
	4	0.977	**0.008**	**0.034**
	5	0.913	**0.003**	0.982
F4				
	1	**0.015**	**0.000**	**0.001**
	2	0.213	**0.000**	**0.014**
	3	0.897	**0.005**	0.998
	4	0.598	**0.000**	**0.043**
	5	**0.027**	**0.003**	**0.004**

Supplementary Table 7. Tukey HSD post-hoc test for β-glucosidase activity between CTR and treatments with Azamethiphos in the different size fractions (TF, F1, F2, F3 and F4) for site 1, 2 and 3, with p <0.05 of significance in bold letters.

	Tukey HSD (*p*)			
	CTR			
Fraction	Treatment	Site 1	Site 2	Site 3
FTotal				
	10	0.657	0.992	0.998
	100	0.954	0.243	1.000
	1000	0.971	1.000	0.277
F1				
	10	0.945	0.616	0.386
	100	0.903	0.057	0.710
	1000	0.966	1.000	0.993
F2				
	10	1.000	0.881	**0.032**
	100	0.925	**0.005**	0.134
	1000	0.191	**0.039**	0.134
F3				
	10	0.918	0.954	0.831
	100	0.116	0.965	0.497
	1000	0.733	0.208	0.672
F4				
	10	0.999	0.729	**0.013**
	100	0.582	**0.025**	**0.002**
	1000	0.936	0.090	**0.013**

Supplementary Table 8. Two-way ANOVA analysis on Malate Dehydrogenase activities of the TF, between the different concentrations of Azamethiphos (CTR, 10, 100 and 1000 µg L^{-1}) and the 5 days of experimentation for sites 1 and 3, with $p < 0.05$ of significance in bold letters.

Site	Two ways ANOVA	
Site 1		
	Treatment:	F (3, N=82) =12.686, p=**0.000**
	Day:	F (5, N=82) =14.707, p=**0.000**
	Treatment*Day:	F (15, N=82) =15.450, p=**0.000**
Site 3		
	Treatment:	F (3, N=82) =8.331, p=**0.000**
	Day:	F (5, N=82) =144.605, p=0.123
	Treatment*Day:	F (15, N=82) =1.484, p=0.149

Supplementary Table 9. Tukey HSD post-hoc test performed for the MDH activities of the total fraction between the control (CTR) and the different concentrations of Azamethiphos (10, 100 and 1000 µg L^{-1}) for sites 1 and 3, with $p < 0.05$ of significance in bold letters.

	Tukey HSD (*p*)	
Site	Treatment	CTR
Site 1	ANOVA: F (3, N=40) =0.165, p=0.919	
	10	**0.001**
	100	0.999
	1000	0.335
Site 3	ANOVA: F (4, N=60) =5.105, p=0.001	
	10	0.999
	100	0.743
	1000	**0.004**

Supplementary Table 10. Tukey HSD post-hoc test performed on the MDH activities for the 5 days of experimentation for sites 1 and 3. with p <0.05 of significance in bold letters.

		Tukey HSD (*p*)					
Site	Day	0	1	2	3	4	5
Site 1							
	0		1.000	**0.018**	0.998	0.128	**0.000**
	1	1.000		**0.020**	0.998	0.117	**0.000**
	2	**0.017**	**0.020**		**0.006**	**0.000**	0.680
	3	0.998	0.998	**0.006**		0.271	**0.000**
	4	0.128	0.117	**0.000**	0.271		**0.000**
	5	**0.000**	**0.000**	0.680	**0.000**	**0.000**	
Site 3							
	0		0.370	0.581	0.123	0.910	0.187
	1	0.370		0.999	0.990	0.928	0.998
	2	0.581	0.999		0.932	0.989	0.975
	3	0.123	0.990	0.932		0.627	0.999
	4	0.910	0.928	0.989	0.627		0.752
	5	0.187	0.998	0.975	0.999	0.752	

Supplementary Table 11. Kruskal-Wallis test for differences in AWCD values of microplates for sites 2 and 3 between the experimental control (CTR) and the different concentrations of Azamethiphos (2, 10, 100 and 1000 µg L^{-1}). The analysis also includes the environmental control (CTRA). Bold numbers indicate p <0.05.

		z' Value *(p)*	
		CTR	
Site	Treatment	ECOPLATE	FF MICROPLATE
Site 2			
	CTRA	0.809 (1.000)	**4.655 (0.000)**
	2	1.449 (1.000)	**3.528 (0.006)**
	10	0.406 (1.000)	0.322 (1.000)
	100	**3.021 (0.038)**	0.799 (1.000)
	1000	**3.157 (0.023)**	0.877 (1.000)
Site 3			
	CTRA	1.630 (1.000)	1.275 (1.000)
	2	**3.849 (0.002)**	**3.658 (0.003)**
	10	**4.674 (0.000)**	1.073 (1.000)
	100	2.149 (0.474)	**4.838 (0.000)**
	1000	**5.752 (0.000)**	0.974 (1.000)

Supplementary Table 12. Kruskal-Wallis test performed for differences in the use of carbon substrates (grouped in amines, amino acids, carbohydrates, carboxylic acids, miscellaneous, polymers) by bacterioplankton and mycoplankton at sites 2 and 3, after 5 days of experimentation at different concentrations of Azamethiphos (CTR, 2, 10, 100 and 1000 µg L^{-1}). Bold numbers indicate p <0.05.

	z' Value (p) CTR					
	Amines	Amino acids	Carbohydrates	Carboxylic acids	Miscellaneous	Polymers
ECOPLATE						
Site 2						
CTRA	0.300 (1.000)	2.334 (1.000)	0.453 (1.000)	1.279 (1.000)	0.746 (0.034)	0.847 (1.000)
2	1.212 (1.000)	1.422 (1.000)	3.334 (0.055)	2.090 (1.000)	0.810 (1.000)	2.066 (1.000)
10	1.506 (1.000)	2.363 (1.000)	1.033 (1.000)	**3.729 (0.013)**	2.692 (0.468)	0.616 (1.000)
100	0.126 (1.000)	1.458 (1.000)	1.358 (1.000)	1.576 (1.000)	1.344 (1.000)	0.875 (1.000)
1000	0.241 (1.000)	2.640 (0.546)	3.331 (0.057)	3.084 (0.134)	2.156 (1.000)	3.220 (0.084)
Site 3						
CTRA	0.273 (1.000)	1.535 (1.000)	0.344 (1.000)	1.575 (1.000)	2.119 (0.510)	0.356 (1.000)
2	0.890 (1.000)	1.207 (1.000)	**3.139 (0.025)**	1.788 (1.000)	0.943 (1.000)	1.804 (1.000)
10	1.493 (1.000)	2.250 (0.366)	1.242 (1.000)	**3.491 (0.007)**	2.487 (0.193)	0.468 (1.000)
100	0.013 (1.000)	0.345 (1.000)	1.225 (1.000)	1.428 (1.000)	1.288 (1.000)	0.760 (1.000)
1000	2.096 (0.541)	2.524 (0.174)	**2.955 (0.047)**	2.296 (0.325)	2.015 (0.658)	**3.125 (0.027)**
FFMICROPLATE						
Site 2						
CTR A	0.395 (1.000)	2.200 (0.416)	2.681 (0.109)	2.687 (0.107)	1.863 (0.935)	0.281 (1.000)
2	0.219 (1.000)	1.582 (1.000)	1.798 (1.000)	1.738 (1.000)	2.04 (1.000)	0.371 (1.000)
10	0.321 (1.000)	0.555 (1.000)	0.404 (1.000)	0.156 (1.000)	0.986 (1.000)	0.371 (1.000)
100	0.633 (1.000)	0.208 (1.000)	0.124 (1.000)	0.681(1.000)	0.74 (1.000)	0.563 (1.000)
1000	0.097 (1.000)	0.609 (1.000)	0.406 (1.000)	0.180 (1.000)	0.422 (1.000)	0.358 (1.000)
Site 3						
CTR A	0.980 (1.000)	0.044 (1.000)	0.190 (1.000)	1.324 (1.000)	1.847 (0.969)	1.872 (0.916)
2	1.341 (1.000)	0.983 (1.000)	1.967 (0.736)	1.750 (1.000)	2.606 (0.137)	0.709 (1.000)
10	0.092 (1.000)	0.630 (1.000)	0.210 (1.000)	0.898 (1.000)	0.940 (1.000)	0.727 (1.000)
100	2.096 (0.539)	2.653 (0.119)	1.653 (1.000)	**3.030 (0.036)**	2.345 (0.285)	1.236 (1.000)
1000	0.268 (1.000)	0.509 (1.000)	0.739 (1.000)	0.854 (1.000)	0.018 (1.000)	0.109 (1.000)

6. DISCUSIÓN

A medida que aumenta el uso de pesticidas, también es probable que aumente el impacto de sus residuos en los ecosistemas acuáticos. Es particularmente importante comprender el efecto de los pesticidas en las comunidades microbianas, dado su papel ecológico y biogeoquímico en estos ecosistemas (Verma *et al*., 2014; Staley *et al*., 2015). La presente Tesis de Grado incluyó experimentos con microcosmos de comunidades microbianas de dos áreas, una sin salmonicultura (Bahía Coliumo, sitio 1) y la otra con un nivel intensivo de cultivo de salmón (Achao, Chiloé, sitios 2 y 3), con el objetivo de estudiar la respuesta metabólica de los microorganismos acuáticos (<100 µm) a cinco días de exposición al pesticida Azametifos, en condiciones controladas.

6.1 Condiciones ambientales

Las condiciones ambientales tanto en el sitio 1 de Bahía Coliumo, como en los sitios 2 y 3 de Achao, se encuentraron dentro de los rangos esperados para la época del año (Schneider *et al*., 2017; Narváez *et al*., 2019). La temperatura superficial muestra diferencias de hasta 2 ºC entre los sitios 1, 2 y 3; sin embargo, estas diferencias son mucho menores a los 5 y 10 m (<1,2 ºC). Las diferencias en la salinidad entre los sitios 1, 2 y 3 fueron menores (~ 1,4) a 10 m de profundidad. Las diferencias observadas en salinidad y temperatura no son relevantes considerando la variabilidad ambiental natural en las áreas estudiadas, incluso en una escala de tiempo diaria.

Las concentraciones de nutrientes en las aguas superficiales de la región costera del norte de la Patagonia (Chiloé) son más bajas (Silva *et al*., 1997; Iriarte *et al*., 2007; Jacob *et al*., 2014; Martínez *et al*., 2015) que las de Chile central (Quiñones *et al*., 2010; Morales y Anabalón, 2012). Las relaciones de nutrientes indican deficiencia de silicato (Si: N <1; y Si: P <10; Souchu *et al*., 2010) en los tres sitios estudiados, lo que

podría explicar por qué las concentraciones de clorofila-*a* no fueron más altas en primavera-verano (Martínez *et al*., 2015; Anabalón *et al*., 2016)

6.2 Degradación de Azametifos

Se encontraron altas concentraciones de Azametifos en los microcosmos a los 5 días en todos los tratamientos de Bahía Coliumo (sitio 1) debido a la baja degradación, la cual fue de ~26,1%, cercana al ~28% esperado por hidrólisis en 5 días. La degradación fue mucho mas alta en los sitios 2 y 3, en el área de Achao, siendo la degradación de ~72,4% en todos los tratamientos. La mayoría de los organofosforados son degradados por microorganismos como fuente de nutrientes limitantes como el carbono y/o el fósforo (Kumar *et al*., 2018; Serasinghe y Manage, 2019). Esto se logra mediante la hidrólisis de los enlaces PO de alquilo y arilo con la ayuda de enzimas tales como hidrolasas (Cui *et al*., 2001; Kapoor y Rajagopal, 2011; Gao *et al*., 2012; Lu *et al*., 2013), fosfotriesterasa y fosfatasa (Ortiz-Hernández *et al*., 2001; Bhadbhade *et al*., 2002; Sogorb y Vilanova, 2002; Iyer *et al*., 2013). La degradación de Azametifos ocurrió en los sitios de Chiloé, a pesar de que no hubo potencial deficiencia de nutrientes o desviaciones de la razón N: P de la estequiometría de Redfield durante el estudio.

Sugerimos que la comunidad microbiana es responsable de una degradación inespecífica por las enzimas liberadas, que depende de: 1) diferencias en la composición de la comunidad microbiana porque las dos áreas estudiadas son oceanográficamente distintas (De Lorenzo *et al*., 2001); y 2) la posible adaptación de las comunidades microbianas a la exposición de pesticidas organofosforados, dado que los sitios estudiados en Achao han estado expuestos a décadas de actividad de cultivo de salmón. Los tratamientos de inmersión con organofosforados (metrifonato y diclorvos) se aplicaron en Chile de 1981 a 2001 (Bravo *et al*., 2014; Urbina *et al*., 2018), y posteriormente el organofosforado Azametifos ingresó al mercado chileno en 2013 (Helgesen *et al*., 2014). Varios autores han sugerido la adaptación de

comunidades microbianas en áreas expuestas a pesticidas organofosforados, estas comunidades serían más eficientes para degradar los pesticidas (Racke y Coats, 1988; Coppola *et al*., 2007; Itoh *et al*., 2014). De acuerdo con Itoh *et al*. (2014), después de la aplicación de pesticidas organofosforados en los suelos, se observaron cambios en la actividad enzimática y la biodiversidad de las comunidades microbianas residentes. Estudios han demostrado efectos adversos de los pesticidas en la biomasa microbiana y la respiración del suelo (Pampulha y Oliveira, 2006; Zhou *et al*., 2006). En el presente estudio, solo observamos un aumento significativo en el consumo de oxígeno en el tratamiento del sitio 3 con 10 µg L^{-1}. Aunque no hay información previa sobre el efecto de los pesticidas organofosforados en la respiración microbiana marina, se ha sugerido que la mayoría de los insecticidas organofosforados no afectan negativamente la respiración microbiana (Jail *et al*., 2014). Una posible explicación para el aumento de la respiración es que los microorganismos oxidan los insecticidas (Tu, 1970; Jones y Ananyeva, 2001; Eisenhauer *et al*., 2009; Jail *et al*., 2014).

6.3 Actividad enzimática

Detectamos diferencias en la actividad de la β-glucosidasa en los tratamientos observando un aumento en la actividad principalmente en las fracciones de menor tamaño F2 (5-25 µm; nanoplancton) y F4 (0.22-3 µm; picoplancton). En el sitio 2, el aumento en la actividad de la β-glucosidasa en el nanoplancton tuvo lugar en los tratamientos de 100 y 1000 µg L^{-1} y en la fracción de picoplancton en el tratamiento de 100 µg L^{-1}, mientras que en el sitio 3 el aumento fue en el nanoplancton en el tratamiento10 µg L^{-1} y en la fracción de picoplancton en todos los tratamientos. La fracción F2 incluye una gran variedad de organismos como hongos, dinoflagelados, flagelados y ciliados, que dominan la zona de estudio, principalmente durante los períodos de menor productividad (Alves de Souza *et al*., 2008; Montero *et al*., 2011, 2017). Junto con la fracción de picoplancton (bacterias, arqueas), los hongos pueden beneficiarse con los aportes de fósforo. Czypionka *et al*. (2011), trabajando en el

fiordo de Aysén, indican la complejidad de los efectos de la variación de nutrientes en el nanoplancton; Los cambios en las concentraciones de fósforo pueden producir cambios en el estado trófico de los nanoflagelados. Estudios anteriores han demostrado que varíos grupos de bacterias (Itoh *et al*., 2014) y hongos (Pandey y Singh, 2004) del suelo, aumentan sus tasas metabólicas cuando se exponen a pesticidas.

La actividad enzimática específica (MDH/ATP) disminuyó en la fracción <100 µm en el tratamiento de 10 µg L^{-1} en el sitio 1 y aumentó en el tratamiento de 1000 µg L^{-1}, mientras que en los otros tratamientos la actividad fue cercana a la del CTR. Una mayor actividad de MDH indica una mayor remineralización de la materia orgánica (Martínez *et al*., 2002), y es un indicador de actividad microbiana para las comunidades de microplancton (Quiñones *et al*., 2006; González y Quiñones 2009). La actividad de MDH/ATP para los sitios 1, 2 y 3 fue 1,673; 1,428 y 2,082 pmol de NADH min^{-1} pg ATP^{-1}, respectivamente, mayor que la encontrada por González y Quiñones (2009). Esto se debería a que las concentraciones de ATP (i.e. biomasa microbiana) encontradas en los tres sitios (promedios Fracción total (FT): Coliumo = 0,021; Achao-sitio 2 = 0,022; Achao-sitio 3 = 0,023 µg de ATP L^{-1}) fueron menores que los 0,2 µg de ATP L^{-1} en la zona sur-central y 0,05 µg de ATP L^{-1} en el norte de Chile reportados por González y Quiñones (2009), lo que demuestra que hubo menos biomasa microbiana en nuestro estudio.

Nuestros resultados indican que el pesticida Azametifos podría causar un aumento en la actividad enzimática extracelular y, por lo tanto, aumentar la remineralización de la materia organica. La actividad intracelular en microorganismos marinos es más difícil de evaluar, debido a la variabilidad de las respuestas observadas (aumento y/o disminución). Estudios anteriores han demostrado que los pesticidas pueden perturbar el metabolismo local y/o la actividad enzimática en suelos (Topp *et al*., 1997; Engelen *et al*., 1998; Liu *et al*., 2008; Gill y Garg, 2014), lo que indica el impacto negativo de pesticidas sobre la actividad de hidrolasas, oxidorreductasas y deshidrogenasas (Schuster y Schroeder, 1990; Tu, 1992; Perucci y Scarponi, 1994; Ismail *et al*., 1998;

Monkiedje y Spiteller, 2002; Menon *et al*., 2005). También hay evidencia de que la actividad enzimática y el contenido de ATP aumentan con algunos pesticidas (Shukla, 1997; Megharaj *et al*., 1999).

6.4 Utilización de sustratos

Los sustratos incluidos en los pocillos de microplacas EcoPlates y FF representan los encontrados en muestras ambientales (Insam, 1997; Prakash-Singh, 2009; Atanasova y Druzhinina, 2010). Se observaron respuestas opuestas en los sitios 2 y 3, en la utilización de sustratos de carbono por parte del bacterioplancton (Ecoplate) con Azametifos. Hubo una disminución en la utilización de sustratos de carbono por hongos marinos expuestos a Azametifos (FFmicroplate) en los dos sitios. Mishra y Pandey (1989) y Murage *et al.* (2007) sugirieron que el efecto tóxico de los pesticidas sobre los microorganismos en el medio ambiente está influenciado por el tipo de materia orgánica disponible, ya que la adición de fuentes de carbono como glucosa, acetato y aminoácidos (glutamina, arginina, serina y triptófano) aumenta la resistencia a la toxicidad de pesticidas para algunas especies de hongos. Esto coincide con nuestros resultados y con los reportados por Fuentes y Quiñones (2016) en cepas de hongos aisladas de ambientes marinos, en los que los sustratos de carbono más utilizados por bacterioplancton y hongos marinos fueron los del metabolismo de carbohidratos. También se determinó en la presente Tesis, un aumento en el uso de ácidos carboxílicos y aminoácidos en los tratamientos con concentraciones más altas de Azametifos.

Estudios previos en suelo usando Biolog Ecoplates, han detectado cambios en la diversidad funcional de las bacterias y un aumento en el uso de N-acetil-D-glucosamina con la aplicación del antibiótico Sulfamethoxazole (Liu *et al*., 2012) y los pesticidas Vinclozolin (fungicida) y λ- Cyhalothrin (insecticida) (Lupwayi *et al*., 2009). Esto concuerda con nuestros resultados, ya que la N-acetil-D-glucosamina fue uno de los 4 sustratos más utilizados en los tratamientos de 100 y 1000 µg L^{-1} del sitio

2, y el sustrato más utilizado en tratamientos con 2, 10 y 1000 µg L^{-1} del sitio 3. Lupwayi *et al.* (2009) sugirieron que el uso de N-acetil-D-glucosamina aumenta porque en los tratamientos con pesticidas, las bacterias pueden catabolizarlo a partir de la biomasa muerta de hongos y artrópodos, ya que forma parte de la pared celular del hongo (Cousin, 1995) y el esqueleto de los insectos (Togawa *et al*., 2004).

En los tratamientos con más altas concentraciones, en el sitio 2, los hongos usaron el sustrato ácido α-cetoglutarico (ácido carboxílico), un componente del ciclo de Krebs que se ha identificado en las vías de biodegradación xenobiótica (vías metabólicas de Keggs; www.genome.jp). El ácido α-cetoglutarico se utilizó en todos los tratamientos con Azametifos en el sitio 3, lo que puede indicar una perturbación previa por un xenobiótico diferente, ya que no se encontró Azametifos en el agua antes del estudio. Se ha detectado la presencia de varios pesticidas cerca de los centros de cultivo de salmón en el sur de Chile (Tucca *et al*., 2018; Quiñones *et al*., 2019). Sugerimos que el aumento en el uso de ácido α-cetoglutarico en concentraciones más altas de Azametifos ocurrió porque el pesticida es un xenobiótico que contiene fósforo en su estructura. Los microorganismos solubilizadores de fosfato utilizan ácido α-cetoglutarico para convertir las formas insolubles de fosfato en solubles (Mardad *et al*., 2013). Se ha propuesto que el mecanismo principal para la solubilización de fosfato mineral sería la liberación de metabolitos microbianos como los ácidos orgánicos (Cunningham y Kuiack, 1992; Singh y Amberger, 1997; Gadd, 1999; Whitelaw, 2000; Lin *et al*., 2006) tales como α-ceto-glutárico, cuyos grupos hidroxilo y carboxilo quelan los cationes unidos al fosfato, convirtiéndolos en formas solubles (Kpomblekou y Tabatabai, 1994; Chen *et al*., 2006).

Cabe señalar que, aunque se encontraron respuestas de las comunidades microbianas en el tratamiento con 1000 µg L^{-1}, esta concentración es 10 veces mayor que la concentración de referencia utilizada en los tratamientos con Azametifos en el cultivo de salmón. Sin embargo, es un indicador de lo que eventualmente podría suceder si las comunidades naturales estuvieran expuestas a mayores concentraciones del pesticida.

7. CONCLUSIONES

- Este estudio encontró una mayor capacidad de degradación del pesticida Azametifos en el área de cultivo de salmón (~ 72,4%) en comparación con un área no expuesta (~ 26,1%).
- Nuestros resultados fueron diversos, indicando que mayores concentraciones de Azametifos puede causar un aumento en la actividad extracelular de la β-glucosidasa en las fracciones de tamaño F2: 5- 25 µm y F4: 0,22- 3 µm, lo que sugiere un aumento en la remineralización de la materia orgánica. **Se rechaza la Hipótesis 1**, la cual sugería una disminución de la actividad extracelular de la β-glucosidasa, a mayores concentraciones de Azametifos.
- Detectamos una alta variabilidad de la actividad intracelular (MDH) en microorganismos marinos, pero en general se observó un aumento de la actividad MDH en las fracciones totales (FT) de los sitios 2 y 3. En cambio, en el sitio 1, el aumento de la actividad a mayores concentraciones de Azametifos fue detectado solo en las fracciones de tamaño F1: 25-100 µm y F2: 5-25 µm al pasar los días y en la fracción F4: 0,22-3 µm, el primer día aumentó la actividad a mayores concentraciones de Azametifos, observando un peak de aumento en el tratamiento 1000 al día 4. **Se rechaza la Hipótesis 2**, la cual sugería una disminución de la actividad intracelular MDH, a mayores concentraciones de Azametifos. El aumento de la actividad intracelular de la MDH tanto en el sitio 2 y 3 estaría relacionada con la utilización de carbohidratos, ácidos carboxílicos y aminoácidos y podría ayudar a disminuir el efecto tóxico de los pesticidas.
- La asimilación de sustratos de carbono por bacterioplancton fue muy variable y opuesta en los sitios 2 y 3. En el sitio 2 se observó un aumento en la utilización de sustratos en el tratamiento 100 µg L^{-1} y una disminución en el tratamiento 1000 µg L^{-1}; mientras que en el sitio 3 en tratamientos ≤10 µg L^{-1} se observó una disminucióny en el tratamiento 1000 µg L^{-1} hubo un aumento de la utilización de sustratos, lo cual coincidió con el aumento en la actividad de la Malato

deshidrogenasa en el mismo sitio. En el caso de la asimilación de sustratos de carbono por hongos marinos, esta disminuyó en los tratamientos con concentraciones ≤100 μg L^{-1} de Azametifos, en los sitios 2 y 3, mientras que en el tratamiento 1000 no se observaron diferencias significativas. Por estas razones **se rechaza la Hipótesis 3**, la cual sugería una disminución de la utilización de sustratos a mayores concentraciones de Azametifos.

- Los sustratos de carbono más utilizados por bacterioplancton y hongos marinos fueron los del metabolismo de los carbohidratos. Se observó un aumento en el uso de ácidos carboxílicos y aminoácidos en los tratamientos con concentraciones más altas de Azametifos.

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Anexo 1. Paneles con sustratos de carbono en Ecoplate para Bacterias

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ- Hydroxy Butyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ- Hydroxy Butyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ- Hydroxy Butyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Keto Butyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Keto Butyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Keto Butyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figura A-1. Fuentes de carbono en la placa Ecoplate para Bacterias

Anexo 2. Paneles con sustratos de carbono en placa FF Microplate para hongos

A1 Water	A2 Tween 80	A3 N-Acetyl-D-Galactosamine	A4 N-Acetyl-D-Glucosamine	A5 N-Acetyl-D-Mannosamine	A6 Adonitol	A7 Amygdalin	A8 D-Arabinose	A9 L-Arabinose	A10 D-Arabitol	A11 Arbutin	A12 D-Cellobiose
B1 α-Cyclodextrin	B2 β-Cyclodextrin	B3 Dextrin	B4 i-Erythritol	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 D-Glucosamine	B12 α-D-Glucose
C1 Glucose-1-Phosphate	C2 Glucuronamide	C3 D-Glucuronic Acid	C4 Glycerol	C5 Glycogen	C6 m-Inositol	C7 2-Keto-D-Gluconic Acid	C8 α-D-Lactose	C9 Lactulose	C10 Maltitol	C11 Maltose	C12 Maltotriose
D1 D-Mannitol	D2 D-Mannose	D3 D-Melezitose	D4 D-Melibiose	D5 α-Methyl-D-Galactoside	D6 β-Methyl-D-Galactoside	D7 α-Methyl-D-Glucoside	D8 β-Methyl-D-Glucoside	D9 Palatinose	D10 D-Psicose	D11 D-Raffinose	D12 L-Rhamnose
E1 D-Ribose	E2 Salicin	E3 Sedoheptulosan	E4 D-Sorbitol	E5 L-Sorbose	E6 Stachyose	E7 Sucrose	E8 D-Tagatose	E9 D-Trehalose	E10 Turanose	E11 Xylitol	E12 D-Xylose
F1 γ-Amino-butyric Acid	F2 Bromosuccinic Acid	F3 Fumaric Acid	F4 β-Hydroxy-butyric Acid	F5 γ-Hydroxy-butyric Acid	F6 p-Hydroxyphenyl-acetic Acid	F7 α-Keto-glutaric Acid	F8 D-Lactic Acid Methyl Ester	F9 L-Lactic Acid	F10 D-Malic Acid	F11 L-Malic Acid	F12 Quinic Acid
G1 D-Saccharic Acid	G2 Sebacic Acid	G3 Succinamic Acid	G4 Succinic Acid	G5 Succinic Acid Mono-Methyl Ester	G6 N-Acetly-L-Glutamic Acid	G7 Alaninamide	G8 L-Alanine	G9 L-Alanyl-Glycine	G10 L-Asparagine	G11 L-Aspartic Acid	G12 L-Glutamic Acid
H1 Glycyl-L-Glutamic Acid	H2 L-Ornithine	H3 L-Phenylalanine	H4 L-Proline	H5 L-Pyroglutamic Acid	H6 L-Serine	H7 L-Threonine	H8 2-Amino Ethanol	H9 Putrescine	H10 Adenosine	H11 Uridine	H12 Adenosine-5'-Monophosphate

Figura A-2. Fuentes de carbono en la placa FF Microplate para hongos