TESIS DOCTORAL

CARACTERIZACIÓN ECOTOXICOLÓGICA DE NUEVOS DISOLVENTES DERIVADOS DEL GLICEROL

ECOTOXICOLOGICAL CHARACTERIZATION OF NEW GLYCEROL-DERIVED SOLVENTS

Eduardo Pablo Perales Sarría

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AGRADECIMIENTOS

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Dra. Dame Jane Morris Goodall

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INTRODUCCIÓN

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Relevancia de los disolventes en reacciones y procesos químicos

Los disolventes son una parte fundamental en muchos procesos químicos a cualquier escala, ya que permiten realizar procedimientos tales como limpieza, extracción, purificación, síntesis de sustancias. También se utilizan como medio para realizar procesos de transferencia de calor y masa (Kerton 2016).

Estas sustancias son de creación antropogénica y/o proceden de la industria del petróleo, siendo muchas de ellas consideradas como compuestos orgánicos volátiles (COVs). Están formadas fundamentalmente por moléculas de carbono, y se caracterizan por su baja presión de vapor en condiciones normales de temperatura y presión atmosféricas. En la Tabla 1 se pueden ver diferentes ejemplos de COVs usados tradicionalmente con algunas de sus propiedades.

Según la legislación de la Unión Europea, un COV es cualquier compuesto orgánico que tenga un punto inicial de ebullición menor o igual a 250 ºC, a una presión atmosférica estándar de 101.3 kPa (Directiva 2004/42/CE). Por ello, su uso está asociado con la producción de contaminación y residuos, que terminan acumulándose en el medio ambiente (Reichardt 2007). No es de extrañar, por tanto, que la búsqueda y el desarrollo de disolventes menos tóxicos o dañinos para el medio ambiente despierte un gran interés en la industria, la comunidad científica y, por extensión, en la sociedad (Anastas & Eghbali 2009; Valavanidis & Vlachogianni 2012).

Disolvente	Punto de ebullición (ºC)	Punto de inflamabilidad (ºC)	Riesgo asociado a su uso
Metanol	64	12	Tóxico, inflamable
Etanol	78	16	Irritante, inflamable
Isopropanol	96	15	Irritante, inflamable
1-Butanol	117	12	Peligroso, inflamable
Acetato de etilo	76	-2	Peligroso, inflamable
Lactato de etilo	154	46	Irritante, inflamable
Tetrahidrofurano	65	-17	Irritante, inflamable
2-Metiltetrahidrofurano	80	-11	Irritante, inflamable
2-Butanona	80	-3	Irritante, inflamable
Diclorometano	40	No tiene	Tóxico, peligroso, sospechoso de ser carcinogénico
Cloroformo	61	No tiene	Posible carcinogénico
Tolueno	110	4	Irritante, teratogénico, inflamable
Hexano	68	-26	Irritante, peligroso para la reproducción, inflamable

Tabla 1: Ejemplos de diferentes COVs (Kerton 2009).

En el plano de la investigación, las preocupaciones medioambientales asociadas con el uso de COVs han derivado en el desarrollo de procesos y sustancias químicas que minimizan el impacto en el medio ambiente (Corrêa et al. 2015). Existen numerosas legislaciones que regulan el uso y distribución de sustancias químicas, siendo progresivamente más exigentes para controlar el uso de estos disolventes, su impacto ambiental y su tratamiento como residuos. Por ejemplo, la Directiva Europea 1999/13/CE (Directiva 1999/13/CE) (actualizada posteriormente en las Directivas 2004/42/CE y 2008/112/CE) tenía como objetivo "prevenir o reducir los efectos nocivos que para las personas y el medio ambiente pudieran derivarse de algunas actividades que utilizan en sus procedimientos de fabricación o de trabajo disolventes orgánicos en cantidades importantes". Posteriormente, aparecieron diversas legislaciones que regulan y controlan las sustancias químicas dentro de la Unión Europea. Una de ellas fue el Reglamento 1907/2006/CE, en el cual se legisla tanto el registro, la evaluación, la autorización y la restricción de sustancias y preparados químicos (REACH), como la creación de la Agencia Europea de Sustancias y Mezclas Químicas (ECHA). Esta agencia se encarga de asesorar a las empresas en el cumplimiento de la normativa REACH, proporcionando información sobre compuestos químicos, y promoviendo su control y uso seguro. Además, en esta legislación se destaca la relevancia de las empresas como responsables de informar sobre los riesgos en el manejo de las sustancias que necesiten para ejercer sus actividades y gestionarlos. Para ello, esta disposición establece rangos límite de utilización de sustancias químicas, en función de los cuales son necesarios unos ensayos mínimos para determinar la toxicidad, tanto para la salud como para el medio ambiente.

El segundo fue el Reglamento 1272/2008/CE, sobre clasificación, etiquetado y envasado de sustancias y mezclas, coordinado con el REACH. Con este documento

legal se pretendió la armonización mundial de las reglas de clasificado y etiquetado de productos químicos. Establece el criterio común con el Sistema Globalmente Armonizado de Clasificación y Etiquetado de Productos Químicos (SGA), de las Naciones Unidas (Naciones Unidas 2015). Su objetivo es cuantificar los valores de toxicidad para la salud humana y el medio ambiente en las sustancias y mezclas peligrosas, estableciendo categorías para facilitar su clasificación y etiquetado.

En un ámbito más nacional, la Directiva 1999/13/EC se incluyó en la legislación española como el Real Decreto 117/2003 (Real Decreto 117/2003/España), sobre limitación de emisiones de COVs debidas al uso de disolventes en actividades tales como fabricación de productos farmacéuticos, limpieza en seco, y fabricación de artículos, entre otras actividades. En estas normativas aparecen los valores umbral de consumo y límites de emisión de disolventes, y se describen las competencias de las autoridades para comprobar su cumplimiento en dichos procesos. En la actualidad, la administración jurídica española dispone del Real Decreto Legislativo 1/2016, perteneciente a la Ley de prevención y control integrados de la contaminación (Real Decreto Legislativo 1/2016/España), cuyo objetivo tiene evitar o reducir y controlar la contaminación de la atmósfera, del agua y del suelo.

Otra ley relevante en la legislación española es el Real Decreto 656/2017, por el cual se aprueba el Reglamento de Almacenamiento de Productos Químicos y sus Instrucciones Técnicas (Real Decreto 656/2017/España). Esta normativa pretende la adaptación de almacenamiento, clasificación y etiquetado de productos químicos de acuerdo a lo establecido en el Reglamento REACH y al Reglamento 1272/2008 de la Comunidad Europea.

Green Chemistry y disolventes respetuosos con el medio ambiente

Como se ha visto, existe una estructura legal y sancionadora que asegura la utilización correcta de toda clase de sustancias químicas, que se ha reflejado también en un interés por parte de la comunidad científica. Desde finales del siglo pasado, grupos investigadores multidisciplinares han desarrollado metodologías y herramientas que permiten minimizar el impacto de los procesos químicos, tanto en la salud humana como en el medio ambiente. Todos estos procedimientos forman parte de la filosofía de la Química Verde (*Green Chemistry*). Esta filosofía se define como el diseño de productos y procesos para reducir o eliminar el uso y la generación de sustancias nocivas para la salud humana y el medio ambiente (Anastas & Eghbali 2009).

La *Green Chemistry* fue inicialmente diseñada por los Profesores Paul T. Anastas y John C. Warner en los años 90, con el objetivo de enfocar la producción química desde un punto de vista más respetuoso con la salud humana y el medio ambiente. Para ello, hicieron hincapié en la eliminación de los procesos y fases tóxicas en las síntesis orgánicas desde su inicio. Ambos investigadores desarrollaron lo que se conoce como los "Doce Principios de la *Green Chemistry*", un compendio de directrices que marcan una hoja de ruta para todos los químicos que deseen trabajar en un marco más sostenible para la salud y el medio ambiente. Estos principios pueden verse esquematizados en la Figura 1 (Anastas & Eghbali 2009).

Figura 1: Doce principios de la *Green Chemistry* (Anastas & Eghbali 2009).

Uno de estos principios especifica la reducción de la utilización de sustancias auxiliares en síntesis químicas, destacando a los disolventes. De no ser posible, se utilizarían compuestos que causen el menor impacto posible en la salud y en el medio ambiente, es decir, disolventes verdes (Capello et al. 2007).

La necesidad de utilizar disolventes verdes que puedan sustituir a otros más contaminantes en síntesis orgánica se basa en el uso extendido que se hace de ellos (Welton 2015). Por ejemplo, según datos conjuntos de la Sociedad Americana de Química, el Instituto de *Green Chemistry* y multinacionales farmacéuticas (*American*

Chemical Society, Green Chemistry Institute-Pharmaceutical Roundtable, ACS GCI-PR), en esta industria se ha estimado que al menos la mitad del material usado para la síntesis de principios activos son disolventes, llegando a valores del 80-90 % (Constable et al. 2007; Jiménez-González et al. 2011). Con este ejemplo del volumen asociado a disolventes en síntesis química, es necesario asegurarse de que estos medios de reacción no sean proclives a causar daños en la salud o en el medio ambiente.

Para lograr este propósito, hay que tener en cuenta tanto las propiedades físicas de los disolventes, como su criterio de evaluación de toxicidad para el medio ambiente, salud y seguridad. En el primer caso, las principales características a tener en cuenta son los puntos de fusión y ebullición, viscosidad, densidad, solubilidad y su capacidad de ser inertes (Kerton 2016). Por otro lado, para evaluar si es realmente verde, hay que tener en cuenta el potencial riesgo de exposición al medio, explosión, inflamabilidad, reactividad, toxicidad aguda y crónica, y persistencia medioambiental (Capello et al. 2007). Por lo general, los disolventes verdes se caracterizan, aparte de por su baja toxicidad ambiental y por ser fácilmente biodegradables, por su alto punto de ebullición, su capacidad de reutilización, y su alta disponibilidad (Li & Trost 2008;Valavanidis & Vlachogianni 2012).

Sin embargo, no existe el disolvente verde universal. Todas las condiciones previamente mencionadas son muy difíciles de conseguir al mismo tiempo en todo tipo de reacciones químicas (Gu & Jérôme 2010). Por ello, en los últimos años se han estudiado diferentes clases de disolventes que puedan ser una alternativa a otros más convencionales y peligrosos para aplicaciones concretas, tales como agua (Chigrinova et al. 2015; Lipshutz & Ghorai 2014), alcoholes (Horváth 2008), fluidos supercríticos (Eckert et al. 1996; Herrero et al. 2013), y líquidos iónicos (Thuy Pham et al. 2010;

Zhao et al. 2005), que puedan ser considerados como una mejora a otros disolventes convencionales.

Glicerol y sus derivados: Origen y utilización

Además de los disolventes alternativos a los provenientes del petróleo que se han nombrado, existen otros con origen en la biomasa. Este término engloba a todo tipo de compuestos lipídicos con origen tanto vegetal (aceites, algas, semillas) como animal (grasas), destacados por su carácter renovable (Corrêa et al. 2015). Tienen relevancia en la industria química por sus numerosas aplicaciones, desde alimentarias hasta energéticas (Gallezot 2012). En la Figura 2 se pueden ver estructuras moleculares de este tipo de disolventes alternativos.

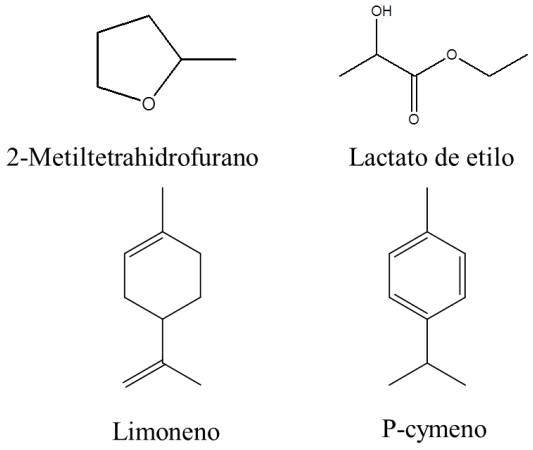

Figura 2: Disolventes alternativos procedentes de la biomasa (Corrêa et al. 2015)

Además de su uso como materia prima para disolventes alternativos, otra de las principales utilidades que tiene la biomasa es la síntesis de biodiesel. A través de reacciones de esterificación y transesterificación, como se esquematiza en la Figura 3, se obtiene un combustible compatible con motores diésel que no depende del petróleo para su síntesis. Esto hace que el biodiesel sea una alternativa viable en comparación a otras gasolinas con respecto al medio ambiente, al rendimiento de motor (Quispe et al.

2013), y la economía (Thanh et al. 2012). En la Figura 4 se pueden ver las principales ventajas del biodiesel con respecto a otros combustibles procedentes del petróleo.

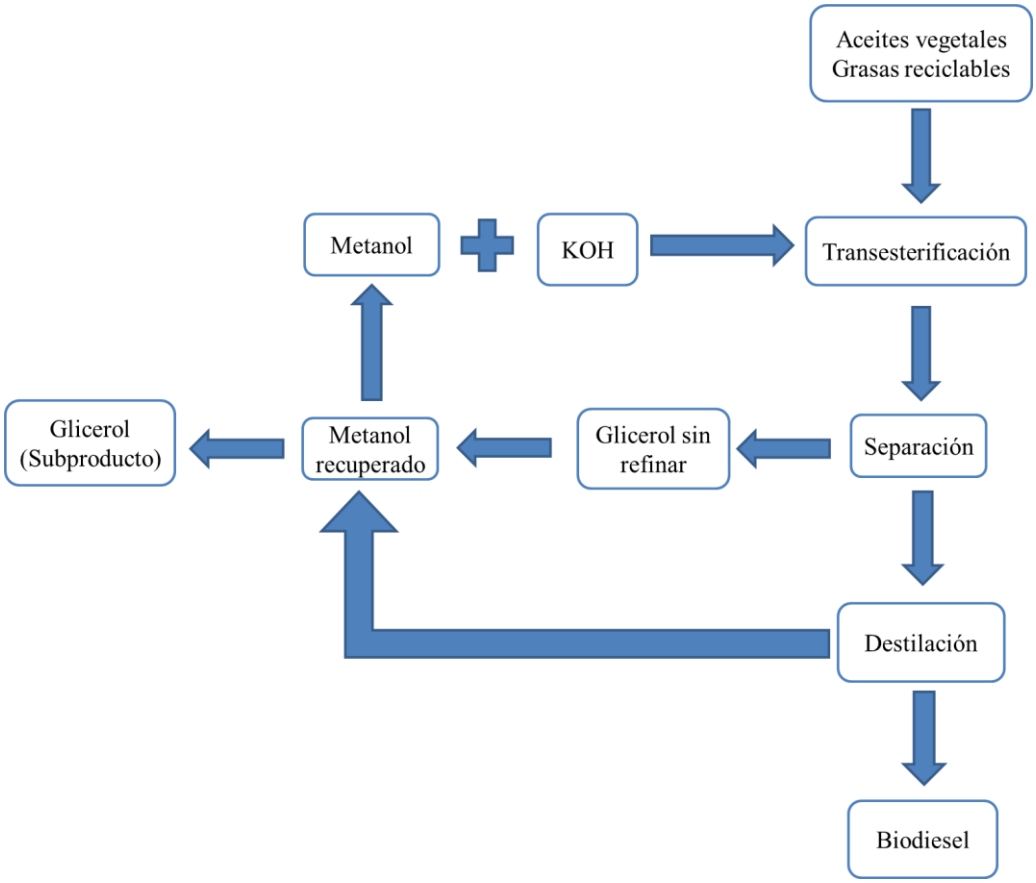

Figura 3: Síntesis de biodiesel y glicerol (Li et al. 2013)

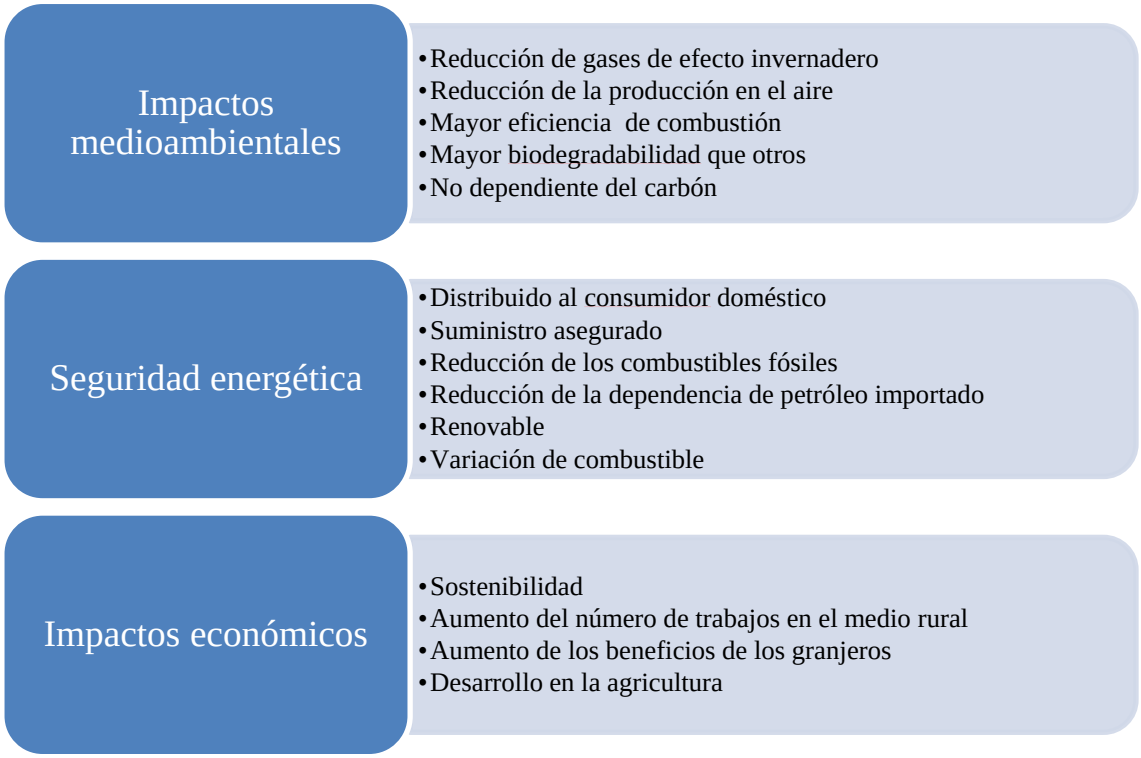

Figura 4: Principales beneficios del biodiesel (Thanh et al. 2012)

En la síntesis del biodiesel se produce glicerol (1,2,3-propanotriol) o glicina (en función de su pureza) en un rendimiento aproximado del 10 %, como sustancia concomitante (Dasari et al. 2005). Es un líquido higroscópico, inodoro, incoloro, viscoso y denso. Sus principales propiedades fisicoquímicas de interés como disolvente pueden verse en la Tabla 2.

Propiedad	Valor
Fórmula molecular	$C_3H_8O_3$
Masa molar (g mol^{-1})	92.09
Densidad relativa (g ml^{-1})	1260
Viscosidad (cP)	1410
Punto de fusión (ºC)	18
Punto de ebullición (ºC ,101.3 kPa)	290
Punto de inflamabilidad (ºC)	177
Calor específico (kJ.kg^{-1},25 ºC)	2435
Conductividad térmica (W.(m K))$^{-1}$	0.28
Entalpía de formación (kJ mol^{-1})	667.8
Tensión superficial (mN m^{-1})	63.4
pH (en disolución)	7
Autoinflamabilidad (ºC)	393

Tabla 2: Propiedades físicas y químicas del glicerol (1,2,3-propanotriol) (Quispe et al. 2013)

Ya en el siglo XIX, el glicerol era sintetizado a escala industrial, obteniendo más adelante una gran relevancia en ambas guerras mundiales por ser materia prima en la producción de explosivos. Por ello, fue considerado como un importante recurso militar (Ciriminna et al. 2014).

No obstante, desde inicio del siglo XXI, la síntesis de glicerol quedó condicionada a la fabricación del biodiesel, debido a la sobreproducción que conllevaba la producción de este combustible (Ciriminna et al. 2014). Con el exceso de oferta disponible del glicerol, y a pesar de que tiene reconocidos más de 2000 usos establecidos en diferentes campos industriales, el mercado no puede absorber tal

volumen (Díaz-Álvarez et al. 2011). Además, existen estimaciones que informan sobre el aumento de la producción de biodiesel (Li et al. 2013; Nanda et al. 2014), por lo que se prevé que se seguirá incrementando en un futuro próximo. Teniendo en cuenta esta sobreproducción, se han realizado investigaciones para buscar nuevas aplicaciones (Bagheri et al. 2015; Behr et al. 2008; Konstantinovic et al. 2016; Leoneti et al. 2012; Li et al. 2013; Pagliaro et al. 2007; Thanh et al. 2012).

Una de las opciones posibles que se han planteado como solución a la sobreproducción ha sido utilizar el glicerol como disolvente alternativo. Precisamente, se han obtenido resultados prometedores como medio de reacción, que permitían no sólo sustituir a otros disolventes convencionales, sino que han incrementado los rendimientos de reacción y la selectividad en reacciones específicas (Corma et al. 2007; Díaz-Álvarez et al. 2013; Gu & Jérôme 2010; Gu & Jérôme 2013; Wolfson et al. 2007).

No obstante, hay que tener en cuenta ciertos inconvenientes en la utilización del glicerol como disolvente. Un problema que tiene es su alta viscosidad, ya que puede causar problemas de transferencia de masa, dificultando que el sustrato se pueda difundir adecuadamente por el medio de la reacción (Gu & Jérôme 2010; Gu & Jérôme 2013).No obstante, este inconveniente puede mitigarse mediante un incremento de temperatura de la reacción (aproximadamente a 50-60 ºC).

Otro inconveniente a la hora de usar el glicerol como disolvente es su elevada reactividad química, producida por los grupos hidroxilo del glicerol. Esto puede causar la síntesis no deseada de productos secundarios (Gu & Jérôme 2010; Thanh et al. 2012).

Por estos motivos diversos grupos de investigación presentaron en el 2005 un proyecto para utilizar glicerol no sólo como medio de reacción, sino como materia prima para sintetizar disolventes que no causen impactos en el medio ambiente. Este

objetivo se estableció en el proyecto SOLVSAFE (*Advanced Safer Solvents for Innovative Industrial Eco-processing*, NMP2-CT-011774) (Gu & Jérôme 2013), una iniciativa de la Unión Europea (UE) que mediante la colaboración entre empresas e investigadores se estableció como finalidad la reducción de los efectos perjudiciales relacionados con COVs.

Entre otros objetivos, este proyecto pretendió encontrar nuevos disolventes derivados del glicerol producido por la síntesis de biodiesel. De esta manera, se podrían solucionar dos problemas medioambientales: por un lado, la reutilización de un producto de desecho de una síntesis química; por otro, la sustitución de disolventes contaminantes por otros que causen menos impacto en la salud y en el medio natural.

Por ello, el grupo Catálisis Heterogénea en Síntesis Orgánicas Selectivas del Instituto de Síntesis Química y Catálisis Homogénea (ISQCH, Grupo Consolidado E11 del Gobierno de Aragón) sintetizó y caracterizó una serie de éteres de glicerol, cuyas características estructurales y fisicoquímicas permitían seleccionar como posibles disolventes verdes alternativos a otros convencionales (García et al. 2010).

Las características mencionadas son:

- Estructura molecular sencilla, para que puedan ser generados en gran cantidad y a bajo coste económico.
- Mantener una presión de vapor y viscosidad bajas, en condiciones líquidas.
- Ser líquidos para su utilización en condiciones normales de trabajo, manteniendo una presión de vapor baja y una baja viscosidad.
- Tener la suficiente inercia química para ser estable y facilitar su almacenado.

Este grupo sintetizó más de 60 compuestos derivados del glicerol que cumplieron estos requisitos previos. La principal diferencia entre ellos fue la presencia de cadenas de alquilo de diferente tamaño y grupos funcionales en los extremos de los grupos hidroxilo de la molécula de glicerol, usada como estructura base.

De todos estos derivados, 19 de ellos, incluyendo el glicerol, han sido seleccionados para realizar un estudio sistemático de ecotoxicidad aguda tras comprobar cuáles podrían ser mejores disolventes industriales por sus propiedades fisicoquímicas (Dearden 2003; García et al. 2010). Se puede ver un listado con las estructuras de los 20 compuestos, junto con sus códigos en la Figura 5. Los derivados contienen cadenas de alquilo (formadas por 1 a 4 hidrocarburos, en disposición lineal, *tert* o *iso*) unidas por los grupos hidroxilo del glicerol. También se han incluido cadenas que contienen átomos de flúor; los cuales han sido incluidos en la batería de ensayos por el interés en sus propiedades fisicoquímicas, ya que tienen valores intermedios de hidrofobia y lipofilia, alta polaridad, capacidad de formar enlaces de hidrógeno y una baja presión de vapor (Aldea et al. 2010; Aldea et al. 2012).

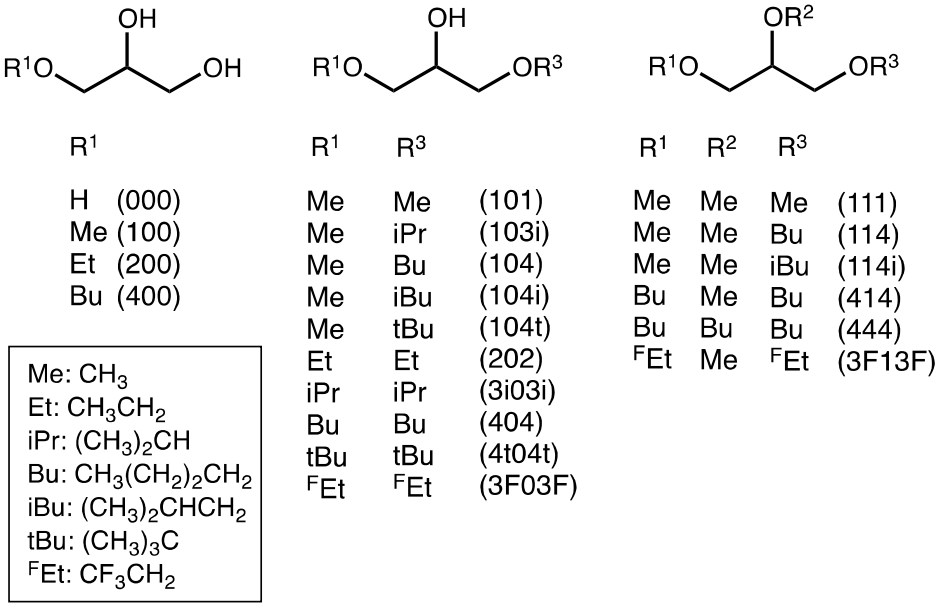

Figura 5: Esquema del glicerol y de sus 19 derivados, junto con la nomenclatura de sus grupos sustituyentes

Obtención de información medioambiental

Aunque la caracterización fisicoquímica ofrece mucha información sobre las propiedades de estos compuestos, se ha de concretar si estos compuestos serán respetuosos con el medio ambiente una vez que hayan sido usados y/o desechados.

Para realizar un estudio ecotoxicológico de manera sistemática, es necesario contar con ensayos estandarizados y sistemas de clasificación globales que permitan valorar de la manera más objetiva posible la toxicidad de los compuestos en el medio ambiente y, por extensión, en el ser humano, tales como SGA (Naciones Unidas 2015).

Estos ensayos se llevan a cabo exponiendo a los bioindicadores a una serie de concentraciones de la sustancia a estudiar, relacionándolas con el efecto cuantificable que producen, denominado *endpoint*. De esta manera, se puede obtener la concentración efectiva/letal 50 (*Effective/Lethal Concentration*, EC/LC_{50}). Este valor es la concentración en la cual el *endpoint* ocurre en la mitad de los individuos expuestos a la sustancia. A través de estos valores se obtiene su clasificación en función de rangos previamente establecidos (Naciones Unidas 2015; Passino & Smith 1987). Inicialmente, es recomendable que los ensayos tengan un periodo de exposición agudo, para poder tener un conocimiento inicial del nivel de peligrosidad con los valores de ecotoxicidad obtenidos para futuros ensayos más complejos.

En la realización de los tests de ecotoxicidad, es preciso seleccionar distintos bioindicadores que permitan evaluar la peligrosidad de la sustancia en el medio donde es más probable que finalice su vida útil. Además, siempre que sea posible, es conveniente comprobar los efectos de las sustancias que se quieran evaluar a diferentes niveles tróficos y complejidad biológica. Debido a la naturaleza y las propiedades de estos compuestos químicos, la opción más probable es que los derivados del glicerol

sean desechados en el medio acuático, por su utilización como disolventes industriales y sus propiedades fisicoquímicas.

Por ello, en la batería de ensayos experimentales realizados en esta tesis se utilizaron cuatro bioindicadores acuáticos con diferente complejidad biológica: la bacteria bioluminiscente *Vibrio fischeri* (*V. fischeri*), el alga unicelular *Chlamydomonas reinhardtii* (*C. reinhardtii*), el crustáceo *Daphnia magna* (*D. magna*), y el pez cebra o *Danio rerio* (*D. rerio*). Aunque todos ellos son acuáticos, *V. fischeri* habita en agua salina y los otros tres en agua dulce. Se escogieron estos bioindicadores porque cubren toda la cadena trófica: *V. fischeri* como descomponedor, *C. reinhardtii* como productor primario, *D. magna* como consumidor primario y *D. rerio* como consumidor secundario (Jos et al. 2003; Lee et al. 2015; Zhu et al. 2010; Zurita et al. 2007).

A continuación, se explicarán con un poco más de detalle cada uno de los bioindicadores utilizados.

V. fischeri

V. fischeri NRRL B 11177, también conocida como *Photobacterium phosphoreum*, con orden *Vibrionales*, es una bacteria gram negativa bioluminiscente de origen marino que tiene la particularidad de emitir luz de manera espontánea. Este microorganismo es la base de los ensayos de ecotoxicidad aguda para el análisis de aguas contaminadas o para la caracterización toxicológica de sustancias solubles en medio acuático (Microtox™, Lumistox™, Biotox™) (Farre & Barcelo 2003; Parvez et al. 2006).

Este ensayo tiene ventajas sobre otro tipo de pruebas ecotoxicológicas, ya que puede realizarse en un periodo de tiempo de entre 15 y 30 minutos, aportando datos fiables y reproducibles (ISO 11348-3 2009). Además, la disponibilidad de poder

realizarlo a través de kits comerciales facilita el mantenimiento y uso del microorganismo (Parvez et al. 2006). Por estas razones, este bioindicador suele considerarse como ensayo inicial en una evaluación ecotoxicológica, por su bajo coste relativo y elevada rapidez (Parvez et al. 2006). Es por ello ha sido estandarizado por diferentes normativas (Kaiser 1998; Parvez et al. 2006).

El mecanismo de acción de este bioensayo se basa en que sustancias exógenas a la bacteria puedan ejercer un efecto tóxico que reduce la bioluminiscencia. *V. fischeri* tiene una enzima llamada luciferasa, que produce la emisión de luz. Este proceso está involucrado en el metabolismo microbiano, de tal manera que se relaciona con la toxicidad de un agente estresor. Otros autores establecen que los efectos nocivos son más variados y complejos, donde diferentes interacciones pueden verse involucradas de manera individual o simultáneamente (Jennings et al. 2001). Por ejemplo, la toxicidad puede influir en fenómenos de interacción con receptores de la superficie celular, disrupción de la función de la membrana citoplasmática, reacciones químicas con componentes celulares, o inhibición/competición con sistemas enzimáticos (Cronin & Schultz 1998; Gustavson et al. 1998; Sixt et al. 1995).

C. reinhardtii

El microorganismo *C reinhardtii* CC125 pertenece al orden *Volvocales*. Son algas de color verde, unicelulares y de forma ovalada, que viven en agua dulce. Poseen una forma ovoidea de aproximadamente 20 µm de largo y 10 de ancho. La célula posee clorofilas de diferentes tipos como pigmentos naturales, los cuales se utilizan para realizar la fotosíntesis.

Desde un punto de vista biológico, las algas microscópicas son un componente clave de las cadenas alimenticias en ecosistemas acuáticos debido a su participación

fundamental en la conversión de energía en la cadena trófica. Por ello, cualquier sustancia que pueda causar daños a este bioindicador puede afectar de manera crítica al resto de un ecosistema (Esperanza et al. 2015). Es considerada como un organismo modelo en experimentación ecotoxicológica acuática, ya que la especie está extendida a nivel mundial, siendo fácil su cultivo y manipulación genética.

La mecánica del análisis consiste en la medición de la emisión de fluorescencia a través de la clorofila "a", mediante la técnica de la fluorometría modulada por amplitud de pulsos (*Pulse Amplitude Modulated*, PAM). Este bioensayo mide la parte de la energía de la luz absorbida que no es usada para las transformaciones fotoquímicas, la cual es disipada como calor o fluorescencia (Phuong et al. 2008). La energía emitida perteneciente a la fluorescencia está directamente relacionada con la luz absorbida por las moléculas de clorofila, lo cual se traduce en un correcto funcionamiento del fotosistema II (PSII). Este complejo proteico es un buen indicador de la tasa total de fotosíntesis y es considerado como la parte más vulnerable del aparato fotosintético ante la influencia de un agente externo que produzca estrés (Martinez et al. 2015).

En los ensayos de ecotoxicidad realizados se comparó el rendimiento fotosintético en fase de luz actínica en el alga *C. reinhardtii*, usando como *endpoint* el rendimiento cuántico fotoquímico efectivo en el PSII [Y(II)]. Una disminución del valor de este parámetro sería indicativo de un descenso en el flujo de electrones que permite la fotosíntesis. Esta inhibición puede ser la consecuencia de diferentes mecanismos de acción, como cambios en la integridad de las membranas fotosintéticas, lo que produciría la separación de los pigmentos captadores de la luz de la cadena de transporte de electrones (Brack et al. 1998; Yamada et al. 1996). También se ha demostrado toxicidad mediante otras alteraciones, como sustitución del átomo de Mg^{2+} en la clorofila por metales pesados (Corcoll et al. 2011; Küpper et al. 2002). No obstante, en

el caso de los compuestos derivados del glicerol, no se han demostrado si tienen un mecanismo de acción más específico.

D. magna

El crustáceo *D. magna*, comúnmente conocido como pulga de agua, es un artrópodo microscópico del orden *Cladocera*. Es un invertebrado lateralmente plano, cuyo cuerpo está cubierto por un exoesqueleto traslúcido, a través de la que se pueden distinguir sus órganos internos usando una lupa o un microscopio óptico. Vive en grandes agrupaciones en medios acuáticos no salados de amplios espacios, cerca de la vegetación de la orilla. Este bioindicador está presente en una amplia extensión de masas acuáticas del planeta, una de las principales razones por las cuales es tan utilizado como organismo medidor de la toxicidad acuática (Persoone et al. 2009). Se alimenta fundamentalmente de algas, protozoos y bacterias y, a su vez, es alimento de otros invertebrados y pequeños peces.

Este bioindicador ha sido ampliamente usado en la evaluación ecotoxicológica, tanto de carácter agudo como crónico, con todo tipo de sustancias que puedan disolverse con facilidad en el agua. Por esta razón, sus protocolos se han estandarizado en diferentes normativas y legislaciones (OCDE 1984; OCDE 2004; ISO 6341 2012). Además, tienen la ventaja de ser una especie sensible a compuestos sintetizados artificialmente, lo cual permite detectar efectos dañinos de sustancias a concentraciones inferiores que otros biomodelos (Giesy & Hoke 1989).

Este ensayo ha sido muy utilizado para valorar si la toxicidad de las sustancias a evaluar tienen una relación directa con su lipofilia, ya que se ha visto previamente buenas relaciones en tóxicos más complejos (Cleuvers 2003; Mínguez et al. 2014).

D. rerio

El *D. rerio* o pez cebra es un miembro de la familia *Cyprinidae*, originario del sur de Asia. Es un vertebrado de agua dulce de pocos centímetros de longitud en su etapa adulta, que alcanza en tres o cuatro meses. Aunque prefiere aguas cálidas, es ubicuo por todo el planeta. Este pez es uno de los modelos animales más utilizados en investigación biológica. Recientemente ha ganado popularidad como modelo en biología del desarrollo, toxicología y embriología (Zhang et al. 2005)

Como sujeto de estudio, el pez cebra es fácil de manipular y relativamente barato de mantener. Por ello, tanto los individuos adultos como los embriones han sido ampliamente utilizados en ensayos de toxicidad agudos y crónicos con metales, compuestos orgánicos y nanopartículas (Maes et al. 2012; Rocco et al. 2015). Maes et al. 2012; Rocco et al. 2015). También han sido utilizados para la monitorización de diferentes aguas residuales (Emelogu et al. 2014; Hallare et al. 2005)**.**

En el caso de individuos adultos, el ensayo de toxicidad consiste en mantener durante un periodo de tiempo agudo o crónico a grupos de individuos en peceras a diferentes concentraciones de la sustancia que queramos evaluar. La respuesta a la toxicidad que se pretende obtener suele ser la LC_{50}, o bien malformaciones o alteraciones en los patrones de conducta en ensayos crónicos. Estos ensayos están estandarizados por la OCDE (OCDE 1992).

Por otro lado, el ensayo de toxicidad aguda en el embrión también se encuentra estandarizado por la OCDE (OCDE 2013). Precisamente, debido a que tienen la particularidad de ser transparentes, son utilizados para determinar anomalías ante efectos de tóxicos y mutaciones inducidas.

Estudio sistemático de la ecotoxicidad: QSAR

Una vez que la información ecotoxicológica esté disponible, se ha de comprobar si existe correspondencia entre sus propiedades y/o estructura molecular y sus valores de EC/LC_{50}, estableciendo relaciones cuantitativas estructura-actividad (*Quantitative Structure-Activity Relationship*, QSAR). De esta manera, se puede llevar a cabo el estudio de manera sistemática, para poder predecir su comportamiento en otros compuestos similares. Este tipo de herramientas *in silico* han tenido un gran interés en los últimos años en diferentes campos experimentales (Dearden 1985; Mackay et al. 2014; Verma et al. 2010). Se basan en el diseño de un modelo matemático que relacione la estructura de una molécula o un conjunto de éstas, usando datos sobre una propiedad u actividad biológica, mediante métodos estadísticos (Gozalbes et al. 2002). Esto se consigue mediante la premisa de que sustancias químicas con similares estructuras poseen similares actividades (Nikolova & Jaworska 2003).

En el caso concreto de los compuestos derivados del glicerol, al ser un grupo de sustancias que tienen la misma estructura básica inicial, se realizó una serie de ensayos QSAR para estimar una relación entre su estructura y sus propiedades relacionadas con la ecotoxicidad, a través de parámetros topológicos, DARC-PELCO (*Description, Acquisition, Retrieval and Computer-aided design-Perturbation of an Environment which is Limited, Concentric and Ordered*), y propiedades fisicoquímicas, tales como la lipofilia. De hecho, García (García et al. 2013) ya utilizó en estos disolventes estos métodos, mediante regresiones lineales múltiples que relacionaban diferentes propiedades fisicoquímicas con la presencia de diferentes cadenas de alquilo en los grupos hidroxilo del glicerol.

En la experimentación realizada, se aplicaron los QSAR mencionados en los valores de EC_{50} de *V. fischeri* y *D. magna* a los diecinueve derivados del glicerol, junto con este compuesto.

Evaluación de riesgo del medio ambiente, salud y seguridad (EHSA)

Finalmente, ya obtenidos los valores EC/LC_{50} de interés, se pueden reunir junto con todos los datos disponibles sobre su impacto en el medio ambiente y/o en la salud, y utilizar una metodología evaluadora para valorar la toxicidad de la sustancia**.** Esta información es fundamental tanto para las empresas industriales como para organismos reguladores, tomando por ejemplo la ECHA en Europa. Esto permite decidir el diseño de la síntesis química y determinar qué sustancias, en función de sus características y propiedades, son las más apropiadas.

Con el objeto de hacer una estimación del grado de toxicidad de los compuestos en el medio ambiente lo más ajustada posible, se han diseñado diferentes metodologías para que los responsables de gestión medioambiental puedan valorar la idoneidad de una sustancia con un margen de seguridad (Chapman et al. 1998).

Una de las metodologías usadas para comprobar la peligrosidad de estos químicos sería la evaluación de riesgos medioambientales, de salud y de seguridad (*Environmental, Health, and Safety Approach*, EHSA) durante el proceso inicial de desarrollo, propuesto por Koller (Koller et al. 2000). En la Figura 6 se puede apreciar el esquema que lo ilustra. Esta metodología permite identificar o estimar la problemática de procesos industriales en fases tempranas de diseño. Se centra en diferentes niveles de impacto (medioambiental, salud humana y seguridad), que a su vez quedan clasificados en varias categorías, que al ser evaluadas y calificadas nos dan la información correspondiente sobre su peligrosidad.

Esta metodología es la que se ha utilizado para evaluar a cinco compuestos derivados del glicerol (200, 202, 400, 404, 444) en los ámbitos de salud, seguridad y medio ambiente, y en la comparación de ecotoxicidad medioambiental del compuesto 3F03F con el líquido iónico [BMIM][PF_6] (1-butil-3-metilimidazolio hexafluorofosfato).

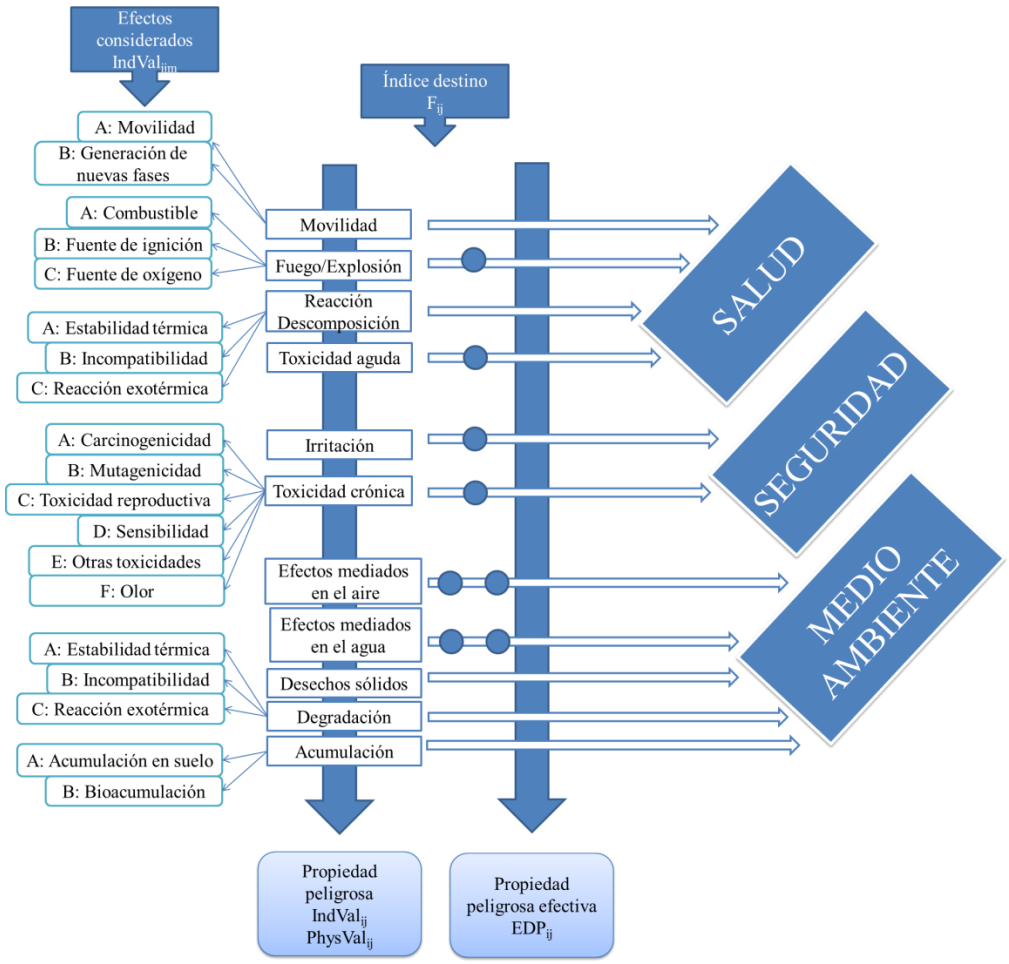

Figura 6: Esquema simplificado de la metodología EHSA establecida por Koller (Koller et al. 2000)

Summary

Relevance of solvents in chemical reactions and processes

Solvents are part of many chemical processes at any scale, since they allow performing procedures such as cleaning, extraction, purification, synthesis of substances. They are also used to perform heat and mass transfer processes (Kerton 2016).

These anthropogenic substances are created from the oil industry, and many of them are considered as volatile organic compounds (VOCs). Mainly, they are formed by carbon atoms, and they are distinguished by their low vapor pressure under normal conditions of temperature and atmospheric pressure.

Environmental concerns associated with the use of VOCs have resulted in the development of processes and chemicals that minimize the impact on the environment (Corrêa et al. 2015). Internationally, there are numerous laws regulating the use and distribution of chemical substances, and through the years, they are being increasingly restrictive to improve the control in these solvents, their environmental impact and their treatment as waste.

One of the more relevant legislations about environment is the Regulation 1907/2006/EC, which establishes the registration, evaluation, authorization and restriction of chemical substances (REACH), and also the establishment of the European Chemical Agency (ECHA). This agency is responsible for advising companies on compliance with REACH legislation, providing information about chemical compounds, and promoting their control and safe use. In addition, this normative highlights the obligation of the companies for reporting on the risks in

handling their chemicals. Finally, this legislation establishes limit ranges for the use of substances.

Another relevant legislation is the Regulation 1272/2008/EC about classification, labeling and packaging of substances and mixtures, coordinated with the REACH Regulation. It establishes criteria agreement with the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (Naciones Unidas 2015). The purpose of this regulation is to quantify the value of the properties of the dangerous substances and their mixtures, establishing categories to facilitate their classification and labeling.

Green chemistry and environmentally friendly solvents

According to the previous paragraphs, there are legislations that ensure the correct use of all kinds of chemical substances, which has also been reflected as an interest in the scientific community. Since the end of the last century, researchers have focused on the development of methodologies and tools to minimize the impact of chemical processes. All these procedures have been compiled in the so-called philosophy of Green Chemistry. This philosophy is defined as the design of products and processes to reduce or eliminate the use and generation of harmful substances to human health and the environment (Anastas & Eghbali 2009).

Green Chemistry was initially designed by Professors Paul T. Anastas and John C. Warner in the 1990s, with the aim of focusing chemical production from a point of view more respectful for human health and the environment. They emphasized about the elimination of dangerous processes in organic synthesis from the outset. Both researchers developed the "Twelve Principles of Green Chemistry," a compendium of

guidelines that set a roadmap for all chemists wishing to work in a more sustainable framework for health and the environment (Anastas & Eghbali 2009).

One of these principles highlights the elimination of auxiliary substances in chemical synthesis, including solvents. If this premise is not possible, compounds that cause the least possible impact would be preferred ("green solvents"). These are any compound that allows minimizing the environmental impact in a chemical synthesis (Capello et al. 2007). In general, green solvents are characterized for their low environmental toxicity, readily biodegradability, reusability, high availability and low price (Li & Trost 2008; Valavanidis & Vlachogianni 2012).

In recent years, different types of alternative solvents have been studied, such as water (Chigrinova et al. 2015, Lipshutz & Ghorai 2014), alcohols (Horváth 2008), supercritical fluids (Eckert et al. 1996; Herrero et al. 2013), and ionic liquids (Thuy Pham et al. 2010; Zhao et al. 2005), which can be considered as an improvement to other conventional solvents.

Glycerol and its derivatives: Origin and use

Nowadays, a lot of attention has been paid to the use of solvents from biomass (Corrêa et al. 2015). One of its most notable applications as raw material is the synthesis of biodiesel. In the synthesis of this fuel, glycerol (1,2,3-propanetriol) or glycine is produced in an approximate 10% yield, among other waste compounds (Dasari et al. 2005). Unless it contains impurities, it is a hygroscopic, odorless, colorless, viscous and dense liquid.

There are reports which explain the increasing diesel production during the last years (Li et al., 2013; Nanda et al., 2014), so it is expected that indirect production of glycerol will continue to increase in the future. Considering this overproduction,

research has been carried out to look for new applications and thus avoid the accumulation of glycerol (Bagheri et al. 2015; Behr et al. 2008; Konstantinovic et al. 2016; Leoneti et al. 2012; Li et al. 2013; Pagliaro et al. 2007; Thanh et al. 2012).

One of the most plausible solutions is the usage of glycerol as an alternative solvent. Indeed, positive results have been obtained in the use of this substance as a reaction medium, increasing reaction yields and selectivity in specific reactions (Corma et al. 2007; Díaz-Álvarez et al. 2013; Gu & Jérôme 2010; Gu & Jérôme 2013; Wolfson et al. 2007).

However, certain drawbacks have to be taken into account about the use of glycerol as a solvent. One of them is its high viscosity, since it can cause mass transfer problems, making it difficult for the substrate to diffuse properly through the medium of the reaction (Gu et al. 2010, Gu & Jérôme 2013). This drawback can be mitigated by an increase in reaction temperature (about 50-60 ° C).

Another disadvantage about using glycerol as a solvent is its high chemical reactivity, produced from the hydroxyl groups of the glycerol. This may cause undesired synthesis of by-products (Gu & Jérôme 2010; Thanh et al. 2012).

For these reasons, glycerol was presented by several research groups, with the collaboration of the EU, as a raw material to synthesize solvents that do not cause an impact on the environment. This proposal was established in the SOLVSAFE project (Advanced Safer Solvents for Innovative Industrial Eco-processing, NMP2-CT-011774) (Gu & Jérôme 2013), an EU initiative for business collaboration and academic research aimed at reducing the harmful effects related to VOCs.

This project sought to find new solvents derived from glycerol, produced by the synthesis of biodiesel. In this way, two environmental problems could be solved: on the

one hand, the reuse of a waste product from a chemical synthesis; on the other, the substitution of polluting solvents for others that have less impact on health and the environment.

For these reasons, the heterogeneous catalysis in selective organic synthesis group of Instituto de Síntesis Química y Catálisis Homogénea (ISQCH, Consolidated Group E11 of the Government of Aragon) synthesized and characterized a series of glycerol ethers, whose characteristics allowed alternative green alternatives to conventional ones (García et al. 2010).

Of all these derivatives, nineteen of them, including glycerol, have been selected to carry out a systematic study of acute ecotoxicity. Although the previously physicochemical characterization of these solvents provided a lot of information about these compounds, it is necessary to specify if they will be respectful to the environment once they have been used or discarded.

Obtaining environmental information

To carry out an ecotoxicological study in a systematic way, it is necessary to have standardized tests and global classification systems that allow evaluating as objectively as possible the toxicity of the compounds in the environment and, by extension, in humans, such as GHS (Naciones Unidas 2015).

These tests are carried out in the laboratory, exposing the bioindicators to several concentrations of the studied substance, and relating them to their measurable effect, called the endpoint. In this way, the effective/lethal concentration 50 (EC/LC_{50}) can be obtained. This value is the concentration at which the endpoint occurs in half of the individuals exposed to the substance. Through these values the designation of hazard is obtained according to established classifications (Naciones Unidas 2015; Passino &

Smith 1987). Initially, it is recommended that the trials have an acute exposure period, in order to have an initial knowledge of the level of danger.

About the ecotoxicity tests, it is necessary to establish a set of bioindicators that allow evaluating the hazardousness of the substance in living organisms belonging to the environment where the compound is most likely to end. In the battery of experimental tests carried out, four aquatic bioindicators with different biological complexity were used: the bioluminescent bacteria *Vibrio fischeri* (*V. fischeri*), the unicellular algae *Chlamydomonas reinhardtii* (*C. reinhardtii*), the crustacean *Daphnia magna* (*D. magna*), and the zebrafish or *Danio rerio* (*D. rerio*). Although all of them are aquatic organisms, *V. fischeri* lives in salt water and the other three live in fresh water. These bioindicators were chosen because they cover the whole trophic chain: *V. fischeri* as decomposer, *C. reinhardtii* as primary producer, *D. magna* as primary consumer and *D. rerio* as secondary consumer (Jos et al. 2003; Zhu et al. 2010; Lee et al. 2015; Zurita et al. 2007).

Systematic study of ecotoxicity: QSAR

Once the ecotoxicological information is available, it must be checked whether there are relationships between its properties and/or molecular structure and its EC/LC_{50} values, establishing quantitative structure-activity relationships (QSARs)

In the case of glycerol-derived compounds, as a group of substances which have the same initial basic structure, a series of QSAR tests were performed to estimate a relationship between their structure and their properties related to ecotoxicity, through DARC-PELCO (Description, Acquisition, Retrieval and Computer-aided design-Perturbation of an Environment which is Limited, Concentric and Ordered), topological parameters, and physicochemical properties, such as lipophilicity. In fact, García

(Garcia et al., 2013) already used these methods by multiple linear regressions that related different physicochemical properties with the presence of different alkyl chains in the hydroxyl groups of glycerol.

Environmental, health and safety risk assessment (EHSA)

Finally, all the obtained values can be combined with all available data and use them in an evaluation methodology to assess the toxicity of the substances.

In order to estimate the degree of toxicity of the compounds in the environment as closely as possible, different methodologies have been designed to establish a classification on which environmental decision makers can choose a specific solvent with a margin of safety (Chapman et al., 1998).

One of the methodologies used to test the hazards of these chemicals would be the Environmental, Health and Safety Approach (EHSA) during the initial development process proposed by Koller (Koller et al. 2000). This methodology allows identifying or estimating the problem of industrial processes in the early stages of design. It focuses on different levels of impact (environmental, human health and safety), which are classified into several categories, giving us the corresponding information about its dangerousness.

JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICACIÓN Y OBJETIVOS

Justificación

La justificación de esta evaluación de ecotoxicidad en el glicerol y en diecinueve de sus derivados se basa en poder disponer de la información suficiente para establecer si estas sustancias serían buenos disolventes verdes en síntesis industriales. Además, con las herramientas utilizadas y validadas durante la experimentación, se podría predecir la toxicidad acuática de otros compuestos derivados del glicerol que no hayan sido analizados. De esta manera, las futuras síntesis realizadas en este grupo homólogo de compuestos podrían estar más dirigidas en derivados que se prevé que no sean peligrosos para la salud humana y ambiental. Esto permite un gran ahorro de tiempo y costes de experimentación, fundamentalmente a la hora de realizar futuros ensayos en un tiempo de exposición crónico.

Objetivos

El objetivo principal de esta tesis fue establecer si las variaciones estructurales en los derivados del glicerol estudiados afectan a sus propiedades ecotoxicológicas, confirmando la posibilidad de que estos compuestos puedan ser disolventes verdes.

Para corroborarlo, el primer objetivo secundario en esta tesis ha sido determinar la ecotoxicidad de estas sustancias en exposición aguda en los biomodelos *V. fischeri*, *D. magna*, *C. reinhardtii* y *D. rerio*, determinando si eran peligrosas para el medio ambiente a través de clasificaciones de ecotoxicidad acuática.

El siguiente objetivo fue, mediante los valores de ecotoxicidad hallados, realizar ensayos QSAR para estimar las relaciones entre las propiedades estructurales y

fisicoquímicas de estos compuestos. Además, estos datos han sido empleados en un sistema evaluador de riesgo toxicológico, que permite valorar estas propiedades**.**

Finalmente, se propuso un último objetivo, que es la comparación ecotoxicológica de dos disolventes utilizados paras las mismas aplicaciones; uno de ellos procedente de la biomasa derivado del glicerol, el 1,3-bis (2,2,2-trifluoro-etoxi)propan-2-ol (3F03F), y el otro un líquido iónico, el 1-butil-3-metilimidazolio hexafluorofosfato ([BMIM][PF_6]). Ambas sustancias han sido utilizadas como disolventes en catálisis enantioméricas bifásicas.

Summary

The justification for this evaluation of ecotoxicity in these glycerol derivatives would be to verifiy if these substances would be good green solvents in industrial synthesis. In addition, with an established methodology, the aquatic toxicity of other glycerol-derived compounds could be predicted. In this way, future syntheses performed in this homologous group of compounds could be more targeted in derivatives that are expected to be not hazardous to human and environmental health. This allows a great saving of time and costs in experimentation.

The main objective of this thesis was to establish whether the structural variations in the studied glycerol derivatives affect their ecotoxicological properties, confirming the possibility that these compounds may be green solvents.

The first secondary objective in this thesis was to determine the ecotoxicity of these substances in acute exposure in the biomodels *V. fischeri*, *D. magna*, *C. reinhardtii* and *D. rerio*, determining if they were dangerous for the environment to aquatic ecotoxicity classifications.

The following objective was, through the ecotoxicity values, to perform QSAR tests to estimate the relationships between the structural and physicochemical properties of these compounds. In addition, these data have been used in a toxicological risk assessment system, which allows evaluating these properties.

Finally, a final objective was proposed, which is the comparison of two solvents used for the same applications; one of them from the biomass derived from glycerol, 1,3-bis (2,2,2-trifluoro-ethoxy) propan-2-ol (3F03F), and the other an ionic liquid, 1-

butyl-3 -methylimidazolium hexafluorophosphate ([BMIM] [PF6]). Both substances have been used as solvents in biphasic enantiomeric catalysis.

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METODOLOGÍA

METODOLOGÍA

Estudios de ecotoxicidad con bioindicadores

V. fischeri

En la Figura 7 se puede ver un esquema simplificado del ensayo de ecotoxicidad con el bioindicador *V. fischeri*.

Previamente a los ensayos, la bacteria liofilizada (cepa NRRL-B-11177) (Macherey-Nagel, Düren, Alemania) fue rehidratada usando el correspondiente medio adjuntado por el fabricante, siendo mantenida previamente hasta el momento de su uso en un rango de temperatura de 2 a 8ºC.

La bioluminiscencia fue medida mediante un luminómetro Biofix® Lumi-10 (Macherey-Nagel, Düren, Alemania), usando el modo agudo (Biotox B) mediante un contador ultra-rápido de protones (*Ultra Fast Single Photon counter*), con un rango espectral de 380-660 nm.

Para la preparación de las muestras, se determinan previamente las concentraciones de la sustancia a evaluar y se preparan en agua al 2 % de NaCl. El pH se corrige mediante disoluciones HCl (0.1 M) o NaOH (0.1 M), para situarlo entre 7.0 y 7.5. Tanto control negativo (Agua al 2% NaCl) como control positivo (Sulfato de zinc, 2.5 mg L^{-1}; fenol, 42.5 mg L^{-1}) fueron utilizados en cada ensayo.

Se midió la bioluminiscencia inicial de la bacteria activada en suspensión después de transferir 0.5 ml a cada pocillo en una temperatura de 15ºC. A continuación, se añaden otros 0.5 ml de la dilución del compuesto a estudiar. La toxicidad se refleja en

el ratio del descenso en la bioluminiscencia bacteriana. La luminiscencia se midió de nuevo a los 30 minutos de exposición. El test al menos fue repetido dos veces.

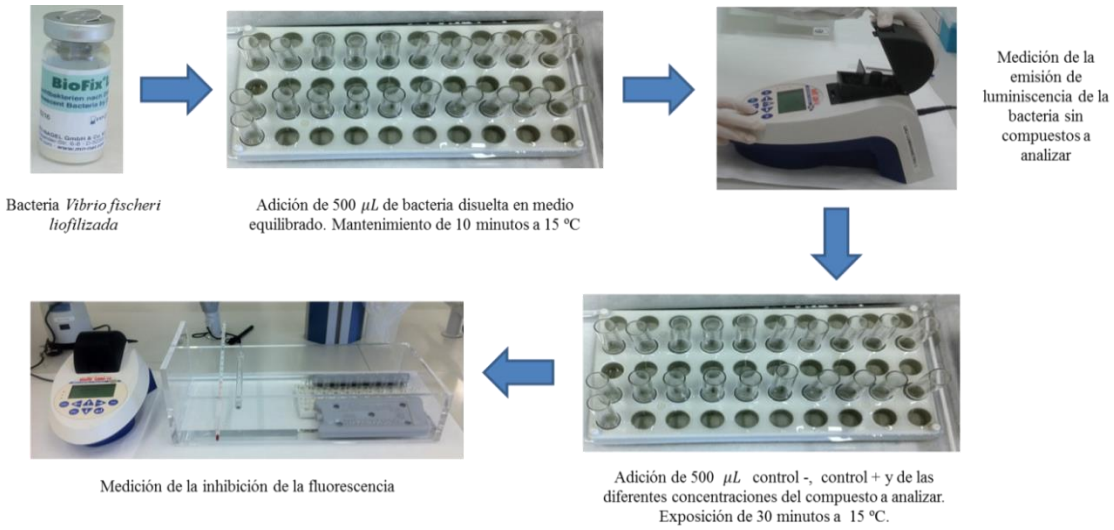


Figura 7: Esquema del protocolo experimental realizado en *V. fischeri*

C. reinhardtii

En la Figura 8 se puede ver un esquema simplificado del protocolo de cultivo de *C. reinhardtii* para la utilización de este ensayo de ecotoxicidad.

Para la realización de este ensayo, se han de tener preparadas previamente las algas [*C. reinhardtii* (CC125)] en el cultivo madre, dispuestas en placa de Petri, bajo iluminación continua de130 µE PAR m^{-2} s^{-1} en un incubador a 25ºC. Para proceder a su activación, han de ser cultivadas en tubos Falcon de 50 ml durante la primera fase (Fase A), y en frascos Erlenmeyer en las siguientes (Fases B-D), en medio Talaquil (Szivák et al. 2009) hasta llegar a la cantidad necesaria de células para poder obtener el cultivo experimental utilizado en el ensayo. Este periodo de preparación dura entre 12 y 15 días, y es necesario realizar intercambios de medio de cultivo mediante centrifugado (3000 rpm a 10 minutos en temperatura ambiente). A su vez, requiere un control de la población celular mediante espectrometría en el rango visible, a 685 nm.

En la iluminación necesaria para el cultivo de algas previo a la experimentación, se necesitan tubos fluorescentes T5HO, 39 W 10 000 K^{-1} (Blau Aquaristic, Barcelona, España), mientras se mantienen en un agitador orbital (Spinette Cell Stirrer, Starna, Hainault, Reino Unido). Para la medición del Y(II) se ha utilizado un fluorómetro Mini-PAM (Walz, Effeltrich, Alemania)

Para la preparación de las muestras, se disponen alícuotas de las algas en frascos Erlenmeyer que contengan MOPS (ácido 4-morfolinpropanosulfónico). Este medio es un *buffer* que permite mantener constante el pH durante los ensayos preparado con agua milli-Q, previamente ajustado a 7.5 mediante una disolución de KOH. Para cada concentración, se pone al menos dos alícuotas, que se exponen durante 1 hora, mientras son agitadas de manera continua.

En la configuración del fluorómetro Mini-PAM se han utilizado los siguientes ajustes:

- ML (*Measuring light*): 0.15 µmol m^{-2} s^{-1}
- SP (*Saturating pulse*): 1577 µmol m^{-2} s^{-1}, de 0.8 segundos de duración.
- Aclimatación previa de las muestras: Aproximadamente 30 segundos a 50-60 15 µmol m^{-2} s^{-1}.

Tras la exposición a la sustancia de estudio durante 1 hora y agitada de manera continua a 90 rpm, se realizan las mediciones del Y(II). En el ensayo se registraron por cada alícuota 4 o 5 mediciones consecutivas con intervalos de 10 segundos. El control negativo es el propio medio MOPS.

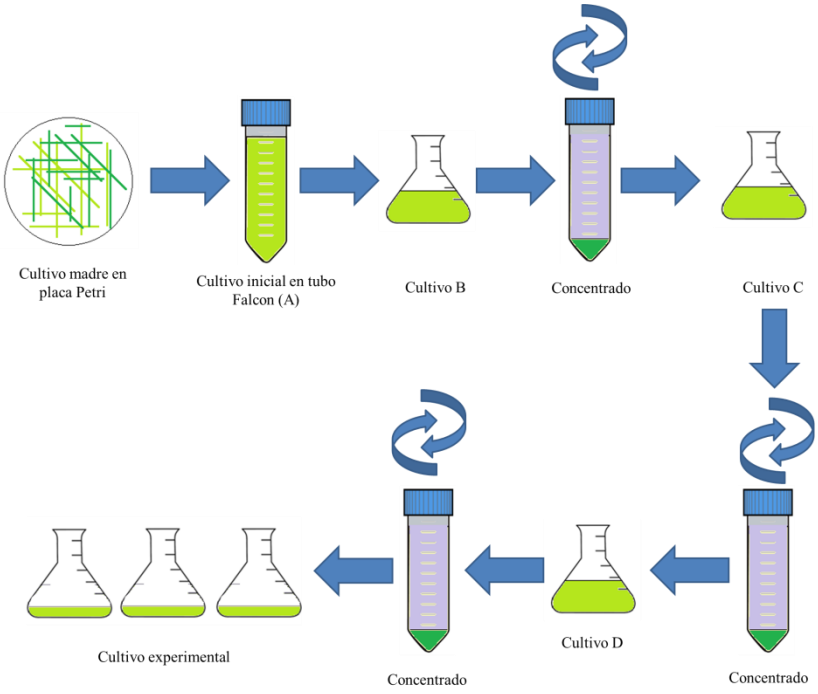


Figura 8: Esquema del protocolo para el cultivo previo realizado en *C. reinhardtii*

D. magna

En la Figura 9 se muestra un esquema simplificado del cultivo y ensayo de inmovilización con *D. magna.*

Los efipios de *D. magna* han sido obtenidos de Vidrafoc (Toxkit, Daphtokit F Magna, Barcelona, España). Su incubación ha sido realizada en un incubador TOXKIT CH-0120D-AC/DC (ECOTEST, Valencia, España).

Se mantienen guardados los efipios de 2 a 8ºC hasta su uso. Para cada ensayo, es necesario extraer un nuevo lote, ya que los individuos son desechados tras cada ensayo. Se prepara su incubación con el medio de cultivo proporcionado por el proveedor durante 72 horas a 22 ± 2ºC, bajo iluminación continua a 6000 lux. Este medio se ha de cambiar cada 24 horas. 2 horas antes del ensayo, los neonatos han de ser alimentados con alga *Spirullina*.

Las concentraciones de la sustancia a analizar se han de preparar mediante el mismo medio donde se han mantenido los efipios. Previamente a su utilización se ha de airear en agitación durante al menos 30 minutos. El pH de las disoluciones se ha de mantener entre 7 y 7.5. El control negativo es el mismo medio utilizado anteriormente y el control positivo se prepara con $K_2Cr_2O_7$.

Tras haber esperado 2 horas después de alimentar a los neonatos con *Spirulina*, se sitúan en cuatro alícuotas de cada concentración en grupos de cinco, controles positivo y negativo incluidos (20 individuos por concentración). Tras cubrirlos para que no se evaporen las muestras, se colocan en un incubador a 20 ± 2ºC en completa oscuridad durante 24 horas. Una vez transcurrido este tiempo, se cuantifican las daphnias inmovilizadas de cada uno de los pocillos. Se considera que un individuo está inmovilizado cuando, tras una agitación leve, no se mueve durante 15 segundos.

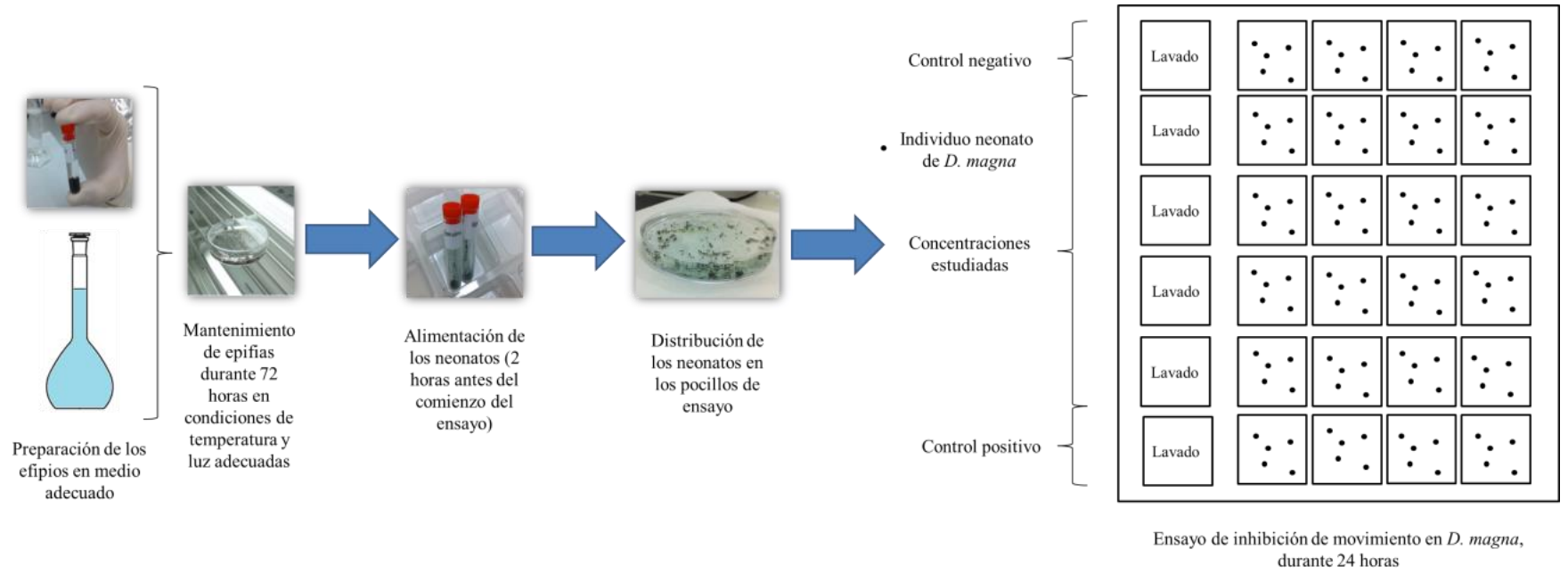


Figura 9: Esquema del protocolo para el cultivo y ensayo realizado en *D. magna*

D. rerio

En la Figura 10 se puede un esquema simplificado del protocolo de preparación del ensayo en embrión de pez cebra.

Los datos obtenidos en los ensayos de ecotoxicidad con *D. rerio* han sido obtenidos en dos centros de investigación diferentes. El ensayo de toxicidad aguda en pez adulto fue realizado en el Centre de Recerca i Innovació en Toxicología de la Universitat Politècnica de Catalunya (Tarrasa, España), según las especificaciones del protocolo de la OCDE 203 (Ensayo de toxicidad aguda en peces). Por otro lado, los ensayos en embrión de *D. rerio* fueron realizados en la empresa de biotecnología ZFBiolabs, (Madrid, España), en una adaptación del ensayo de la OCDE 236 (Ensayo de toxicidad aguda en embrión de pez). La exposición fue de 24 y 48 horas.

Test de toxicidad aguda en pez adulto (OCDE 203)

Los test fueron llevados a cabo en acuarios con agua potable desclorada. Se realizaron a una temperatura de 22±2ºC. Se utilizaron 7 peces en cada concentración, y el mismo número en el control. Se estableció un patrón de iluminación de 16 horas de luz y 8 de luz difusa. La concentración de oxígeno siempre fue superior al 60 % y el pH se mantuvo entre 8.3 y 8.5. Los peces no fueron alimentados durante el periodo de ensayo y tanto su mortalidad como sus cambios de comportamiento fueron registrados a las 3, 24, 48, 72 y 96 horas.

Los tests de toxicidad aguda de 96 horas se realizaron en un sistema de exposición estática (sin renovación del tóxico). Los criterios de validación del ensayo fueron el mantenimiento de las condiciones del ensayo, mortalidad de los controles por debajo del 10 %, y la concentración de saturación de oxígeno en el medio acuático.

Test de toxicidad aguda en embrión de pez (OCDE 236)

Los embriones de *D. rerio* fueron obtenidos mediante fecundación *in vitro* y fueron seleccionados, siendo su porcentaje de eclosión superior al 80%. Una disolución de DMSO (dimetilsulfóxido) al 0.25 % fue usada para disolver los compuestos. La temperatura se mantuvo entre 24 y 26ºC, mientras que la saturación de oxígeno entre 60 y 100 %. El pH fue ajustado entre 6.5 y 8.5. El control positivo fue paracetamol (4155 mg L^{-1}).

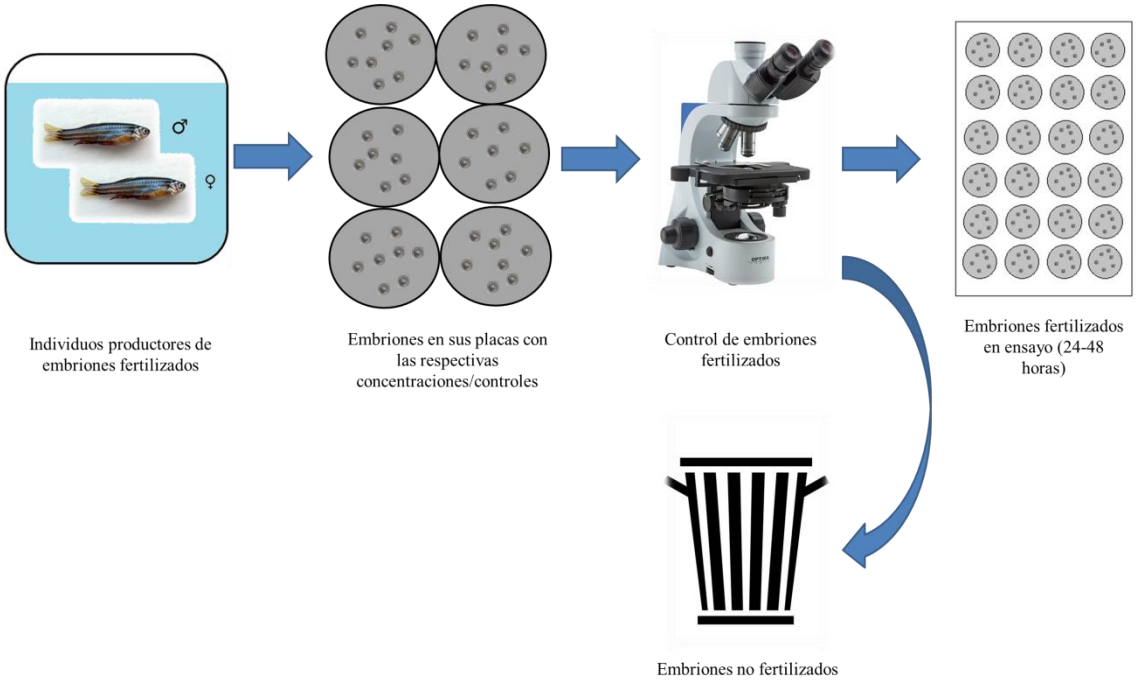


Figura 10: Esquema del protocolo de preparación del ensayo en embrión de pez cebra.

Desarrollo de la experimentación realizada

En la Figura 11 se puede ver un esquema de la metodología seguida en la evaluación ecotoxicológica de los disolventes derivados del glicerol. En la parte superior del esquema se encuentra una imagen de la estructura básica del glicerol, junto a una tabla donde se indican los grupos sustituyentes que pueden existir en cada grupo hidróxilo. En la parte central se puede ver una curva de concentración frente al porcentaje de respuesta, para la obtención de la EC/LC_{50} en un bioindicador expuesto a una sustancia. En el extremo izquierdo, derecho e inferior se muestra el trabajo realizado en esta tesis, clasificado por los artículos obtenidos durante el transcurso de esta tesis.

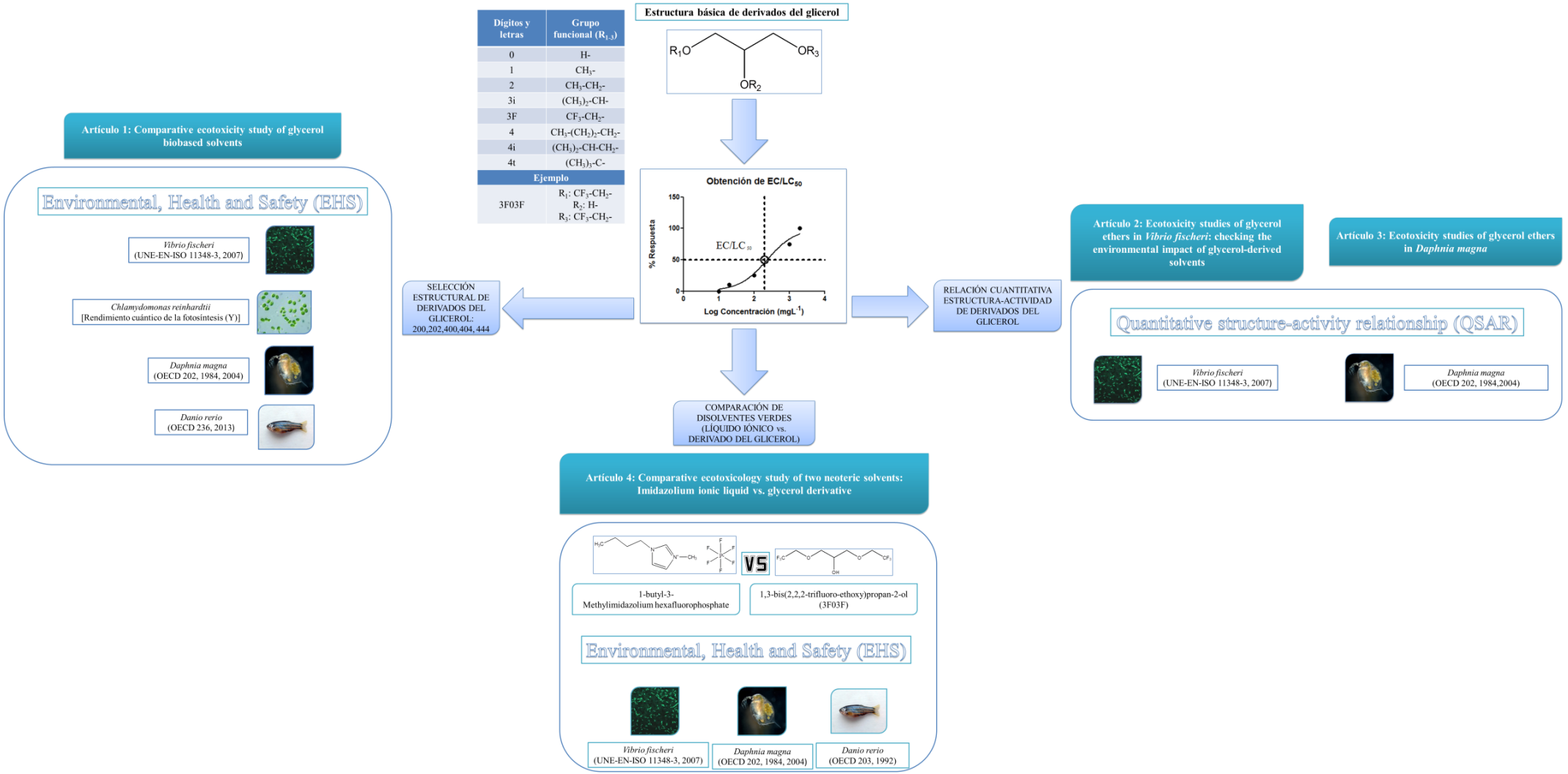


Figura 11: Esquema de la metodología seguida en la elaboración de esta tesis doctoral

RESULTADOS

RESULTADOS

En este capítulo se resumen los resultados que se han obtenido en el transcurso de la tesis, así como los artículos publicados o aceptados.

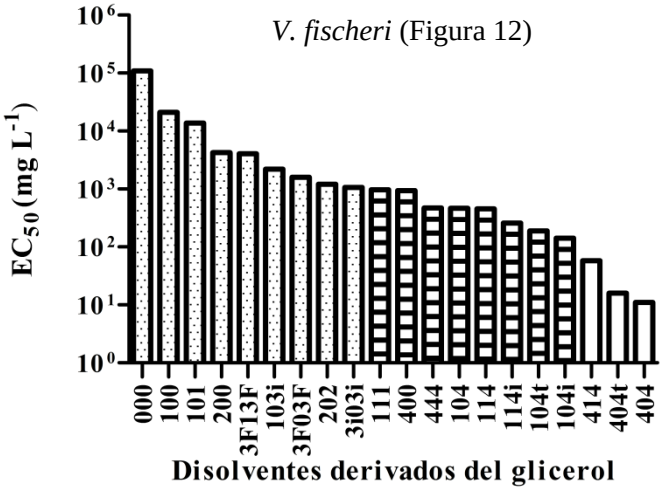
Los resultados de ecotoxicidad obtenidos en esta tesis se pueden ver resumidos en la Tabla 3 y en las Figuras 12-15, donde se indican los valores de EC/LC_{50} obtenidos en la experimentación de los bioindicadores *C. reinhardtii*, *D. magna*, *V. fischeri* y *D. rerio.* Además, en cinco de los compuestos derivados del glicerol (200, 202, 400, 404 y 444), se realizó una evaluación de salud, seguridad y medio ambiente, para determinar cuál de estos compuestos es menos peligroso para la salud y el medio acuático.

Además, se han realizado estudios QSAR con los valores de EC_{50} en *V. fischeri* y *D. magna* de los veinte compuestos, mediante la metodología DARC-PELCO, parámetros topológicos y la lipofilia.

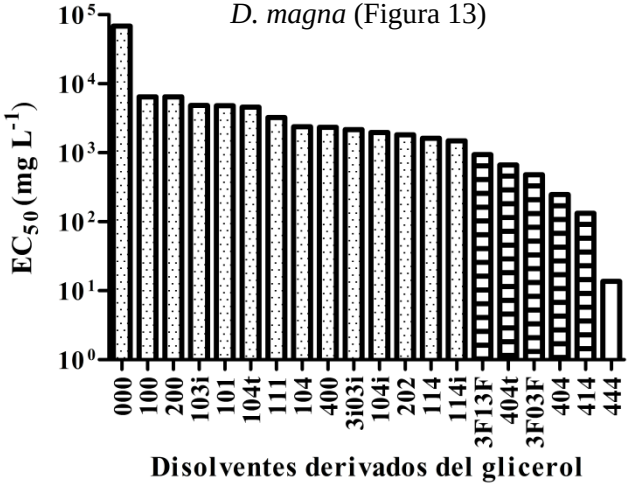
Por último, se realizó una comparación ecotoxicológica del líquido iónico [BMIM][PF6] con el compuesto derivado del glicerol 3F03F, en *V. fischeri*, *D. magna*, y *D. rerio* adulto. Se realizó también una evaluación medioambiental para determinar cuál de estos compuestos puede causar menos daño al medio acuático.

Compuesto	Peso molecular	EC_{50} *V. fischeri* (mg L^{-1})	SD	EC_{50} *D. magna* (mg L^{-1})	SD	EC_{50} *C. reinhardtii* (mg L^{-1})	SD	LC_{50} *D. rerio* (mg L^{-1})[a]	SD
000	92.09	108421	4.8	68784	7.7				
100	106.1	21052	3.6	6478	7.7				
200	120.1	4240	3.9	6458	5.3	52811	0.028	36700	2.6
400	148.1	941	1.8	2332	3.2	4445	0.052	4300	9.1
101	120.1	13702	3.8	4790	6.1				
111	134.1	969	4.0	3240	5.8				
202	148.2	1215	4.1	1819	5.6	8613	0.053	2800	8.7
404	204	11	1.8	248.1	4.1	631	0.043	17	3.7
114	176.1	453	5.4	1617	6.0				
104	162	464	3.5	2388	4.4				
414	218.2	58	2.9	133.3	5.9				
444	260.2	473	3.6	13.7	4.8	64	0.030	2700	7.1
103i	148.2	2188	3.8	4828	6.5				
104i	162.1	142	2.9	1975	5.5				
3i03i	176	1064	2.5	2170	5.3				
114i	176.1	258	3.9	1496	2.4				
104t	162.1	189	3.4	4568	4.3				
404t	204	16	3.5	667.6	8.1				
3F03F	256	1597	2.4	479.9	5.9			353[b]	0.773
3F13F	270.1	4033	3.8	943.7	4.2				

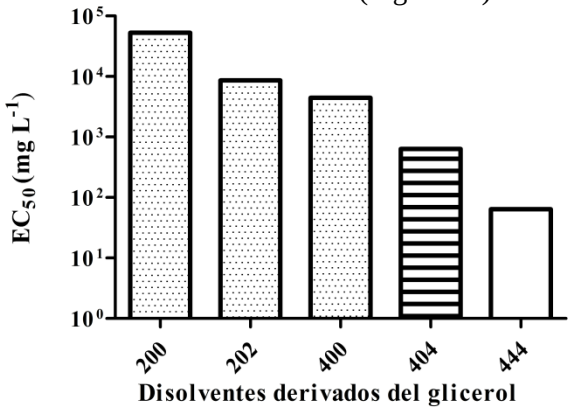
Tabla 3: Valores de peso molecular y ecotoxicidad aguda en los biomodelos *V. fischeri*, *D. magna*, *C. reinhardtii* y *D. rerio*. [a]OCDE 236 (2013) Test de toxicidad aguda en embriones de peces (FET).[b]OCDE 203 (1992) Test de toxicidad aguda en peces.



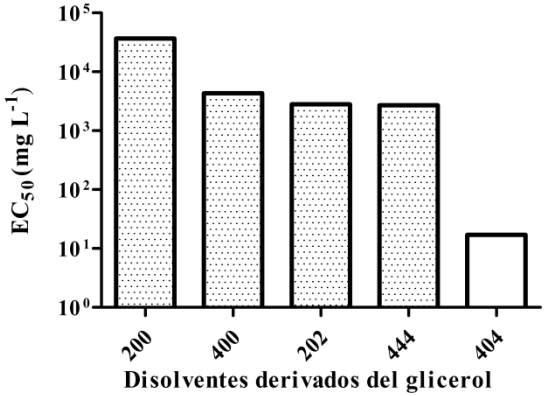
V. fischeri (Figura 12)



D. magna (Figura 13)



C. reinhardtii (Figura 14)



D. rerio (Figura 15)

Figuras 12-15: Representación gráfica de los valores de EC/LC_{50} derivados del glicerol, ordenados de menor a mayor toxicidad. Los patrones de las barras están dispuestos según su rango en la clasificación de Passino y Smith (Passino & Smith 1987). Barras punteadas: no tóxicos (EC_{50} mayor que 1000 mg L^{-1}); barras con rectas horizontales: prácticamente no tóxicos (EC_{50} 100-1000 mg L^{-1}); barras blancas: ligeramente tóxicos (EC_{50} = 10-100 mg L^{-1}).

Artículos publicados

Artículo 1: Comparative ecotoxicity study of glycerol biobased solvents

Autores: Eduardo Perales[A], Cristina Belén García[A], Laura Lomba[A], José Ignacio García[B], Elísabet Pires[B], Mari Carmen Sancho[C], Enrique Navarro[C], Beatriz Giner[A].

[A]*Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, 50830, Zaragoza, España.*

[B]*Instituto de Síntesis Química y Catálisis Homogénea (ISQCH) y Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Zaragoza– Consejo Superior de Investigaciones Científicas-(CSIC), Calle Pedro Cerbuna 12, 50009 Zaragoza, España.*

[C]*Instituto Pirenaico de Ecología (CSIC), Avenida Montañana 1005, 50059 Zaragoza, España.*

Revista: *Environmental Chemistry* (CSIRO *Publishing*). (Artículo aceptado, pendiente de publicación)

Factor de impacto: 3.52 (JCR, 2016)

Áreas temáticas: Química (miscelánea), química medioambiental, geoquímica y petrología.

En este artículo se realizó una comparación de la ecotoxicidad aguda de cinco de los disolventes del glicerol mediante los indicadores *D. magna*, *V. fischeri*, *C. reinhardtii* y *D. rerio*. A continuación, se utilizaron los valores obtenidos, junto con otros previamente calculados y/o estimados para determinar su adecuación como disolventes verdes según la metodología EHSA.

Decision Letter (EN17082.R1)

From: en@theeditorialhub.com

To: eperales@usj.es

CC:

Subject: Environmental Chemistry - Decision on Manuscript ID EN17082.R1

Body: 22-Jun-2017

Dear Mr Perales:

Thank you for sending the revised version of this paper (Comparative ecotoxicity study of glycerol biobased solvents) and for dealing so thoroughly with the referees' comments. I have now had the opportunity to examine your revised manuscript and I am pleased to accept it for publication in Environmental Chemistry.

Thank you for your excellent contribution. On behalf of the Editors of Environmental Chemistry, we look forward to your continued contributions to the Journal.

You will hear in due course from the Production Editor regarding the copyedited manuscript, page proofs, etc.

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Sincerely,

Dr Jason Unrine
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Date Sent: 22-Jun-2017

Running head: Acute ecotoxicological study of glycerol-derivatives

Title: Comparative ecotoxicity study of glycerol biobased solvents

Eduardo Perales[a], Cristina Belén García[a], Laura Lomba[a], José Ignacio García[b], Elísabet Pires[b],Mari Carmen Sancho[c],Enrique Navarro[c],Beatriz Giner[a]

[a]Address:

Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, 50830, Zaragoza, Spain.

Telephone number: 0034 976 060100

Fax number: 0034 976 077584

[b]Address:

Instituto de Síntesis Química y Catálisis Homogénea (ISQCH) y Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Zaragoza–CSIC. Calle Pedro Cerbuna, 12. 50009 Zaragoza, Spain.

Telephone number: 0034 976 762271

[c]Address:

Instituto Pirenaico de Ecología (CSIC). Av. Montañana, 1005, 50059, Zaragoza, Spain.

Telephone number: 0034 976 369393

Corresponding author:

Name: Beatriz Giner.

Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, 50830, Zaragoza, Spain.

Email address: bginer@usj.es

Telephone number: 0034 976 060100

Fax number: 0034 976 077584

Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Glycerol biobased ethers have a high potential as solvents due to their chemical inertness and diversity that allows modulate their properties, such as polarity, hydrophobicity or viscosity, depending on the specific needs in each case. Despite their renewable source, the environmental goodness of these solvents has to be checked. In this case, the acute ecotoxicity of five glycerol-derivative solvents (3-ethoxi-1,2-propanodiol [2.0.0],1,3-diethoxi-2-propanol [2.0.2], 3-butoxi-1,2-100 propanediol [4.0.0], 1,3- dibutoxi-2-propanol [4.0.4], and 1,2,3-tributoxipropane [4.4.4]) has been evaluated in a systematic study using several bioindicators as aquatic models covering the trophic chain (the crustacean *Daphnia magna*, the fish *Danio rerio* and the green algae *Chlamydomonas reinhardtii*). These results have been compared with the previously studied bioindicator *Vibrio fischeri*. As a general trend and according to the hypothesis of this work, the toxicity of these solvents increased as a function of their lipophilicity, being related with the increase of the alkyl chains in the basic structure; accordingly, the least toxic compound for all the aquatic organisms was 3-ethoxi-1,2-propanodiol and the most toxic solvent was 1,2,3-tributoxipropane, excepting in the case of *D. rerio* and *V. fischeri*, with 1,3-dibutoxi-2-propanol as the most toxic chemical. Furthermore, with the intention of evaluating the potential damage caused by eventual emissions, we have used the bases of the *Environmental Health and Safety Approach* –EHSA, a methodology used in the early phases of chemical process design for detecting risks related with the environment and the human health. Using available physicochemical and toxicity data, each chemical compound receives a score for category-Health, Safety and Environment-. According to this evaluation, the best candidates to be considered as less dangerous for a short exposition time according the studied biomodels are 3-ethoxi-1,2-propanodiol, 3-butoxi-1,2-propanediol and 1,3-diethoxi-2-propanol.

Keywords: Ecotoxicology, chemical toxicology

1. Introduction

Organic solvents are one of the main sources of anthropogenic volatile organic compounds (VOC). At present, most organic solvents come from petroleum and are used in huge amounts for industrial and household applications, so they constitute an important concern for air and water pollution, because their toxicity and ecotoxicity effects. Harmful VOCs are not acutely toxic in most cases, but they often have long-term effects on health and environment, and also are not considered as renewable mediums. A more sustainable chemistry requires new solvents, coming from new sources, able to provide the concrete features needed for the application in which will be used but, above all, being more respectful with the environment than those derived from petroleum. In this context, chemicals derived from renewable sources (as biomass) are attracting a great interest in the last years.

Current agricultural and industrial activities generate huge amounts of raw materials, which can be employed to produce useful chemicals (either commodities or fine chemicals). One of the most promising platform molecules that has received much attention in the last years is glycerol (1,2,3-propanetriol)[1-3]. Although in the past it was also produced from fossil sources, nowadays glycerol appears as an outcome in the production of biodiesel (ca. 10% weight of the total production) and also in oleochemical industry. In recent years, the world production of glycerol coming from vegetable oil transformations has surpassed 2 million metric tons, which may pose a problem if the surpluses have to be disposed of. On the other hand, glycerol constitutes a valuable starting point to obtain bio-based chemicals that are used in high amounts, and solvents are a good example[4-7].

Glycerol ethers have a high potential for chemical diversity, given that mono-, di, and trialkylation, either symmetrical or unsymmetrical at positions 1 and 3, leads to 1,2-diols, alcohols and trialkyl ethers, respectively. These possibilities allow modifications in the structure of the molecules depending on the solvent properties needed, such as polarity, hydrophobicity or viscosity[8-9]. Concerning the harmlessness of these bio-based solvents, the low risk of being

dangerous for the environment for glycerol ethers is generally taken for granted[5,10]. The pertinent question arises, however, as to whether solvents coming from glycerol can be considered environmentally benign or not, given the lack of systematic experimental evidences on its toxicity and ecotoxicity. Our group has recently published the EC_{50} acute value of a series of glycerol-derived ethers in a typical bioindicator, the bacteria *Vibrio fischeri*[11] (*V. fischeri*) as a first approximation to the toxic effect of these chemicals. Only those ethers with long substituents were considered as slightly toxic for this bioindicator. Furthermore, we also have published the EC_{50} values in a short time of exposure of a fluorinated glycerol derivative and a commercially available ionic liquid, comparing these data with three different bioindicators: *V. fischeri*, *Daphnia magna* (*D. magna*),and *Danio rerio* (*D. rerio*)[12]. These previous results show that most of the 20 glycerol derivatives could cause little harm in the environment during a short time of exposure. However, more investigations are needed, since toxic substances can cause effects at different levels of biological organization [13]. Thus, legislations as REACH established the need for evaluating with several biomodels to ensure the adequacy of the glycerol derivatives to the aquatic environment[14].

Although it is well known that *V. fischeri* is a good and accepted environmental model, ecotoxicity results cannot be extrapolated to more complex biomodel. At this point, it is interesting to note the saline medium can influence the ecotoxicity. Higher salinities can exclude neutral organic molecules due to the strong ionic interactions among water molecules and the major salted ions, resulting in reduced solubility in salt water (salting out). Thus, this phenomenon influences the solubility and, therefore, the chemical activity of more lipophilic compounds[15]. The differences in species sensitivities detected in previous studies point to the need of expanding the range of species and trophic levels to improve the environmental risk assessment for glycerol derivatives[16]. Thus, we analyze the effect of the chemicals in three different aquatic organisms [*D. magna*, *V. fischeri* and *Chlamydomonas reinhardtii* (*C. reinhardtii*)] to evaluate if any of them have an increased sensibility that can be considered as a risk indicator of toxicity for the aquatic biota. Based on our previous results [11,12], the working

hypothesis of this study is based on the relationship between chemical structure and the ecotoxicological effects: an increase in the lipophilic character of the molecule may lead to an increase in its toxicity. The selection of these environmental biomodels covers the whole trophic chain.

2. Experimental

2.1 Solvents

Monoalkylated glycerol derivatives (3-ethoxi-1,2-propanodiol, [2.0.0] and 3-butoxi-1,2-propanediol, [4.0.0]) were obtained by ring opening of glycidol (2,3-epoxi-1-propanol or oxyranylmethanol) using the appropriate alcohol (ethanol or butanol) and catalytic amounts of potassium hydroxide. The alcohol is also used as reaction solvent, so it is in great excess with regard to glycidol. Dialkylated glycerol derivatives (1,3-diethoxi-2-propanol, [2.0.2] and 1,3-dibutoxi-2-propanol, [4.0.4]) were obtained by reaction of epichlorohydrin [2-(chloromethyl)oxirane] with the appropriate amount of potassium alkoxide (ethoxide or butoxide) using the corresponding alcohol as solvent. Finally, the trialkylated glycerol derivative (1,2,3-tributoxipropane, [4.4.4] was obtained from the corresponding dialkylated glycerol derivative, by reaction of the central free hydroxyl group with sodium hydride, followed by addition of butyl iodide. Their structures, including their names and corresponding number codes are showed in Table 1. Details on the synthetic procedures can be found in previous works[8,11]. The log P of the different glycerol derivatives would be considered, for discussion purposes, as an estimate of their overall lipophilicity, *i.e.* something that modulates its behavior in relevant biological processes such solubility, permeability through biological membranes, hepatic clearance[17].

2.2 Ecotoxicity studies

2.2.1 C. reinhardtii culturing and exposure to solvents

The unicellular algae *C. reinhardtii* CC125 in exponential phase were used for the experiments. Algae were growing for 72 hours in an incubator at 25ºC, on an orbital shaker at

90 rpm under a continuous illumination of 130 µE PAR·m^{-2}·s^{-1}, given by four fluorescent tubes (Blau aquaristic T5HO, 39 w·10000·K^{-1}). The culture medium "Talaquil" was prepared as described in Szivák et al[18], using $CuCl_2 \cdot 2H_2O$ and $ZnCl_2$ instead of the corresponding sulphates.

Different concentrations of the studied solvents were tested in order to obtain dose-response curves, with two replicates (flasks) for each one. Exposure media was prepared by adding the appropriate amount of solvents into Millipore filtered water buffered with 10 mM MOPS (3-N-morpholino propanesulfonic acid) adjusted to 7.5 pH, and then adding the algae. In case of [2.0.0] concentrations were ranged between 5000-90000 mg·L^{-1}, for [2.0.2] between 1250-50000 mg·L^{-1}, for [4.0.0] between 500-10000 mg·L^{-1}, for [4.0.4] between 20-2000 mg·L^{-1}, and for [4.4.4] between 50-500 mg·L^{-1}. Negative controls (two replicates consisting in water with MOPS 10 mM, 7.5 pH, with the same algae concentration) were also tested, and the dose-response curves were repeated at least 3 times. The 72-hour-old algae were centrifuged (10 min, 3000 r.p.m.) and the concentrate were used to obtain an optical density (OD_{685}) of 0.3, equivalent to 1150000 cells·mL^{-1}.

As toxicity endpoint, the photochemical quantum yield –Y- (i.e. the efficiency in transforming light energy into biochemical energy by the photosynthesis), was used and measured using a Mini-PAM fluorometer (from WALZ, Effeltrich, DE). The settings used were a ML level of 0.15 µmol·m^{-2}·s^{-1}, SP of 1577 µmol·m^{-2}·s^{-1}, and 0.8 s length pulses. Fluorescence parameters were measured on 2 ml algal suspension after 1 hour of exposure. After 30 s of acclimatization to measuring light conditions (≈ 50-60 µmol·m^{-2}·s^{-1}), 4-5 consecutive Y measures were registered, with a 10-second interval. Suspension was kept under agitation using a micro stirrer (Spinette Cell Stirrer from STARNA, Essex, UK).

2.2.2 V. fischeri culturing and exposure to solvents

Test conditions and the operating protocol of the *V. fischeri* acute toxicity of the experiments carried out are in accordance with the UNE-EN-ISO 11348-3 protocol[19]. A description of the complete experimental procedure can be found in Lomba *et al.*[20]. The

lyophilized *V. fischeri* (strain NRRL-B-11177) used for the tests were purchased from Macherey-Nagel (ref. 945 006).

A 2% NaCl stock solution was used to prepare the dilutions of each of the studied solvents, adjusting the pH to 7-7.5 using either 0.1 M HCl or 0.1 M NaOH solutions in 2% NaCl. For [2.0.0], concentrations ranged between 625-20000 $mg \cdot L^{-1}$, for [2.0.2] between 475-10000 $mg \cdot L^{-1}$, for [4.0.0] between 100-3500 $mg \cdot L^{-1}$, for [4.0.4] between 3-100 $mg \cdot L^{-1}$, and for [4.4.4] between 50-2500 $mg \cdot L^{-1}$ Positive controls[21] were zinc sulphate (2.2 $mg \cdot L^{-1}$) and phenol, (42.5 $mg \cdot L^{-1}$). Negative controls were culturing medium(Biofix® Lumi, medium for freeze-dried luminous by DIN EN USO 11348-3, Macherey-Nagel, Düren, Germany). Two replicates for each control were tested.

The time exposition of bacteria to the solvents was 30 min at constant temperature (15ºC). Luminescence measurements were obtained with a Biofix® Lumi-10 luminometer (Macharey-Nagel) in acute mode (Biotox B). The test was repeated at least twice.

2.2.3 D. magna culturing and exposure to solvents

This test was performed according the guidelines of the OECD 202 test conditions[22,23]. Once again, the complete and detailed description of the experimental protocols can be found elsewhere[12]. The *D. magna* ephippia used were purchased from Vidrafoc (Toxkit, Daphtokit F Magna, ref. DM090812, Barcelona, Spain) and stored at 4°C until their use. The tests were carried out with new batch each time. The preparation of the eggs consisted in their incubation with culturing medium prepared according to the specifications of the supplier during 72 hours at 22±2°C with 6000 lux in a TOXKIT CH-0120D-AC/DC incubator (supplied by ECOTEST, Valencia, Spain) and fed with *Spirulina* algae 2 hours prior of starting the experiment. Positive controls with $K_2Cr_2O_7$ and negative controls were also tested.

Concentrations tested were prepared using culturing medium as solvent. The range for [2.0.0], [2.0.2], [4.0.0], [4.0.4] and [4.4.4] were 550-15000 $mg \cdot L^{-1}$, 1000-3000 $mg \cdot L^{-1}$, 1000-4000 $mg \cdot L^{-1}$, 50-500 $mg \cdot L^{-1}$ and 0.5-75 $mg \cdot L^{-1}$, respectively. The pH of the dilutions was measured and adjusted between 7 and 7.5 prior to the exposition. A total of 20 newborn daphnids (aged < 24 hours), were exposed to the test compounds in complete darkness for 24 h

at 20°C per concentration and compound. The crustaceans were separated into four groups of five organisms, four replicates per concentration exposure. The test was repeated at least three times. The immobilization of the organisms was measured through observation. Crustaceans that were not able to swim during 15 s after gentile agitation were considered as immobilized.

2.2.4 D. rerio culturing and exposure to solvents

Fish embryo acute toxicity (FET) test in zebrafish was developed in ZFBiolabs and consisted of an adaptation of FET approved as OCDE 236[24]. Embryos of *D. rerio* were obtained by *in vitro* fecundation and hatchings were selected when percentage of viability was over 80%.

A 0.25% DMSO solution was used to dilute the compounds. DMSO was used to increase the permeability of embryo chorion. The temperature was maintained between 24 and 26ºC, and oxygen saturation between 60 and 100%. The pH was adjusted to 6.5-8.5 if necessary. For [2.0.0], concentrations ranged between 2300-300000 $mg \cdot L^{-1}$, for [2.0.2] between 78-10000 $mg \cdot L^{-1}$, for [4.0.0] between 156-20000 $mg \cdot L^{-1}$, for [4.0.4] between 1-312 $mg \cdot L^{-1}$, and for [4.4.4] between 78-10000 $mg \cdot L^{-1}$. Positive controls consisted of 4-acetaminophen (paracetamol) (4155 $mg \cdot L^{-1}$). Negative controls were also included. Twelve embryos were exposed to each concentration with a dilution factor of 2. When toxicity range was low, a dilution factor of 1.5 was used to repeat the protocol ([2.0.0], [2.0.2] and [4.0.0]).

Signs of lethality of embryos were observed after 24 and 48 hours of exposure to the chemicals to determine LC_{50} at 48 hours. Assays were repeated at least twice.

2.2.5 Statistics and graphical representation

To obtain the half maximal effective or lethal concentration (EC/LC_{50}) values and the dose-response curves the following procedures were carried out: for *C. reinhardtii* results were fitted using R and *drc* package to a four parameter logistic curve while the "compPAR" function was used to perform comparison tests. The null hypothesis is that the ratio obtained dividing EC_{50} values equals 1; if it significantly differs from 1, null hypothesis was rejected because those values are significantly different ($p<0.05$). Analogously, for the rest of the bioindicators,

Tukey method for multiple comparisons was applied. In all cases, results were all significantly different.

Experimental data for *V. fischeri*, *D. magna*, and *D. rerio* were fitted to obtain the corresponding EC/LC_{50} values and standard deviations (SD) using the least squares method:

$$\%I = 100/(1 + 10^{(a-logc)b}) \quad (1)$$

where *%I* denotes % bioluminescence inhibition for *V. fischeri*, % immobilization for *D. magna* and % death for *D. rerio*,*c* is for concentration (in $mg \cdot L^{-1}$) in all the cases and *a* and *b* are adjustable parameters.

3. Results and Discussion

3.1 Ecotoxicity results

All the glycerol ethers studied showed concentration-dependent toxicity to the organisms tested in acute exposition. Figs. 1 to 3 show the experimental dose-response representation obtained for *C. reinhardti*, *D. magna* and *D. rerio*, and Table 2 shows their corresponding EC/LC_{50} and standard deviations (SD) values. For comparison, their corresponding EC_{50} and log P values in previous work for the studied compounds in *V. fischeri* are gathered in Table 3 and Figura 4[11].

The acute EC_{50} values of the 1,2,3-propanetriol (glycerol) in the environment has been well established in the previous bibliography, including for *V. fischeri*[11] and *D. magna*[25]. Thus, glycerol can be considered as clearly harmless for the aquatic environment, according Passino and Smith classification[26], a logarithmic hazard ranking for aquatic biomodels which allows classify soluble chemical substances in acute exposures. Comparing with our results, the EC_{50} values of the original compound is higher than the studied glycerol derivatives. As far as we know, there are few studies regarding the toxicities of this group of compounds. Just Sutter et *al.*[27] have evaluated one of these chemicals, 1,2,3-trimethoxypropane, in several toxicity studies, including algae, crustaceans and fishes.

As a general trend and according to the hypothesis of this work, the toxicity of these solvents increased as the lipophilic character does (Figura 5), which is also related with the presence and the increase of the length of alkyl chains attached to hydroxyl groups in the glycerol molecule[28]. Mainly, for *D. magna and C. reinhardtii*, there was a correlation between log P and log EC_{50} values. Organic compounds without toxically active functional groups have an action mechanism called "narcosis". This action mechanism involves non-specific non-covalent interactions of the organic molecule with the lipophilic cell membrane of the biomodel. The final result of this interaction is the reversibly altered structure and function of the membrane, causing the toxic effect[14, 29]. However, results indicate that *D. rerio* and *V. fischeri* did not show a direct relationship between log P and log EC_{50}.

According to our results, the higher EC/LC_{50} for all the aquatic organisms was presented by [2.0.0]. The most toxic solvent in *V. fischeri* and *D. rerio* was [4.0.4], whereas [4.4.4] was the most toxic chemical in *D. magna* and *C. reindhartii*. In line with the Passino and Smith classification[26], only [4.0.4] (in the case of *V. fischeri* and *D. rerio*) and [4.4.4] (for *D. magna* and C. *reinhardtii*) can be considered as slightly harmful for these bioindicators, since their EC/LC_{50} values are below 100 mg L^{-1}. [4.0.0] and [4.4.4] can be considered as practically harmless for *V. fischeri* having an EC_{50} value between 100 and 1000 mg L^{-1}, and the rest of the studied solvents, displaying EC/LC_{50} values over 1000 mg L^{-1}, are clearly harmless for the aquatic environment in the same classification ([2.0.0] and [2.0.2]) (Figura 6).

Focusing on the structure of the studied compounds, it is remarkable that for *D. magna* and *D. rerio*, the effect on the toxicity of two ethyl substituents in positions 1 and 3 of the glycerol derivative is higher than the presence of only one butyl substituent. Furthermore, we have detected another anomaly in the trend; when the position 2 is substituted with the biggest substituent (butyl) toxicity does not increase in all the cases: for *D. rerio*, toxicity clearly decreases. This result has been previously observed for the bioindicator *V. fischeri*[11]. In that case, when molecular size of substituent in position 2 was methyl, the extra radical at this position seemed to affect only slightly the toxicity. Here, for the case of *D. rerio*, this effect is

particularly clear: toxicity of [4.0.4] is ca. 160 times higher than that of [4.4.4]. Effectively, the size of the substituent in position 2 is a key factor in toxicity depending on the bioindicator studied and tailoring opportunities arise from this fact.

The algal toxicity followed the expected trend for the studied chemicals: [2.0.0] is the less toxic chemical in acute exposition, followed by [2.0.2], [4.0.0], [4.0.4] and [4.4.4] (Figura 1). Good correlation between log EC_{50} and log P is observed (correlation coefficient, r = 0.988) and the values of EC_{50}decreased as the length and number of alkyl chains in the glycerol series increased (Figura 5). The toxicity in the algae is measured as the decrease of the yield of Photosystem II, Y (II), indicating that these compounds can affect the electron flow in the photosynthesis. Previous studies have demonstrated that this effect is usual in several chemicals and herbicides, like atrazine[30]. The inhibited electron transfer in Y (II) results in oxidative stress, photooxidation of chlorophyll and cell necrosis. Another reason of the toxicity of these compounds to the algae could be explained to the harm of the photosynthetic membranes, due to their lipophilicity[31]. On the other hand, the log P is considered as an estimate of a compound's overall lipophilicity, i.e. something that modulates its behavior in relevant biological processes such solubility, permeability through biological membranes or hepatic clearance[17]. In this case, according to the linear relationship found between log P and log EC_{50}, this type of glycerol-derivative compounds could be harmful for algae if log P value is higher than 3.

In the *D. magna* bioassay, the toxicity rank of the studied chemicals decreased as follows: [2.0.0] followed by [2.0.2], [4.0.0], [4.0.4] and [4.4.4] (Figura 2). In this case also a good correlation between lipophilic character and toxicity has been observed (correlation coefficient, r = 0.994, Figura 5). For this crustacean, according their linear relationship between log P and log EC_{50}, a glycerol-derivative compound with log P value higher than 3, may become a serious threat for both crustacean and algae. This is because compounds with higher log P values present the lower EC_{50} concentrations (see Figure 5), in the environmental range[28] of the concentrations found in natural systems.

Results in *D. rerio* (Figura 3) showed that the most toxic compound was [4.0.4] in acute exposition, according their LC_{50} values. As in the previous biomodels, the less toxic was [2.0.0], followed by [4.0.0], [2.0.2] and [4.4.4]. There were significant differences among all these values. Because of the inversion of the toxicities of [4.0.4] and [4.4.4], LC_{50} values did not correlate well with the lipophilic character. Again, LC_{50} experimental values of [2.0.2] were lower than those of [4.0.0], as it was already found in *D. magna*. Acute ecotoxicity of [4.0.4] was higher than that of [4.4.4], as it was previously found in *V. fischeri*. The reason for this apparent lack of coherence with the toxicity trend, may be explained by the complexity of biological processes of the tested organism (fish) compared to algae or cladocerans. Analogously, log P values between 1 and 2 (and the [4.0.4] presents a 1.88 while [4.4.4] presents a 3.48) is often considered optimal to achieve a compromise between permeability and first-pass clearance in vertebrates[32].

3.2 Environmental, Health and Safety approach

Furthermore, in order to estimate the potential damage to the local and regional environment caused by eventual emissions from the chemical processes in which these new solvents could be involved, we have used the bases of the Environment, Health and Safety Approach (EHSA)[33] and some other information has been gathered (Tables 4 and 5) [8, 34-37] and analyzed.

To check several hazards associated with the mobility of the solvent during its handling and use, two properties have been selected at room temperature: volatility and boiling point. These physicochemical characteristics inform about the probability of generating new phases and being released to the atmosphere. Although associated hazards for both properties will be linked to the temperature and pressure process, we can provide several conclusions. The higher the vapor pressure, the higher the environmental risk: in this case, values were quite low (below 0 in an index scale from 0 to 1, being 1 the highest risk indicator), and none of them can be considered as potential hazards, according the methodology. On the other hand, boiling points

were really high and thus, in most of the cases, when using these solvents, at room temperature, the probability of generate new vapor phases was rather low, due to the index value below or very close to 0 in the scale for all the compounds. Therefore, the risk of overpressure in experimental devices was also small as well as the probability of substances escaping form the system in case of equipment failure.

Hazards related with fire or explosions have been analyzed by means of the flashpoint. Once again, the process temperature will determine the potential risk in this sense. However, to get a general idea of the fire/explosion risk associated to the studied solvents we can compare the raw flash point values at a process temperature of 25 ºC. According to the EHSA, all of these solvents could be dangerous and the higher risk of fire or explosion is found in [2.0.2] (0.76 in a scale from 0 to 1), while [2.0.0] and [4.0.4] (0.56 and 0.555, respectively) showed the lowest risk in comparison. It is important, however, to put these values into context, since common organic solvents structurally related to glycerol alkyl ethers have, in general, much lower flash point values: diethyl ether (–45 ºC,1.35), ethanol (13 ºC, 1.06), 1-butanol (37 ºC, 0.94), methyl *tert*-butyl ether (–28 ºC, 1.265), diglyme (67 ºC, 0.81). Only ethylene glycol displays a similar value (111 ºC, 0.57)[38].

To assess human acute toxicity, we have selected oral LC_{50}. High values estimated for this property indicates that the risk associated with toxicity was quite low, below to 0.0001 in an index scale from 0 to 1 for all the compounds. On the other hand, environmental toxicity was assessed by means of the mean value obtained in all of available aquatic acute toxicity experiments. In this case, we have used our own experimental values to evaluate this environmental aspect. Mean values above the threshold 1000 mg L^{-1} (a 0 value in the index scale) were considered very low risk. Thus, only [4.4.4] (0.023) and [4.0.4] (0.161) showed environmental risk and therefore, specific waste purification processes should be proposed**.**

Another important environmental property is the ease with which the substances are degraded once they are in the environment. Potential for degradation has been assessed with

ready biodegradability. It should be mentioned that this estimation depends strongly on the conditions of degradation, particularly in waste treatment plants. In this case, only [4.4.4] could be more persistent in the environment, according to the predictions of the Biowin model[34,37].

Finally, the potential of these chemicals to accumulate through the soil or the chain food has also been analyzed. The selected property to check this risk has been log bioconcentration factor (log BCF). BCF is defined as the ratio of the concentration of a chemical in an organism to the concentration in the surrounding environment at steady state. It is a valuable indicator of the bioaccumulation potential of a substance, and hence has become an essential environmental property required for regulatory purposes. The log BCF values used in this work were calculated using the Meylan et al. model[39] included in the EPI suite of programs[34]. Basically, this model employs different equations relating the experimental BCF values from a large data base with calculated log P values, taking into account the ionic or not ionic nature of the solutes, as well as their classification in the log P scale and other correction factors. In this case, only [4.4.4] (0.11 in an index scale from 0 to 1) showed certain risk. Its calculated log BCF is 2.22, which is above the threshold of the Chemical Safety Assessment (2.00), but still below the threshold established by the REACH regulation for bioaccumulative substances (3.30)[40].

To sum up, the comparison of the EC_{50} values of five glycerol alkyl ethers to three different aquatic bioindicators, along with .*V. fischeri*[11], as well as the application of the EHSA approach, allows establishing some interesting conclusions. First of all, the glycerol ethers bearing either the shortest alkyl chains ([2.0.0], [2.0.2]) or just one long chain ([4.0.0]) can be classified as harmless for the environment in acute exposition, according Passino and Smith classification[26]. Those ethers bearing two or more butyl substituents ([4.0.4] and [4.4.4]) raise more concern, since they display moderate toxicity for two out of four bioindicators for the same classification. These results cannot be directly connected with the lipophilicity of these compounds in the case of *V. fischeri*[11]and *D. rerio*. About *C. reinhardtii* and *D. magna*, a strong linear relationship is found between their log P and EC_{50} values, and an approximate estimation of their range of toxicity according their lipophilia is reported. In general, values of

log P higher than 3 in these types of chemicals could lead to harmful environmental effects (because its lower EC_{50}).

None of the glycerol ethers studied seems to be of concern regarding human toxicity, and only [4.4.4] collects several negative indices, such as higher persistence in the environment, moderate toxicity for some aquatic bioindicators, and relatively low flash point (but still much higher than those of common organic solvents).

In the light of the results described in this work, the best candidates for solvent substitution appear to be [2.0.0], [2.0.2] and [4.0.0]. Further studies are being conducted to improve the information about the overall greenness of these bio-based solvents.

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Tables

Table 1 Solvent code of each glycerol derivative compound with their corresponding chemical name and group (R_1-R_3) for each position in 1,2,3-propanetriol (glycerol) molecule.

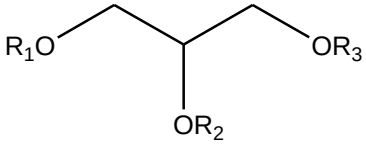
Solvent code	Chemical name	R_1	R_2	R_3	Molecular structure
200	3-ethoxi-1,2-propanodiol	Ethyl	-	-	
202	1,3-diethoxi-2-propanol	Ethyl	-	Ethyl	
400	3-butoxi-1,2-propanediol	Butyl	-	-	
404	1,3-dibutoxi-2-propanol	Butyl	-	Butyl	
444	1,2,3-tributoxipropane	Butyl	Butyl	Butyl	

Table 2 Experimental $EC_{50}(mg \cdot L^{-1})$ values for *C. reinhardtii*, *D. magna* and *D. rerio* obtained for studied solvents with their correspondent standard deviation (SD).

	C. reinhardtii		*D. magna*		*D. rerio*	
Solvent code	EC_{50} $(mg \cdot L^{-1})$	SD	EC_{50} $(mg \cdot L^{-1})$	SD	LC_{50} $(mg \cdot L^{-1})$	SD
[2.0.0]	52811	0.03	6458	5.3	36700	2.6
[2.0.2]	8613	0.05	1819	5.6	2800	8.7
[4.0.0]	4445	0.05	2332	3.2	4300	9.1
[4.0.4]	631	0.04	248	4.1	17	3.7
[4.4.4]	64	0.03	13.7	4.8	2700	7.1

Table 3 Experimental $EC_{50}(mg \cdot L^{-1})$ values for *V. fischeri* previouslyobtained with their correspondent log P.[11]

Solvent code	*V. fischeri* EC_{50} $(mg \cdot L^{-1})$	Log P
[2.0.0]	4240	-0.63
[2.0.2]	1215	0.07
[4.0.0]	941	0.28
[4.0.4]	11	1.88
[4.4.4]	473	3.48

Table 4 EHSA physico-chemical properties of the studied solvents.

Solvent code	P^o (bar, 25ºC)[34]	T_b (ºC)	Flash point (ºC)
[2.0.0]	$1.7 \cdot 10^{-5}$	221[8]	113[36]
[2.0.2]	$7.9\ 10^{-5}$	202-205[35]	73[36]
[4.0.0]	$1.9 \cdot 10^{-6}$	249[8]	105[37]
[4.0.4]	$6.4 \cdot 10^{-6}$	248[8]	114[37]
[4.4.4]	$2.8 \cdot 10^{-6}$	270[8]	99[37]

Table 5 EHSA toxicological properties of the studied solvents.

Solvent code	LC_{50} (mg/kg)[37]	Mean EC_{50}/LC_{50} (mg L^{-1})[A]	Ready biodegradability[34]	Log BCF[34]
[2.0.0]	5219	25214	YES	0.50
[2.0.2]	5264	3758	YES	0.50
[4.0.0]	3946	3010	YES	0.50
[4.0.4]	3812	228	YES	0.78
[4.4.4]	7682	812	NO	2.22

[A] This work.

Artículo 2: Ecotoxicity studies of glycerol ethers in *Vibrio fischeri*: checking the environmental impact of glycerol-derived solvents.

Autores: Jose Ignacio García[A], Elisabet Pires[A], Luis Aldea[B], Laura Lomba[B],Eduardo Perales[B], Beatriz Giner[B].

[A]Instituto de Síntesis Química y Catálisis Homogénea (ISQCH) y Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Zaragoza– Consejo Superior de Investigaciones Científicas-(CSIC), Calle Pedro Cerbuna 12, 50009 Zaragoza, España.

[B]Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, 50830, Zaragoza, España.

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En este artículo se evaluó la ecotoxicidad aguda del glicerol y diecinueve de sus derivados mediante el bioindicador *V. fisheri*. Los valores de EC_{50} obtenidos se relacionaron con las propiedades estructurales de los derivados del glicerol, establecidas por sus parámetros topológicos y por la metodología DARC-PELCO. También se estableció una relación entre la lipofilia y los valores de ecotoxicidad entre estos compuestos, con notables excepciones en 3 de ellos.

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Ecotoxicity studies of glycerol ethers in *Vibrio fischeri*: checking the environmental impact of glycerol-derived solvents†

J. I. García,*[a] E. Pires,[a,b] L. Aldea,[c] L. Lomba,[c] E. Perales[c] and B. Giner*[c]

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The toxicities of a series of glycerol mono-, di-, and trialkyl ethers against *Vibrio fischeri* bacteria have been determined. A systematic study has been carried out and the possible structure–toxicity relationships have been discussed using different QSAR models. Inhibition of bioluminescence after 30 minutes of exposure shows relatively low toxicity of many of the glycerol derived chemicals studied. Results indicate that, as a general rule, the ecotoxicity increases with the length and number of substituents. However, if the size of the molecule increases, an extra substituent at position 2 makes the toxicity lower than that of the corresponding analogues.

Introduction

The search for new solvents, coming from new sources and/or able to provide special features (often known as neoteric solvents), is a field of growing interest, especially in connection with the possibility of using renewable raw materials to produce harmless solvents, more respectful with the environment than those derived from petroleum (the so-called green solvents).[1] In this context, biomass-derived chemicals have attracted a great deal of attention in the last few years, in connection with the development of the biorefinery concept. Agricultural and some industrial activities are able to generate huge amounts of raw materials, capable of being used to produce commodity and fine chemicals.[2] In this sense, glycerol is one of the platform molecules that has received much attention in recent years.[3–5] Glycerol appears as a concomitant product in the production of biodiesel, amounting to *ca.* 10% weight of the total output. At present, the world production of glycerol coming from vegetable oil transformations surpasses 2 million metric tons, so it constitutes a valuable starting point to obtain bio-based chemicals, useful as, for instance, solvents.[6–9]

Our research group[10–14] and others[15–18] have described the synthesis and application as solvents of glycerol ethers. These kinds of solvents have the additional advantage of being much more chemically inert than other glycerol-derived solvents, such as esters, acetals or carbonates. Some of these glycerol ethers, namely those bearing fluoroalkyl chains, exhibited special physical–chemical features, in some way similar to those displayed by some ILs: high polarity, low vapour pressure at room temperature, and immiscibility with both hydrocarbons and water. The most prominent example of these compounds is 1,3-bis(2,2,2-trifluoroethoxy)propan-2-ol, which can be efficiently prepared from trifluoroethanol and epichlorohydrin (a commodity chemical currently produced from glycerol using the Solvay procedure).[12] Other authors have also recognized the special characteristics of these new fluorinated solvents.[19]

Apart from this particular case, glycerol ethers have a high potential for chemical diversity, given that mono-, di, and trialkylation, either symmetrical or unsymmetrical at positions 1 and 3, leads to 1,2-diols, alcohols and trialkyl ethers. These possibilities allow tuning important solvent properties, such as polarity, hydrogen-bonding ability, hydrophobicity or viscosity. Some QSPR studies have been conducted to develop models able to predict some key physical–chemical properties of glycerol ethers, prior to their synthesis.[20]

Concerning the greenness of glycerol-derived solvents, not only their renewable origin must be taken into account, but also other aspects of their life cycle, including their fate and the consequences on the environment. Being solvents coming from glycerol and short chain alkyl alcohols, which can in turn be obtained from biomass to a large extent, the low toxicity of glycerol ethers is generally taken for granted. The pertinent

[a]Instituto de Síntesis Química y Catálisis Homogénea (ISQCH). Facultad de Ciencias. CSIC-Universidad de Zaragoza, C/Pedro Cerbuna, 12, E-50009 Zaragoza, Spain. E-mail: jig@unizar.es
[b]Dept. Organic Chemistry, Facultad de Ciencias, Univ. de Zaragoza, Pedro Cerbuna, 12, E-50009 Zaragoza, Spain
[c]Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, E-50830 Zaragoza, Spain

†Electronic supplementary information (ESI) available: Tables with the molecular descriptors used and additional QSAR analyses. Analyses of the predictive ability of the QSAR equations. Experimental details of the synthesis of the compounds tested. See DOI: 10.1039/c5gc00857c

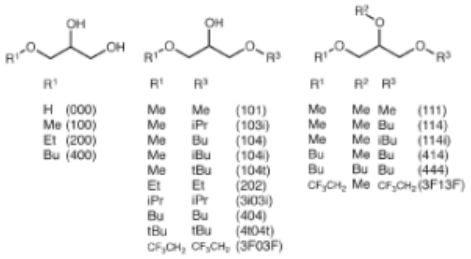

Fig. 1 Structures and codes of the 20 glycerol derivatives included in this study.

question arises, however, as to whether this family of solvents can be considered environmentally benign or not, given the lack of systematic experimental evidence for its toxicity and ecotoxicity.

In this paper, we present our first results on the ecotoxicity of a series of glycerol-derived ethers. In this case, we have studied the inhibition of the bioluminescence of *Vibrio fischeri* (*V. fischeri*) bacteria. This bioindicator has been frequently used,[21,22] since it is a well-known organism and operating protocols are standardized. Inhibition bioluminescence tests offer robust, easy handling and cost-effective responses.[22] Finally, this study provides some clues to the structure–toxicity relationships found in the studied solvent set (Fig. 1). This solvent set, though not quite large, has been chosen to cover a wide range of structural and physical–chemical features, such as the number of free hydroxyl groups, length and ramification of the alkyl chains, hydrophobicity, solubility in water, and the presence of fluorinated groups, among others.

Results and discussion

EC_{50} values obtained in the biotests using *V. fischeri*, with their respective adjustable parameters and standard deviations, are gathered in Table 1. Furthermore, results are graphically represented in Fig. 2–9.‡

As far as we know, there is no previous experimental ecotoxicological information on any of the studied glycerol derivatives. Regarding glycerol itself (000), there are no experimental data of ecotoxicity in *V. fischeri*. However, previous studies indicate that glycerol is of low toxicity towards microorganisms; in a 16 hour test with *Pseudomonas putida* no inhibition of bac-

‡Concentration units in the UNE-EN-ISO 11348-3 2007 acute toxicity test used are expressed in mg L^{-1}. Given the different molar masses (M) of the solvents studied, this could be an issue when comparing relative toxicities. Of course, in the case of compounds with similar M, these differences tend to be negligible. However, for the sake of completeness, we have repeated all the QSAR analyses with EC_{50} toxicities expressed in mM units, without noticeable changes in the conclusions reached. The results of these analyses are available as the ESI.†

Table 1 Experimental EC_{50} values for *V. fischeri* obtained for studied solvents with their adjustable parameters for eqn (1) and standard deviations

Solvent code	EC_{50} (mg L^{-1})	a	b	SD
000	108 421	0.01544	0.09568	4.791
100	21 052	0.01698	0.05195	3.587
200	4240	0.01333	0.05534	3.910
400	941	0.00873	0.02610	1.839
101	13 702	0.01795	0.10270	3.798
111	969	0.01655	0.10070	4.003
202	1215	0.02520	0.07998	4.082
404	11	0.00777	0.01893	1.831
114	453	0.01967	0.13460	5.365
104	464	0.02099	0.06516	3.468
414	58	0.01066	0.03351	2.937
444	473	0.01796	0.04461	3.564
103i	2188	0.01553	0.10940	3.764
104i	142	0.01280	0.03896	2.876
3i03i	1064	0.00980	0.03596	2.523
114i	258	0.02083	0.05875	3.905
104t	189	0.03629	0.03283	3.391
404t	16	0.02154	0.04298	3.544
3F03F	1597	0.01006	0.05688	2.375
3F13F	4033	0.02849	0.06963	3.845

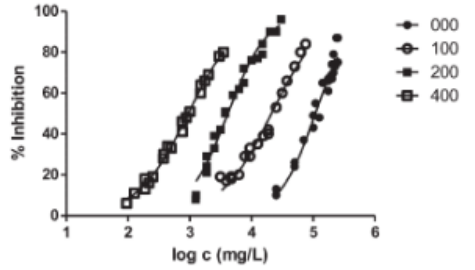

Fig. 2 Dose–response curves for studied solvents for *V. fischeri*: ● 000, ○ 100, ■ 200, □ 400.

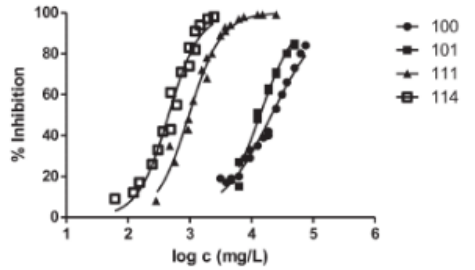

Fig. 3 Dose–response curves for studied solvents for *V. fischeri*: ● 100, ■ 101, ▲ 111, □ 114.

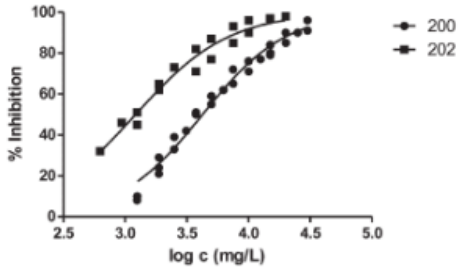

Fig. 4 Dose–response curves for studied solvents for *V. fischeri*: ● 200, ■ 202.

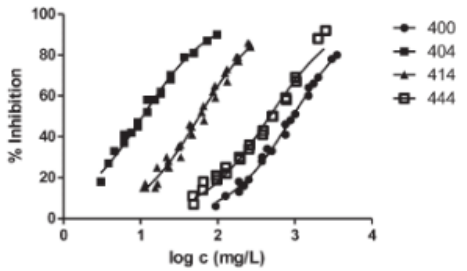

Fig. 5 Dose–response curves for studied solvents for *V. fischeri*: ● 400, ■ 404, ▲ 414, ▼ 444.

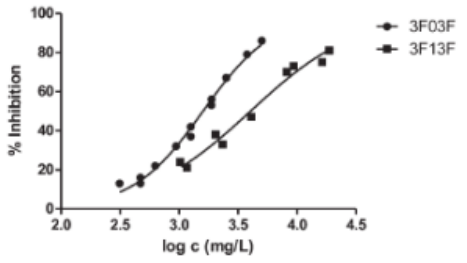

Fig. 6 Dose–response curves for studied solvents for *V. fischeri*: ● 3F03F, ■ 3F13F.

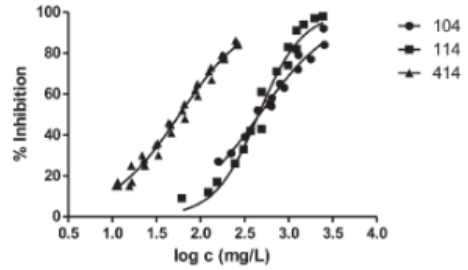

Fig. 7 Dose–response curves for studied solvents for *V. fischeri*: ● 104, ■ 114, ▲ 414.

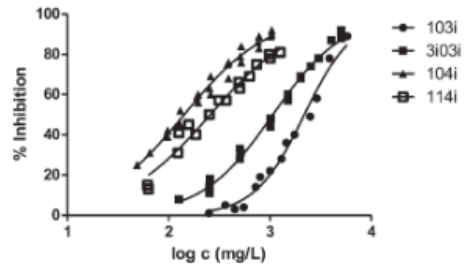

Fig. 8 Dose–response curves for studied solvents for *V. fischeri*: ● 103i, ■ 3i03i, ▲ 104i, □ 114i.

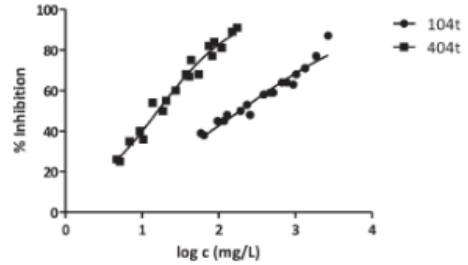

Fig. 9 Dose–response curves for studied solvents for *V. fischeri*: ● 104t, ■ 404t.

terial growth was found at concentrations between 100 and 10 000 mg L^{-1} (ref. 24 and 25) and similar results are found for other microorganisms such as *Entosiphon sulcatum* or *Clostridium* sp.[26,27] This information supports the conclusion that, overall, glycerol is of low toxicity to microorganisms. In this study, EC_{50} of glycerol for *V. fischeri* is higher than 100 000 mg L^{-1}, indicating the low toxicity of this chemical for this bioindicator.

In view of the results, ecotoxicity of the studied solvents is higher than that of glycerol in all the cases. However, taking into account the results obtained for the bioindicator *V. fischeri*, only a few solvents can be considered hazardous for the environment. According to the Passino and Smith classification,[28] just 404, 404t and 414 are slightly harmful, while 111, 104, 114, 444, 104i, 114i and 104t can be considered practically

harmless. The rest of the studied solvents (100, 200, 400, 101, 202, 103i, 3i03i, 3F03F, 3F13F) are clearly harmless for the environment.

Although the action mechanism is still unidentified, it is known that bacterial bioluminescence reactions are coupled to the electron transport system in cellular respiration. Consequently, bioluminescence reactions are indicative of cellular metabolism; *i.e.*, lower bioluminescence implies a decreased cellular respiration. Thus, the presence of the studied solvents in the culture medium affects, to a greater or lesser extent, the cellular respiration by the modification of both lipid and protein biosynthetic pathways and therefore alters the bioluminescence emission.[29]

In general, the alteration of the bioluminescence emission and thus the ecotoxicity increases with the length of the radical. This trend can be easily observed in Fig. 2, where we show the results obtained for the series 000–100–200–400 and the ecotoxicity for *V. fischeri* increases substantially with the length of the substituent at position 1.

The presence of a substituent at position 3 makes toxicity for *V. fischeri* higher compared with the corresponding analogous solvent. This is the case of the pairs 100–101, 200–202 and 400–404, whose behaviour is illustrated in Fig. 3–5 respectively.

Fig. 3 shows the representation of the 100–101–111–114 series. Results indicate that toxicity increases with the length of the alkyl chain and also with the number of substituents, independently of the position of the radical. However, if the size of the molecule increases, an extra substituent at position 2 makes the toxicity lower than the corresponding analogues. Fig. 5, 6 and 8 exemplify this behaviour. For example, the toxicity for 404 is *ca.* 5 times higher than 414, while for 3F03F is *ca.* 2.5 times higher than 3F13F.‡ It is worth mentioning that in the case of the pairs 104–114 and 104t–114t when the molecular size is between that of 101–111 and 404–414, the ecotoxicological behaviour is quite similar and the extra radical at position 2 seems to affect only slightly the toxicity (Fig. 7 and 8).

Finally, to analyse the effect of the structure of the substituents, we have studied derivatives with branched radicals. The obtained results are graphically represented in Fig. 8 and 9. Once again, the higher the size of radicals, the higher the ecotoxicity, in the case of both iso- and *tert*-substituents.

In Table 2, *V. fischeri* ecotoxicity data of a selection of some conventional solvents and ionic liquids (ILs) are shown for comparative purposes.[20–22] Ecotoxicity of most of the studied glycerol derivatives is smaller than that of some traditional solvents, such as toluene or phenol. Smaller compounds (100, 200 or 101) show values of EC_{50} similar to those of low molecular weight alcohols (methanol, ethanol or propan-2-ol) or acetone, known for being innocuous for *V. fischeri*. Only glycerol derivatives with large substituents (404, 414 and 404t) are as ecotoxic as *o*-xylene or phenol, which are solvents traditionally known for being toxic for the environment.

Regarding the comparison with ILs, it is interesting to note that ionic liquids have been catalogued as "green solvents" in

Table 2 Several EC_{50} values for different traditional solvents in the *V. fischeri* bioassay during 30 minutes of exposure

Molecular solvents	EC_{50} (mg L^{-1})
Methanol	101 068[20]
Propan-2-ol	35 383[21]
Ethanol	23 089[21]
Acetonitrile	21 172[21]
Acetone	19 311[20]
Dichloromethane	2532[20]
Chloroform	1199[20]
Ethylene glycol	621[20]
Benzene	108[20]
Toluene	32[20]
Phenol	31[20]
o-Xylene	9[20]
Ionic liquids	
1-Methyl-3-propylimidazolium tetrafluoroborate	1850[22]
1-Pentyl-3-ethylimidazolium tetrafluoroborate	350[22]
1-Butyl-3-ethylimidazolium tetrafluoroborate	151[22]
1-Butylpyridinium dicyanamide	98[22]
1-Heptyl-3-methylimidazolium tetrafluoroborate	74[22]
1-Ethyl-3-hexylimidazolium tetrafluoroborate	38[22]
1-Methyl-3-octylimidazolium tetrafluoroborate	7[22]
1-Hexyl-3-methylpyridinium bromide	2[22]

several occasions,[24,25] although the toxicity of a number of them is quite high to *V. fischeri* and many other bioindicators.[26] Our solvents are generally very eco-friendly from the ecotoxicity point of view than the selected ILs, independently of the cation (Table 2); only in the case of ILs with small substituents, toxicity is comparable to the vast majority of the studied glycerol-derived solvents.

However, it should be mentioned that the comparison made regarding the toxicity of glycerol-derived chemicals and some other solvents is limited to only one bioindicator, *V. fischeri*, in this case. Furthermore, to completely assess the greenness of the studied compounds, not only ecotoxicity data have to be taken into account; the analysis and evaluation of some other important properties such as bioavailability or biodegradability would change this first approach. Finally, the whole lifecycle of the product: production processes, uses and applications, removal rate in depuration processes, the final fate and toxicity of degradation products, are some parameters that should also be taken into account to determine definitively the environmental properties of the solvents.

Quantitative structure–activity relationships

To carry out a deeper analysis of the quantitative relationships between the molecular structure and the measured ecotoxicity, we use the same QSAR approaches previously applied to some physical–chemical properties of a larger family of glycerol-derived solvents.[20]

The first part of a QSAR study is to choose the quantitative description of the molecular structure. To this end, we used two different approaches: local structure descriptors and global structure descriptors. In the first ones, each descriptor indicates the presence or absence of a group of atoms at a

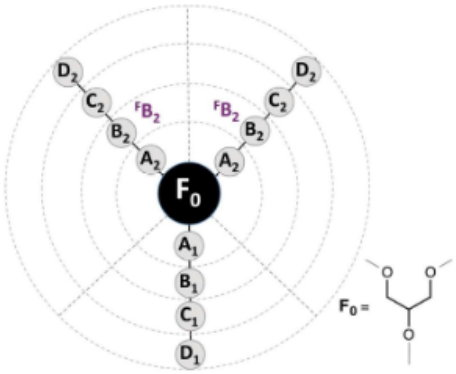

Fig. 10 DARC/PELCO scheme used to describe the molecular structures of the glycerol-derived solvents.

given molecular position. As in our previous study, we used the DARC-PELCO approach,[27] following the definition shown in Fig. 10.

The DARC/PELCO method is particularly suitable for studying families of compounds with a common chemical substructure, and is based on the exhaustive generation of all possible substitution sites around the reference structure (F_0)—corresponding to the glycerol skeleton common to all ethers—and the evaluation of their contribution to the property. In this definition we have incorporated the symmetry of the glycerol derivatives used, by assuming that the contributions of groups occupying equivalent positions (*i.e.* those linked to carbons 1 and 3 of the glycerol moiety) will display the same influence on the property under study. Previous studies have demonstrated that this simplification does not alter the results of the regression analyses.[20]

The second approach to molecular codification is the use of topological parameters,[28,29] which are easily derived from the connectivity and adjacency matrices of each compound. The number of connected components of a graph is a topological invariant that measures the number of structurally independent or disjoint subnetworks. These parameters are excellent descriptors of molecular size, shape and flexibility. They are global parameters in the sense that the whole molecular structure is condensed in a single number. The full list of DARC-PELCO and topological descriptors used in the QSAR analyses is available in the ESI.†

The second part of the QSAR analysis consists in relating the dependent variable (ecotoxicity) with the independent ones (the molecular descriptors), through a quantitative model, derived in most cases from a least-squares fit. In our case, we used Multiple Linear Regression (MRL) analysis, whose detailed description has already been given elsewhere.[20]

We started by applying the MLR analysis to the DARC-PELCO model. Given that not all the molecular descriptors have to be statistically relevant to the final equation, we used a stepwise procedure for variable selection. The final regression equation derived was the following:

$$\log EC_{50} = 4.828(\pm 0.320) + 1.064(\pm 0.460)B_1 - 0.614(\pm 0.192)A_2 - 0.310(\pm 0.0.082)B_2 - 0.685(\pm 0.123)C_2 \quad (1)$$

$N = 20$, $R = 0.93$, $R_{CV} = 0.83$, $\sigma(y) = 0.418F = 22.6$ ($F_{(4.15,\ 0.05)} = 3.1$)

As can be seen, the model fits fairly well the experimental data of ecotoxicity (86% of the experimental variance is explained by the model, as indicated by the R^2 determination coefficient), with a ratio of observations/adjustable parameters = 5, which is quite reasonable. The standard error of the model (0.42) is also close to the experimental value (0.54). However, to have a better knowledge of the true predictive ability of this equation, a cross-validation procedure was applied. In this procedure, the toxicity value of each solvent is predicted by the regression equation derived without using the experimental data of that solvent, so that it becomes a pure prediction. In the case of eqn (1), the toxicity of solvent 444 could not be predicted by this procedure, as the B_1 coefficient cannot be calculated without this toxicity value. Using the remaining 19 solvents a $R_{CV} = 0.83$ was obtained, still quite reasonable for a biological response. Fig. 11 plots the experimental values *vs.* those calculated with the MLR models.

From the QSAR point of view, a positive sign of the regression coefficient in eqn (1) means that the presence of an atom in the corresponding position leads to a decrease in

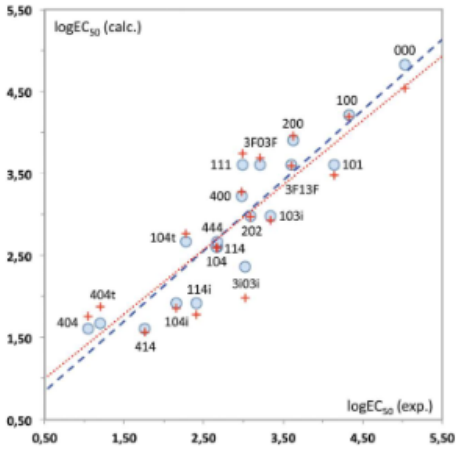

Fig. 11 Plots of predicted *vs.* experimental values of log EC_{50} as calculated through MLR analysis using the DARC/PELCO model. Circles represent the predictions made with the plain regression equation and crosses are the cross-validated predictions. Dashed and dotted lines represent the least-squares fit between both sets of data, respectively.

toxicity, whereas the converse is true for negative coefficients. Thus, enlargement of the substituents at positions 1 and 3 results in a progressive increase of toxicity, which is easier to see by looking at the standardized regression coefficients for A_2, B_2 and C_2 (−0.342, −0.399 and −0.600, respectively). On the other hand, substitution at the central oxygen atom tends to decrease the toxicity of these compounds, although in a less clear manner (standardized coefficient for B_1 = 0.241).

The application of the stepwise MLR procedure to the same data set, using the topological descriptors as structural variables, led to the following equation:

$$\log EC_{50} = -7.715(\pm 2.710) - 1.455(\pm 0.200)\chi_2^v + 4.779(\pm 1.023)\mathrm{Bal}^{\mathrm{PX}} + 0.553(\pm 0.180)\mathrm{HBD}_{\mathrm{count}} \quad (2)$$

$N = 20$, $R = 0.91$, $R_{CV} = 0.85$, $\sigma(y) = 0.436$ $F = 27.0$ ($F_{(2.16,\ 0.05)} = 3.2$)

Again, a fairly good fit is obtained (84% of the total experimental variance explained), which is mostly kept in the cross-validation analysis ($R_{CV} = 0.85$). Fig. 12 plots the experimental values *vs.* those calculated with these MLR models. As can be seen, 444 displays the highest deviation in the predicted toxicity, a fact undoubtedly related to being the sole structure studied with a long alkyl chain at position 2.

The interpretation of results is not straightforward in this case, due to the global character of the topological descriptors. The positive coefficient of the hydrogen bond donor count reflects the fact that the more free hydroxyl groups the solvent has, the lower its toxicity. The other two topological indices are mostly related to the molecular size, with some corrections to atom electronegativities and valence, which mainly affect the fluorinated compounds. Looking at the standardized coefficients for $\mathrm{Bal}^{\mathrm{PX}}$ and χ_2^v (1.157 and −1.513, respectively) it can be seen that molecular size increase roughly results in an increase of toxicity, too.

We finally tested a classical approach in QSAR studies, *i.e.*, the correlation between the measured toxicity and the hydrophobicity of the compounds, as expressed by $\log P$, the logarithm of the octanol/water partition coefficient.[40,41] Fig. 13 plots both sets of values. As can be seen, there are clear deviations from a linear behaviour in three solvents, namely 444 and the two fluorinated solvents. This indicates that hydrophobicity alone cannot account for the variations in toxicity obtained. In fact, it has also been shown that there is not always a direct correlation between lipophilicity and the adsorption, biodegradation rates and therefore toxicity; the persistence of the substance appears to be a key factor when correlating the bioavailability of the substance and the biological effect.[42–45] Leaving aside these three solvents, the remaining seventeen show a good linear response:

$$\log EC_{50} = 3.243(\pm 0.112) - 0.992(\pm 0.110)\log P \quad (3)$$

$N = 17$, $R = 0.92$, $R_{CV} = 0.88$, $\sigma(y) = 0.429$ $F = 81.9$ ($F_{(1.16,\ 0.05)} = 4.5$)

In any case, it is always dangerous to use this kind of correlation to drive conclusions about causality, because of the cross-correlations that exist among different molecular properties. For instance, in the case of $\log P$ in a rather homogeneous molecular set as that considered in this work, there are strong correlations of this property with other molecular properties mainly related with molecular size, such as molar

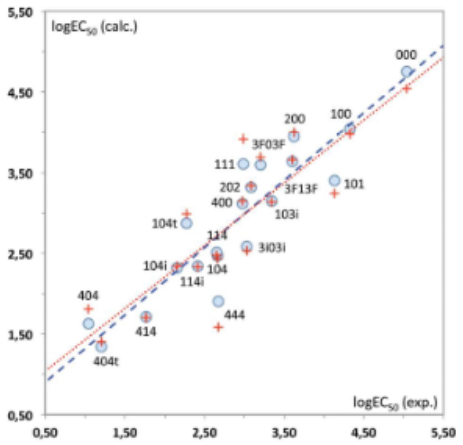

Fig. 12 Plots of predicted *vs.* experimental values of log EC_{50} as calculated through MLR analysis using the topological descriptors. Circles represent the predictions made with the plain regression equation and crosses are the cross-validated predictions. Dashed and dotted lines represent the least-squares fit between both sets of data, respectively.

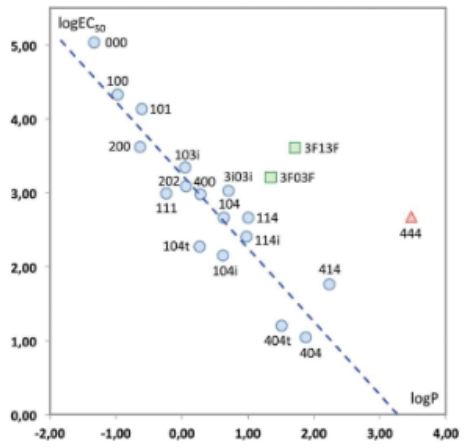

Fig. 13 Plot of log EC_{50} *vs.* log *P*. Only the solvents represented with circles have been used to obtain the least-squares fitting line.

volume (0.984) or solvent accessible surface (0.977). It is clear that a deep knowledge of the mechanisms responsible for the toxicity measured in *V. fischeri*, as well as of the bioavailability of the aqueous solutions of the glycerol derivatives, would be necessary before assigning a clear causality in the SAR analyses. Studies in this direction, as well as the enlargement of the ecotoxicity studies to other bioindicators, are currently in progress in our groups and will be reported in due course.

Concerning the predictive ability of the QSAR equations presented in this work in real-world situations, we have tried to provide some clues in this direction by synthesizing and testing a new compound, namely that coded as 114t (1-*tert*-butoxy-2,3-dimethoxypropane). First results obtained in the ecotoxicity measurements of this compound indicate a value of 1735 ± 3.64 mg L^{-1}, which fits well in the equations derived with the 20 original solvents. Furthermore, if a new solvent is included in the QSAR analyses, the derived equations show little difference with the original ones, indicating their robustness against the inclusion of new data. The corresponding plots and equations can be found in the ESI.†

Experimental

Synthesis of the glycerol derivatives

Monoalkylated glycerol derivatives were obtained by ring opening of glycidol using the appropriate alcohol and catalytic amounts of potassium hydroxide. Non-symmetric dialkylated glycerol derivatives were obtained by reaction of glycidol ethers with the appropriate alkoxide. Symmetric dialkylated glycerol derivatives were obtained by reaction of epichlorohydrin with the appropriate potassium alkoxide. Finally, trialkylated glycerol derivatives were obtained from the corresponding dialkylated derivatives, by alkylation of the central free hydroxyl group. Full experimental details on the synthetic procedures are available in the ESI.†

Ecotoxicity experiments

The evaluation of the ecotoxicity was based on the determination of the inhibition of luminescence of *V. fischeri* bacteria. The experiments were carried out in accordance with the test conditions and the operating protocol of the *V. fischeri* acute toxicity test (UNE-EN-ISO 11348-3 2007).‡ Details of the experiments can be found elsewhere.[46]

Trend analysis and quantitative structure–activity relationship (QSAR) models were applied with the software QSAR Toolbox 2.3 to determine the solvent concentrations to be tested. Once the range of EC_{50} was narrowed, at least 10 dilutions for each of the studied solvents were prepared using 2% NaCl as a stock solution. The pH of the solutions was adjusted to 7–7.5. Additionally, positive controls (phenol 42.5 mg L^{-1} and zinc sulphate, 2.2 mg L^{-1}) and negative controls were also tested.[47] The lyophilized *V. fischeri* (strain NRRL-B-11177) used were provided by Macherey-Nagel (ref. 945 006). Bacteria were exposed to the toxicants for 30 min at 15 °C.

Luminescence measurements were taken with a Biofix® Lumi-10 luminometer (Macherey-Nagel) using Biotox B mode (for acute measurements).

The obtained results have been fitted using the least-squares method using eqn (4) to obtain the corresponding EC_{50} values and standard deviations (SD):

$$\%I = 100/(1 + 10^{(a - \log c)b}) \quad (4)$$

where $\%I$ denotes % bioluminescence inhibition, c is the concentration (in mg L^{-1}) and a and b are adjustable parameters.

Conclusions

We have described for the first time the ecotoxicity of a series of glycerol alkyl ether solvents (including glycerol itself), using the bacteria *Vibrio fischeri* as a bioindicator. Although all the glycerol-derived structures display higher ecotoxicities than glycerol, half of them can be considered harmless for the environment on the basis of this bioindicator, and only three are clearly harmful following the Passino–Smith classification. These results support the often assumed greenness of these bio-based solvents, as far as potential environmental damage is concerned, although more ecotoxicity studies representing different trophic levels are necessary to have a whole view on this topic.

A comparison of the ecotoxicity towards *V. fischeri* between several traditional solvents and glycerol ethers indicates that EC_{50} of some of our solvents is similar to low molecular weight alcohols, traditionally known to be not harmful for these bacteria. Furthermore, in general terms, the ecotoxicity of ionic liquids seems to be higher than that of glycerol derived solvents, especially when the length of substituents increases.

The QSAR studies point to a dependence of the toxicity on the alkyl chain size, manifest in the case of substitution at 1 and 3 positions of glycerol, but less clear in the case of substitution at the central 2 position. An enlargement of the solvent set seems necessary to establish with certainty the latter effect.

Acknowledgements

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Electronic Supplementary Information for

Ecotoxicity studies of glycerol ethers in *Vibrio fischeri*: checking the greenness of glycerol-derived solvents

J. I. García.*[a] E. Pires.[a,b] L. Aldea.[c] L. Lomba.[c] E. Perales[c] and B. Giner*[c]

[a] *Instituto de Síntesis Química y Catálisis Homogénea (ISQCH). Facultad de Ciencias. CSIC-Univ. de Zaragoza. Pedro Cerbuna. 12. E-50009 Zaragoza. Spain. Tel: +34 976762271; E-mail: jig@unizar.es.*

[b] *Dept. Organic Chemistry. Facultad de Ciencias. Univ. de Zaragoza. Pedro Cerbuna. 12. E-50009 Zaragoza. Spain; E-mail: epires@unizar.es.*

[c] *Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, E-50830, Zaragoza, Spain; E-mail: bginer@usj.es.*

Content

Table S1. Topological parameters of 20 glycerol-derived solvents.

Code	HBA	HBD	RB	φ	Bal^{IX}	Bal^{IV}	W	Z	κ_1^{am}	κ_2^{am}	κ_3^{am}	SC_p^0	SC_p^1	SC_p^2	SC_p^3	SC_c^3	$^0\chi$	$^1\chi$	$^2\chi$	$^3\chi_p$	$^3\chi_{cl}$	$^0\chi^{vm}$	$^1\chi^{vm}$	$^2\chi^{vm}$	$^3\chi_p^{vm}$	$^3\chi_{cl}^{vm}$
000	3	3	5	3.02	2.572	2.814	31	20	5.88	3.08	2.88	6	5	5	4	1	4.99	2.81	1.92	1.39	0.29	3.33	1.71	1.02	0.42	0.13
100	3	2	5	3.98	2.620	2.901	50	24	6.88	4.05	3.72	7	6	6	5	1	5.70	3.31	2.30	1.48	0.29	4.29	2.09	1.29	0.57	0.13
200	3	2	6	4.95	2.665	2.926	76	28	7.88	5.03	4.88	8	7	7	6	1	6.41	3.81	2.66	1.75	0.29	5.00	2.68	1.50	0.73	0.13
400	3	2	8	6.91	2.723	2.939	153	36	9.88	6.99	6.88	10	9	9	8	1	7.82	4.81	3.36	2.25	0.29	6.42	3.68	2.26	1.16	0.13
101	3	1	5	4.95	2.686	2.996	75	28	7.88	5.03	4.88	8	7	7	6	1	6.41	3.81	2.68	1.56	0.29	5.26	2.47	1.56	0.72	0.13
103i	3	1	6	5.58	2.915	3.193	143	38	9.88	5.65	6.88	10	9	10	8	2	7.98	4.66	3.87	2.02	0.70	6.83	3.45	2.49	0.98	0.37
104	3	1	8	7.89	2.788	3.033	202	40	10.88	7.98	7.78	11	10	10	9	1	8.53	5.31	3.74	2.33	0.29	7.38	4.06	2.54	1.31	0.13
104i	3	1	7	6.51	2.909	3.162	194	42	10.88	6.58	7.78	11	10	11	9	2	8.69	5.16	4.22	2.26	0.70	7.54	3.91	3.04	1.12	0.54
104t	3	1	6	4.65	3.173	3.444	180	46	10.88	4.70	7.78	11	10	13	9	5	8.91	4.96	4.99	2.17	1.85	7.76	3.76	3.53	1.07	1.24
202	3	1	7	6.91	2.792	3.066	149	36	9.88	6.99	6.88	9.88	9	9	8	1	7.82	4.81	3.39	2.10	0.29	6.67	3.64	1.97	1.03	0.13
3i03i	3	1	7	6.34	3.079	3.334	243	48	11.88	6.40	8.88	12	11	13	10	3	9.56	5.52	5.05	2.47	1.11	8.41	4.43	3.42	1.24	0.60
404	3	1	11	10.86	2.907	3.121	419	52	13.88	10.96	10.88	14	13	13	12	1	10.65	6.81	4.80	3.10	0.29	9.50	5.64	3.51	1.91	0.13
404t	3	1	9	7.15	3.152	3.380	388	58	13.88	7.21	10.88	14	13	16	12	5	11.03	6.46	6.05	2.94	1.85	9.88	5.35	4.51	1.66	1.24
111	3	0	5	5.93	2.907	3.263	102	32	8.88	6.01	4.39	9	8	8	8	1	7.11	4.35	2.85	1.97	0.20	6.22	2.85	1.77	1.04	0.12
114	3	0	8	8.88	2.974	3.260	250	44	11.88	8.97	7.32	12	11	11	11	1	9.23	5.85	3.91	2.74	0.20	8.34	4.44	2.74	1.63	0.12
114i	3	0	7	7.46	3.089	3.383	241	46	11.88	7.53	7.32	12	11	12	11	2	9.40	5.70	4.39	2.67	0.61	8.50	4.30	3.24	1.44	0.53
414	3	0	11	11.86	3.105	3.357	488	56	14.88	11.95	10.17	15	14	14	14	1	11.36	7.35	4.97	3.51	0.20	10.46	6.03	3.72	2.23	0.12
444	3	0	14	14.84	3.443	3.683	789	68	17.88	14.94	13.17	18	17	17	17	1	13.48	8.85	6.06	4.15	0.20	12.58	7.62	4.70	2.74	0.12
3F03F	9	1	7	6.05	3.136	3.615	557	72	15.46	6.26	12.46	16	15	21	14	9	12.82	7.10	8.01	3.25	3.41	7.94	4.07	2.91	1.15	0.49
3F13F	9	0	7	6.80	3.325	3.835	638	76	16.46	7.02	11.72	17	16	22	16	9	13.53	7.64	8.18	3.66	3.33	8.90	4.46	3.12	1.47	0.48

Table S2. DARC/PELCO parameters of 20 glycerol-derived solvents.[a]

Code	A_1	A_2	B_1	B_2	$^FB_2$	C_1	C_2	D_1	D_2
000	0	0	0	0	0	0	0	0	0
100	0	1	0	0	0	0	0	0	0
200	0	1	0	1	0	0	0	0	0
400	0	1	0	1	0	0	1	0	1
101	0	2	0	0	0	0	0	0	0
103i	0	2	0	2	0	0	0	0	0
104	0	2	0	1	0	0	1	0	1
104i	0	2	0	1	0	0	2	0	0
104t	0	2	0	3	0	0	0	0	0
202	0	2	0	2	0	0	0	0	0
3i03i	0	2	0	4	0	0	0	0	0
404	0	2	0	2	0	0	2	0	2
404t	0	2	0	4	0	0	1	0	1
111	1	2	0	0	0	0	0	0	0
114	1	2	0	1	0	0	1	0	1
114i	1	2	0	1	0	0	2	0	0
414	1	2	0	2	0	0	2	0	2
444	1	2	1	2	0	1	2	1	2
3F03F	0	2	0	0	2	0	0	0	0
3F13F	1	2	0	0	2	0	0	0	0

[a] Note that B_1, C_1 and D_1 columns are identical, because there is a single compound with a substituent at 2-position longer than methyl (444). As a consequence, the coefficient for B1 in any regression model will account for the contribution of the whole butyl group, and not only for that of a single carbon at that position.

Table S3. Experimental EC_{50} values for *V. fischeri* expressed in mM units and calculated logP for the solvent set used.[a]

Code	EC_{50} (mM)	$logEC_{50}$	logP
000	1177.34	1177.34	–1.33
100	198.38	198.38	–0.97
200	35.29	35.29	–0.63
400	6.35	6.35	0.28
101	114.04	114.04	–0.60
103i	14.76	14.76	0.05
104	2.86	2.86	0.64
104i	0.88	0.88	0.62
104t	1.17	1.17	0.27
202	8.20	8.20	0.07
3i03i	6.04	6.04	0.71
404	0.05	0.05	1.88
404t	0.08	0.08	1.51
111	7.22	7.22	–0.24
114	2.57	2.57	1.00
114i	1.46	1.46	0.98
414	0.27	0.27	2.24
444	1.82	1.82	3.48
3F03F	6.23	6.23	1.35
3F13F	14.93	14.93	1.71

[a] A. K. Ghose and G. M. Crippen. *J. Chem. Inf. Comput. Sci.*. 1987. 27. 21–35.

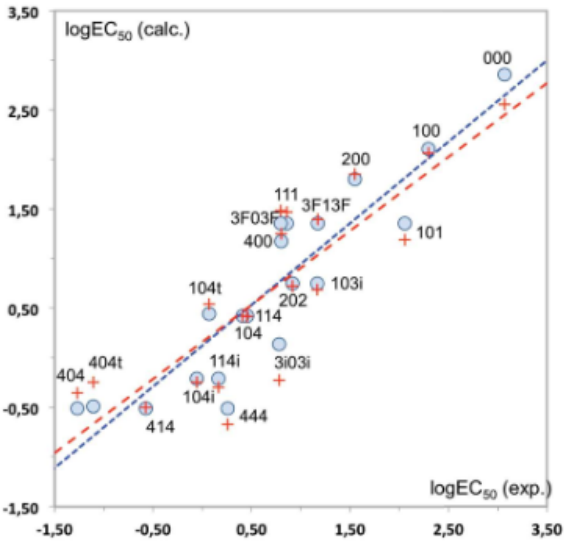

Figure S1. Plots of predicted *vs.* experimental values of $logEC_{50}$ (EC_{50} expressed in mM units) as calculated through MLR analysis using the DARC/PELCO model. Circles represent the predictions made with the plain regression equation and crosses are the cross-validated predictions. Blue and red lines represent the least squares fit between both sets of data, respectively.

$logEC_{50} = 2.856(\pm0.374) - 0.751(\pm0.225)\cdot A_2 - 0.304(\pm0.0.096)\cdot B_2 - 0.630(\pm0.136)\cdot C_2$

$logEC_{50} = -0.387\cdot A_2 - 0.361\cdot B_2 - 0.510\cdot C_2$ Standardized coefficients

$N = 20$, $R = 0.91$, $R_{CV} = 0.84$, $\sigma(y) = 0.490$

$F = 24.7$ ($F_{(3,16,\,0,05)} = 3.2$)

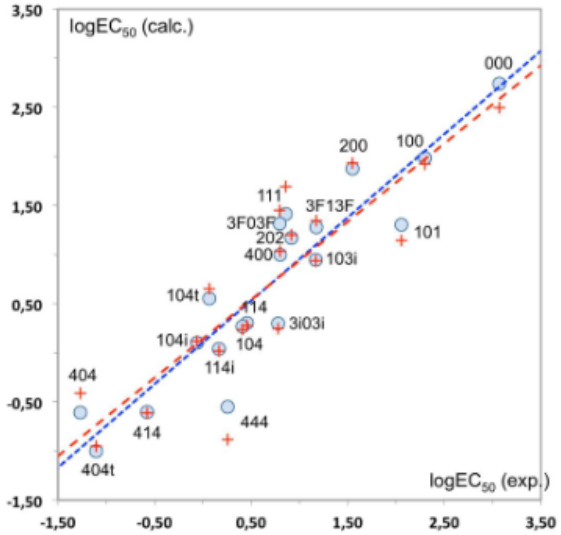

Figure S2. Plots of predicted *vs.* experimental values of $logEC_{50}$ (EC_{50} expressed in mM units) as calculated through MLR analysis using topological descriptors. Circles represent the predictions made with the plain regression equation and crosses are the cross-validated predictions. Blue and red lines represent the least squares fit between both sets of data, respectively.

$$logEC_{50} = -9.000(\pm 2.811) - 1.492(\pm 0.208)\cdot\ {}^{v}\chi_2 + 4.491(\pm 1.061)\cdot Bal^{IX} + 0.571(\pm 0.187)\cdot HBD_{count}$$

$$logEC_{50} = -1.434\cdot\ {}^{v}\chi_2 + 1.004\cdot Bal^{IX} + 0.442\cdot HBD_{count} \qquad \text{Standardized coefficients}$$

$N = 20$, $R = 0.92$, $R_{CV} = 0.85$, $\sigma(y) = 0.452$

$F = 29.9$ ($F_{(3,16,\,0.05)} = 3.2$)

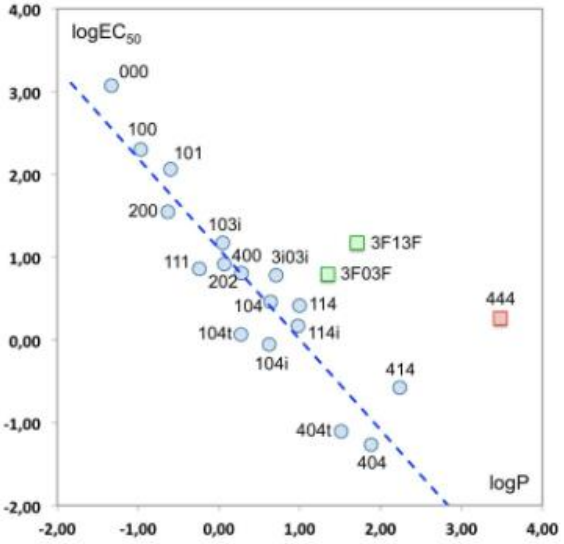

Figure S3. Plot of $logEC_{50}$ (EC_{50} expressed in mM units) vs. logP. Only the solvents represented with circles have been used to obtain the least-squares fitting line.

$logEC_{50} = 1.099(\pm0.115) - 1.094(\pm0.112)\cdot logP$

N = 17, R = 0.93, R_{CV} = 0.90, $\sigma(y)$ = 0.439
F = 95.1 ($F_{(1,15,\,0,05)}$ = 4.5)

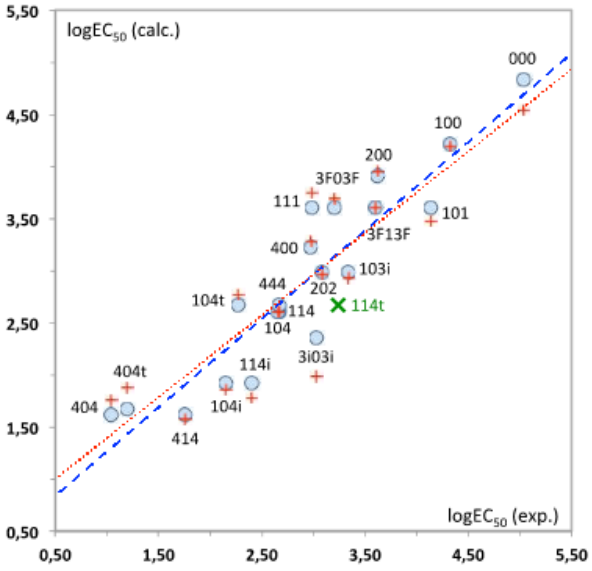

Figure S4. Plots of predicted *vs.* experimental values of $logEC_{50}$ (EC_{50} expressed in $mg \cdot L^{-1}$ units) as calculated through MLR analysis using the DARC/PELCO model. Circles represent the predictions made with the plain regression equation and crosses are the cross-validated predictions. Blue and red lines represent the least squares fit between both sets of data, respectively. Green cross represents the pure prediction of the toxicity of compound 114t made with the same MRL equation.

MLR equation derived with the initial 20 solvent set:

$$logEC_{50} = 4.828(\pm0.320) + 1.064(\pm0.460)\cdot B_1 - 0.614(\pm0.192)\cdot A_2 - 0.310(\pm0.0.082)\cdot B_2 - 0.685(\pm0.123)\cdot C_2$$

N = 20, R = 0.93, $\sigma(y)$ = 0.418
F = 22.6 ($F_{(4,15,\,0,05)}$ = 3.1)

MLR equation derived including the experimental value of 114t (21 solvent set):

$$logEC_{50} = 4.816(\pm0.325) + 1.062(\pm0.467)\cdot B_1 - 0.602(\pm0.195)\cdot A_2 - 0.282(\pm0.0.080)\cdot B_2 - 0.718(\pm0.122)\cdot C_2$$

N = 21, R = 0.92, $\sigma(y)$ = 0.425
F = 21.7 ($F_{(4,16,\,0,05)}$ = 3.0)

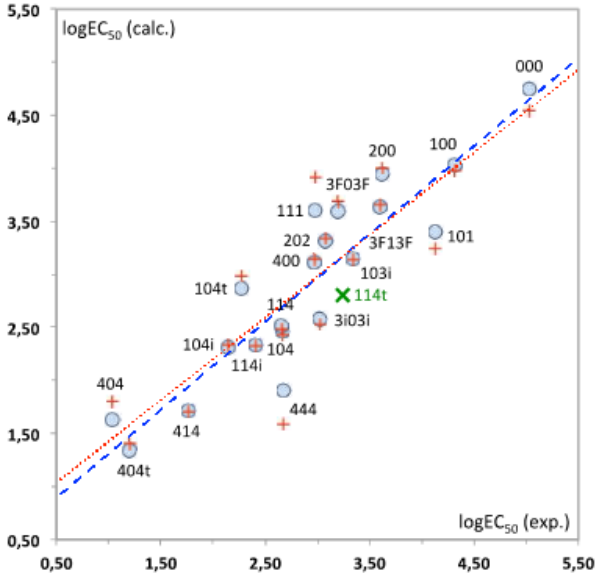

Figure S5. Plots of predicted vs. experimental values of $logEC_{50}$ (EC_{50} expressed in $mg \cdot L^{-1}$ units) as calculated through MLR analysis using topological descriptord. Circles represent the predictions made with the plain regression equation and crosses are the cross-validated predictions. Blue and red lines represent the least squares fit between both sets of data, respectively. Green cross represents the pure prediction of the toxicity of compound 114t made with the same MRL equation.

MLR equation derived with the initial 20 solvent set:

$$logEC_{50} = -7.715(\pm 2.710) - 1.455(\pm 0.200) \cdot {}^{v}\chi_2 + 4.779(\pm 1.023) \cdot Bal^{IX} + 0.553(\pm 0.180) \cdot HBD_{count}$$

N = 20, R = 0.91, σ(y) = 0.436
F = 27.0 ($F_{(3,16,\ 0.05)}$ = 3.2)

MLR equation derived including the experimental value of 114t (21 solvent set):

$$logEC_{50} = -8.421(\pm 2.576) - 1.490(\pm 0.195) \cdot {}^{v}\chi_2 + 5.054(\pm 0.970) \cdot Bal^{IX} + 0.560(\pm 0.179) \cdot HBD_{count}$$

N = 21, R = 0.91, σ(y) = 0.434
F = 27.3 ($F_{(3,17,\ 0.05)}$ = 3.2)

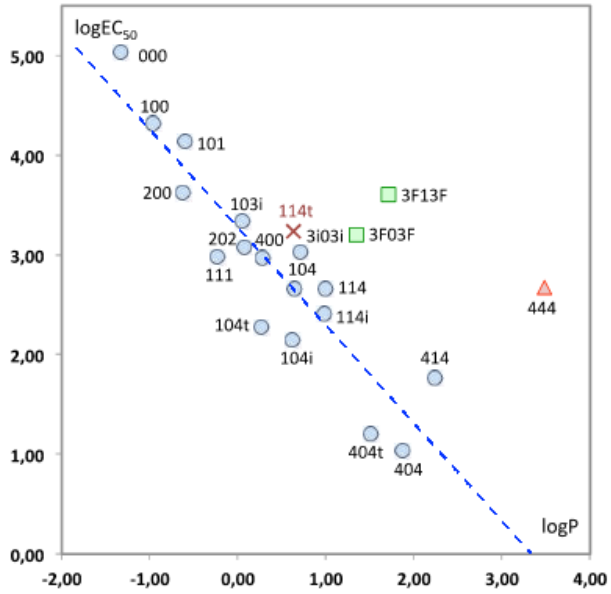

Figure S6. Plot of $logEC_{50}$ (EC_{50} expressed in $mg \cdot L^{-1}$ units) *vs.* logP. Only the solvents represented with circles have been used to obtain the least-squares fitting line. Red cross represents the pure prediction of the toxicity of compound 114t made with the same MRL equation.

MLR equation derived with the initial 20 solvent set:

$$logEC_{50} = 3.243(\pm 0.112) - 0.992(\pm 0.110) \cdot logP$$

$N = 17$, $R = 0.92$, $\sigma(y) = 0.429$
$F = 81.9$ ($F_{(1,16,\ 0.05)} = 4.5$)

MLR equation derived including the experimental value of 114t (21 solvent set):

$$logEC_{50} = 3.274(\pm 0.113) - 0.982(\pm 0.113) \cdot logP$$

$N = 18$, $R = 0.91$, $\sigma(y) = 0.441$
$F = 76.0$ ($F_{(1,17,\ 0.05)} = 4.5$)

General procedure for the synthesis of non-fluorinated 1-alkylglycerols ($R^1$00) from glycidol.

3.28 mol of R^1OH (R^1 =Me, Et, ^{i}Pr, ^{n}Bu) and 0.1 mol of KOH were placed in a round bottom flask. The mixture was heated at 70 °C and 0.5 mol (34 g) of glycidol were added dropwise to the reaction mixture. The reaction was monitored by gas chromatography until no signal of glycidol was observed. After 1 hour the reaction was completed and after cooling down the reaction mixture to room temperature, HCl was added dropwise until neutrality. Salts were filtered off and the excess of alcohol was removed under vacuum. The product was purified by vacuum distillation.

General procedure for the synthesis of fluorinated 1-alkylglycerol (R^f00) from glycidol.

1.1 mol of R^fOH (R^f = CF_3CH_2) and 1 mol (140 g) of potassium carbonate were placed in a round bottom flask. The flask was heated up at 70 °C, and 1 mol of glycidol was added dropwise to the reaction mixture. The reaction was monitored by gas chromatography until no signal of glycidol was observed. After 1 hour the reaction was completed and after cooling down, the reaction was filtered off in order to remove potassium carbonate. The reaction mixture was diluted with 75 mL of water and then extracted with dichloromethane‡ (3 x 50 mL). The organic phase was washed with 50 mL of a saturated solution of NaCl, dried with $MgSO_4$ and the solvent was removed under vacuum. Finally the product was purified by vacuum distillation.

General procedure for the synthesis of non-fluorinated symmetric 1,3-dialkylglycerols ($R^1$0R^1) from epichlorohydrin.

300 mL of R^1OH (R^1 = Me, Et, ^{i}Pr, nBu) were placed in a round bottom flask and cooled in an ice bath. Then 1 mol (24 g) of sodium was added to generate the corresponding alkoxide. When the sodium reacted completely, the flask was heated up to 70 °C, and 1 mol of epichlorohydrin (94 g) was added dropwise to the reaction mixture. The reaction was monitored by gas chromatography until no signal of epichlorohydrin was observed. After 1 hour, the reaction was completed. The solvent was removed under vacuum, the reaction was cooled down in an ice bath and 25 mL of water were added. Extractions with dichloromethane‡ (3 x 50 mL) were carried out. The organic phase was washed with 50 mL of saturated solution of NaCl and finally dried with $MgSO_4$. The solvent was removed under vacuum. Non-fluorinated 1,3-dialkoxyglycerols were purified by vacuum distillation.

General procedure for the synthesis of fluorinated symmetric 1,3-dialkylglycerols (R^f0R^f) from epichlorohydrin

2.2 mol of R^fOH (R = CF_3CH_2) and 1 mol (140 g) of potassium carbonate were placed in a round bottom flask. The flask was heated up at 70 °C, and 1 mol of epichlorohydrin (94g) was then added dropwise to the reaction mixture. The reaction was monitored by gas chromatography until no signal of epichlorohydrin was observed. After 1 hour the reaction was completed and after cooling down, the reaction was filtered off in order to remove potassium carbonate. The reaction mixture was diluted with 75 mL of water and then extracted with dichloromethane‡ (3 x 50 mL). The organic phase was washed with 50 mL of a saturated solution of NaCl, dried with $MgSO_4$ and the solvent was removed under vacuum. Finally the product was purified by vacuum distillation.

General procedure for the synthesis of non-fluorinated non-symmetric 1,3-dialkylglycerols ($R^1$0R^3) from glycidol ethers

3.28 mol of R^1OH (R^1 =Me, Et, ^{i}Pr, nBu) and 0.1 mol of KOH were placed in a round-bottomed flask. The mixture was heated at 70 °C and then 0.5 mol of the corresponding glycidol ether were added dropwise to the reaction mixture (glycidyl isopropyl ether (R^3 = iPr) 59 g; butyl glycidyl ether (R^3 = nBu) 68.5 g; glycidyl isobutyl ether (R^3 = iBu) 67.5 g; tert-butyl glycidyl ether (R^3 = tBu), 67 g). The reaction was monitored by gas chromatography until no signal of glycidol was observed. After 1 hour the reaction was completed and after cooling down the reaction mixture to room temperature, HCl was added dropwise until neutrality. Salts were filtered off and the excess of alcohol was removed under vacuum. The product was purified by vacuum distillation.

General procedure for the synthesis of 1,2,3-trialkylglycerols ($R^1R^2R^3$) from 1,3-dialkylglycerols

1.8 mol of NaH were placed in a round bottom flask together with 250 mL of anhydrous THF. Then 1.8 mol of 1,3-dialkylglycerols (R^10R^1 or R^10R^3) diluted in 50 mL of THF were added dropwise to the reaction flask. The reaction was heated at 40 °C and then 2.2 mol of R^2I were slowly added. When finished, the reaction was cooled down in an ice bath, quenched with 200 mL of water and neutralized with HCl. The organic phase was separated and the water phase was extracted with ether (4 x 100 mL). The combined organic phase was dried with $MgSO_4$ and the solvent was removed under vacuum. The product was purified by vacuum distillation.

‡NOTE ABOUT THE USE OF DICHLOROMETHANE IN THE SYNTHETIC PROCEDURES

As a referee noted, although dichloromethane is a very convenient solvent for the purification steps of some of the glycerol derivatives described, allowing easy phase separation and subsequent elimination in a rotary evaporator, its use is not desirable in the context of a green strategy. In this regard, ethyl acetate has been successfully tested with the same purpose, so the experimental procedure can be modified accordingly. We have, however, maintained the description of the original procedure for the sake of truthfulness.

Artículo 3: Ecotoxicity studies of glycerol ethers in *Daphnia magna.*

Autores: Eduardo Perales[A], José Ignacio García[B], Elisabet Pires[B], Luis Aldea[A], Laura Lomba[A], Beatriz Giner[A].

[A]*Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, 50830, Zaragoza, España.*

[B]*Instituto de Síntesis Química y Catálisis Homogénea (ISQCH) y Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Zaragoza– Consejo Superior de Investigaciones Científicas-(CSIC), Calle Pedro Cerbuna 12, 50009 Zaragoza, España.*

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En este artículo se evaluó la ecotoxicidad aguda del glicerol y diecinueve de sus derivados mediante el indicador *D. magna*. De igual manera que con los ensayos en *V. fischeri*, también se relacionaron sus valores de EC_{50} con sus parámetros topológicos, DARC-PELCO y con su lipofilia. Para confirmar la validez de la función establecida mediante QSAR con esta última propiedad, se comparó la ecuación obtenida con el modelo preestablecido ECOlogical Structure-Activity Relationship Model (ECOSAR, versión 1.11), perteneciente al software EPIweb (versión 4.1).

Accepted Manuscript

Ecotoxicity and QSAR studies of glycerol ethers in *Daphnia magna*

Eduardo Perales, Jose Ignacio García, Elisabet Pires, Luis Aldea, Laura Lomba, Beatriz Giner

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Ecotoxicity and QSAR studies of glycerol ethers in *Daphnia magna*

Eduardo Perales[a], Jose Ignacio García[b], Elisabet Pires[b,c], Luis Aldea[a], Laura Lomba[a] and Beatriz Giner[a]*

a. Facultad de Ciencias de la Salud, Universidad San Jorge. Autovía A-23 Zaragoza-Huesca Km. 299. Villanueva de Gállego, E-50830, Zaragoza, España.

b. Instituto de Síntesis Química y Catálisis Homogénea (ISQCH). CSIC-Universidad de Zaragoza. C/ Pedro Cerbuna, 12. E-50009, Zaragoza, España.

c. Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Zaragoza. C/ Pedro Cerbuna, 12, E-50009, Zaragoza, España.

***Corresponding author:** Beatriz Giner. Facultad de Ciencias de la Salud, Universidad San Jorge. Autovía A-23 Zaragoza-Huesca Km. 299. Villanueva de Gállego, E-50830, Zaragoza, España. Telephone: (+34) 976 060 100. Fax: (+34) 976 077 584. E-mail: bginer@usj.es.

Abstract

Glycerol is currently considered a raw, renewable material, which can be used to synthesize new glycerol derivatives that may be used as green solvents. However, these compounds must be environmentally evaluated before their use. The acute ecotoxicity of a series of mono-, di-, and trialkyl ethers synthesized from glycerol for the crustacean *Daphnia magna* has been studied. The EC_{50} values of these ethers after 24 h of exposure were determined according to the OECD 202 protocol. Their possible structural-toxicity relationships according to different alkyl substituents have been discussed after applying different QSAR models (with the DARC-PELCO approach and topological parameters). The results of the immobilization test show that most of the glycerol derivatives studied exhibit relatively low ecotoxicity. There is a correlation between the lipophilicity and the increase of the toxic effect in the crustacean biomodel. Furthermore, the length and the number of the alkyl substituents and ecotoxicity are highly related.

Keywords

Daphnia magna; ecotoxicity; glycerol derivative; solvent; QSAR.

1. Introduction

The problems associated with the massive use of petroleum-derived organic solvents have been recognized for a while now, and this recognition has led to regulations regarding their use, handling and safety measures (EU Regulation, 2006). Thus, there is an emerging interest in replacing these solvent types with others that have similar functional properties but are simultaneously safer or even harmless from the environmental and health perspectives (Clark et al., 2015; Gu and Jérôme, 2013; Jessop, 2011; Subramaniam, 2010). According to Green Chemistry principles (Anastas and Eghbali, 2010), these so-called green solvents should be nontoxic and easily biodegradable to the extent possible under environmental conditions. Furthermore, green solvents should have low vapour pressures (i.e., fall in the category of non-VOC compounds) and can be easily obtained from renewable raw materials (Subramaniam, 2010).

In this context, biodiesel production has recently emerged as a possible source of renewable green solvents (Li et al., 2013). An important consequence of this industrial process is the production of glycerol as a major by-product. Although biodiesel production has decreased in the recent years, the use of glycerol as a raw material has been considered a highly relevant subject (Ciriminna et al., 2014; Quispe et al., 2013). Thus, new markets or new applications of glycerol, such as converting glycerol into value-added products, have been the object of recent interest (Katryniok et al., 2011; Pagliaro et al., 2007; Tan et al., 2013; Zhou et al., 2008).

The synthesis and applications of glycerol-based solvents have already been described by several authors (Díaz-Álvarez et al., 2011; García et al., 2010; García et al., 2014; Gu and Jérôme, 2010; Sutter et al., 2015). For instance, 1,2,3-trimethoxypropane, 1,3-dimethoxy-2-propanol and 2,3-dimethoxypropan-1-ol, among others, are considered good polar solvents, which can be used as substitutes for other hazardous compounds, such as dimethoxyethane (Sutter et al., 2013a, c).

Another glycerol-derived solvent is 1,3-bis(2,2,2-trifluoroethoxy)propan-2-ol. This compound can be prepared from trifluoroethanol and epichlorohydrin (García et al., 2010), which are obtained from glycerol using the Solvay procedure. This fluorinated derivative has several physicochemical properties that are similar to those of certain ionic liquids (Mallakpour and Rafiee, 2011a, b), which could be replaced in certain applications (Aldea et al., 2010; Aldea et al., 2012).

One of the primary advantages of glycerol-derived solvents is their high potential for chemical diversity, which results in an extensive variation of their corresponding physicochemical properties. Therefore, a broad spectrum of chemicals could be developed according to specific needs. This potential is a relevant point to consider since multiple replacement solvents could be available from the same raw material. However, a renewable origin is not sufficient to label these new solvents as harmless. Likewise, the systematic experimental evidence for their safety would be required to confirm whether these compounds could be considered environmentally friendly.

Thus, the use of solvents must be supervised and regulated (by the EPA in the United States or the ECHA in Europe), and ecotoxicological aquatic toxicity information on different trophic levels must be compiled (Levet, 2016). Bearing this idea in mind, we are studying a family of glycerol derivatives, alkyl glyceryl ethers, which have a high potential for use as solvents from the environmental safety perspective (Fig. 1). In a previous study (García et al., 2015), we evaluated the acute ecotoxicity of a set of these twenty solvents using *Vibrio fischeri* (*V. fischeri*) as a bioindicator, due to its advantages as a standardized aquatic biomodel (Arbuckle and Alleman, 1992; Farré et al., 2004; Salizzato et al., 1998; UNE-EN-ISO 11348-3, 2007). Our results showed that most of these glycerol derivatives were considered harmless to *V. fischeri*, and only three of them were clearly harmful according to the Passino-Smith classification (Passino and Smith, 1987). However, to achieve a better understanding of the ecotoxicological behaviour and to assess the toxicity of the same series of glycerol-derived

ethers (Fig. 1) at a higher trophic level (crustaceans), we have used another bioindicator, the crustacean *Daphnia magna* (*D. magna*).

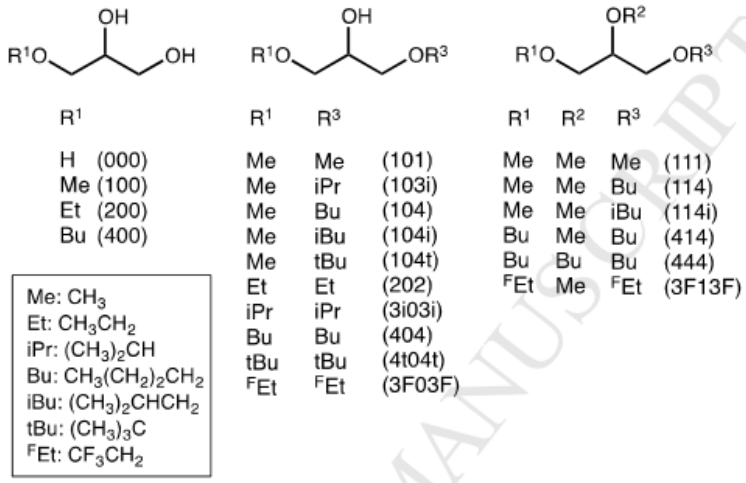

Fig. 1. Structures of the 20 studied glycerol derivatives, and the short codes used to identify them.

The bioindicator *D. magna* is a well-known standardized organism (OC SE TG 202, 2004; OECD 202, 1984) and is considered a relevant link between microorganisms and biologically more complex organisms (Aydin et al., 2015) and one of the bioindicators of choice for the general procedures of Environmental Risk Assessment methodologies (Koller et al., 2000). We hypothesized that there might be a relationship between the lipophilicity of the glycerol derivatives and their ecotoxicity. In this way, we could estimate whether the action mechanism of these compounds is related to a non-polar narcosis action mechanism (Cleuvers, 2003; Levet et al., 2016; Mínguez et al., 2014), which harms the cell membranes in an unspecific way. Additionally, the presence and length of the alkyl chains in different positions of the basic glycerol structure were analysed using quantitative structure-activity relationship (QSAR) studies to relate them to the EC_{50} values in *D. magna*.

2. Materials and methods

97 *2.1. Chemicals*

98 The glycerol derivatives were prepared according to previously described procedures
99 (García et al., 2014, 2015).

100 *2.2. D. magna acute immobilization test*

101 The *D. magna* individuals used in the acute immobilization test were provided in a
102 Daphtoxkit F Magna kit (ref. DM090812) that was purchased from Vidrafoc (Barcelona, Spain).
103 The ephippia vials were stored at 4°C until use. This experiment and its previous preparation
104 were performed according to the guidelines of the OECD 202 test conditions (OC SE TG 202,
105 2004; OECD 202, 1984).

106 First, *D. magna* medium was prepared according to the specifications of the supplier.
107 Next, the ephippia were incubated for 72 hours at 20–22°C with a luminescence of 6000 lux in a
108 TOXKIT model CH-0120D-AC/DC incubator (ECOTEST, Valencia, Spain). The ephippia
109 were then fed with dry *Spirulina* algae 2 hours before starting the experiment (approx. one vial
110 of algae per vial of *D. magna*). The positive controls were tested with $K_2Cr_2O_7$, and the results
111 agree with the reference values in all the cases (the range of concentrations tested: 3.5-0.3 mg L^{-1},
112 average EC_{50} measured: 1.2 mg L^{-1}) (OC SE TG 202, 2004).

113 The negative controls were also tested, and 2.6% of the tests were rejected because they
114 did not meet the established negative control requirements (no more than 10% of the individuals
115 remained unimmobilized). The results have been given for the tests that met these criteria. The
116 pH of the dilutions was adjusted to between 7 and 7.5 after their preparation and before starting
117 the assay.

118 A total of 20 newborn daphnids (aged < 24 hours) were exposed to the test compounds
119 in complete darkness for 24 hours at 20°C per concentration and compound. The crustaceans
120 were separated into four groups of five organisms per concentration. At least six concentrations
121 for each compound were exposed in the assays. A previous range finding test was performed to
122 determine the concentrations of the definitive tests, which were repeated at least three times.

The immobilization of the organisms was measured by direct observation. If daphnids were unable to swim for 15 seconds after gentle stirring, they were considered immobilized.

With the obtained EC_{50} values of the studied compounds, a linear regression was performed, comparing the values with the logarithm of the partition coefficient (log P) to correlate these values in *D. magna* with their lipophilicity. Additionally, QSAR studies using the DARC-PELCO approach (Dubois, 1974; García et al., 2013; García et al., 2015) and topological parameters were determined (García et al., 2015; Katritzky and Gordeeva, 1993; Kier and Hall, 1985).

Concentration units in the utilized OECD 202 acute toxicity test are expressed in mg L^{-1}. Given the different molar masses (M) of the studied solvents, these units could be an issue when comparing relative toxicities. Of course, in the case of compounds with similar M, these differences tend to be negligible. However, for the sake of completeness, we have repeated the QSAR analyses with EC_{50} toxicities expressed in mM units without noticeable changes in the conclusions reached. The results of these analyses are available in the Supplementary Information.

2.3. Statistics and graphical representation

To obtain the half maximal effective concentration (EC_{50}) values and the dose-response curves, the obtained results were fitted using the least squares method for the following function (Eq. 1):

$$\%I = 100/(1 + 10^{(a-logc)b}) \qquad \text{(Eq. 1)}$$

where *%I* denotes the % immobilization, *c* is the concentration (in $mg \cdot L^{-1}$), and *a* and *b* are adjustable parameters.

The QSAR analyses were conducted using multiple linear regression analyses. Toxicity data were fitted to the regression equation using the least squares procedure. In each equation, the standard error of the corresponding regression coefficient is given between brackets. Only

148 statistically significant coefficients ($p<0.05$) have been retained in the final equations. Apart
149 from the multiple correlation coefficient (R) values, in each equation, the cross-validated R
150 coefficients (R_{CV}) are also given. These values were determined by predicting the toxicity of a
151 given derivative using the regression equation fitted with the remaining compounds, i.e., using
152 the predicted residual sum of squares instead of the fitted residual sum of squares. The R_{CV}
153 coefficient yields more realistic information regarding the true predictive ability of the model
154 (see the Supplementary Information for a complete definition of these parameters).

155 ## 3. Results and Discussion

156 *3.1. D. magna acute immobilization test*

157 The EC_{50} values obtained in the biotests using *D. magna* are presented in Table 1. The
158 results of each glycerol-derivative solvent are graphically represented in Fig. 2, which groups
159 each result by the similarity of the position and the length of the substituents for ease of
160 comparison.

161

Solvent code	Nominal concentration range (mg L^{-1})	EC_{50} (mg L^{-1})	SD	EC_{50} (mM)
444	0.5-75	13.7	4.814	0.052
414	6-500	133.3	5.966	0.611
404	50-500	248.1	4.110	1.214
3F03F	30-1,500	477.0	6.978	1.8622
404t	90-1,500	667.6	8.142	3.268
3F13F	50-6,000	943.7	4.181	3.493
114i	425-3,000	1,496.0	2.394	8.487
114	425-3,000	1,617.0	6.055	9.174
202	1,000-3,000	1,819.0	5.622	12.274
104i	500-4,000	1,975.0	5.575	12.174
3i03i	400-5,000	2,170.0	5.331	12.311
400	1,000-6,000	2,332.0	3.211	15.735
104	1,000-5,000	2,388.0	4.400	14.720
111	1,000-5,000	3,240.0	5.756	24.147
104t	2,500-10,000	4,568.0	4.276	28.158

101	1,000-9,000	4,790.0	6.105	39.867
103i	3,000-7,000	4,828.0	6.494	32.578
200	3,000-15,000	6,458.0	5.282	53.916
100	600-15,000	6,478.0	7.680	60.856
000 (glycerol)	10,000-100,000	68,784.0	7.700	746.921

Table 1. Experimental EC_{50} values for *D. magna* obtained for the studied solvents with their corresponding standard deviations (SDs).

According to the Passino-Smith classification (Passino and Smith, 1987), only one compound, 444, can be considered as slightly harmful to *D. magna* since its EC_{50} is below 100 mg L^{-1}. Compounds that have EC_{50} values between 100 and 1,000 mg L^{-1} can be classified as practically harmless (414, 404, 404t, 3F03F, and 3F13F), and the rest of the studied solvents, displaying EC_{50} values over 1,000 mg L^{-1}, are clearly classified as harmless to *D. magna*. In the results, several of these solvents were classified in less harmful categories than they were for *V. fischeri* in our previous study (400, 404, 414, 404t, 111, 114, 104, 104i, 114i, and 104t) (García et al., 2015). In contrast, 444, 3F03F and 3F13F were more harmful and sufficiently harmful to be classified into a higher toxic category (Passino and Smith, 1987).

From previous studies, the basis glycerol compound (compound 000) was indicated as absolutely not harmful to the water flea in acute exposures. For example, in a 24-hour test in the static exposure of the glycerol to this cladoceran, the EC_{50} obtained was greater than 10,000 mg L^{-1} (Bringmann and Khun, 1977). There have been similar results for other organisms such as *V. fischeri* (García et al., 2015), *Carassius auratus* (Goldfish) (Bridie et al., 1979), *Entosiphon sulcatum* (Bringmann and Khun, 1980) and *Clostridium pasteurianum* (Dabrock et al., 1992), which also confirms the low toxicity of glycerol as shown in our own results (Table 1).

Based on an extensive literature review, there are no prior experimental ecotoxicological data on any of the studied glycerol derivatives, with the exception of 111. For this compound, Sutter et al. (2013a) have performed an ecotoxicity study according to different ISO/OECD guidelines, including for *D. magna*. Our findings were verified with this previous information.

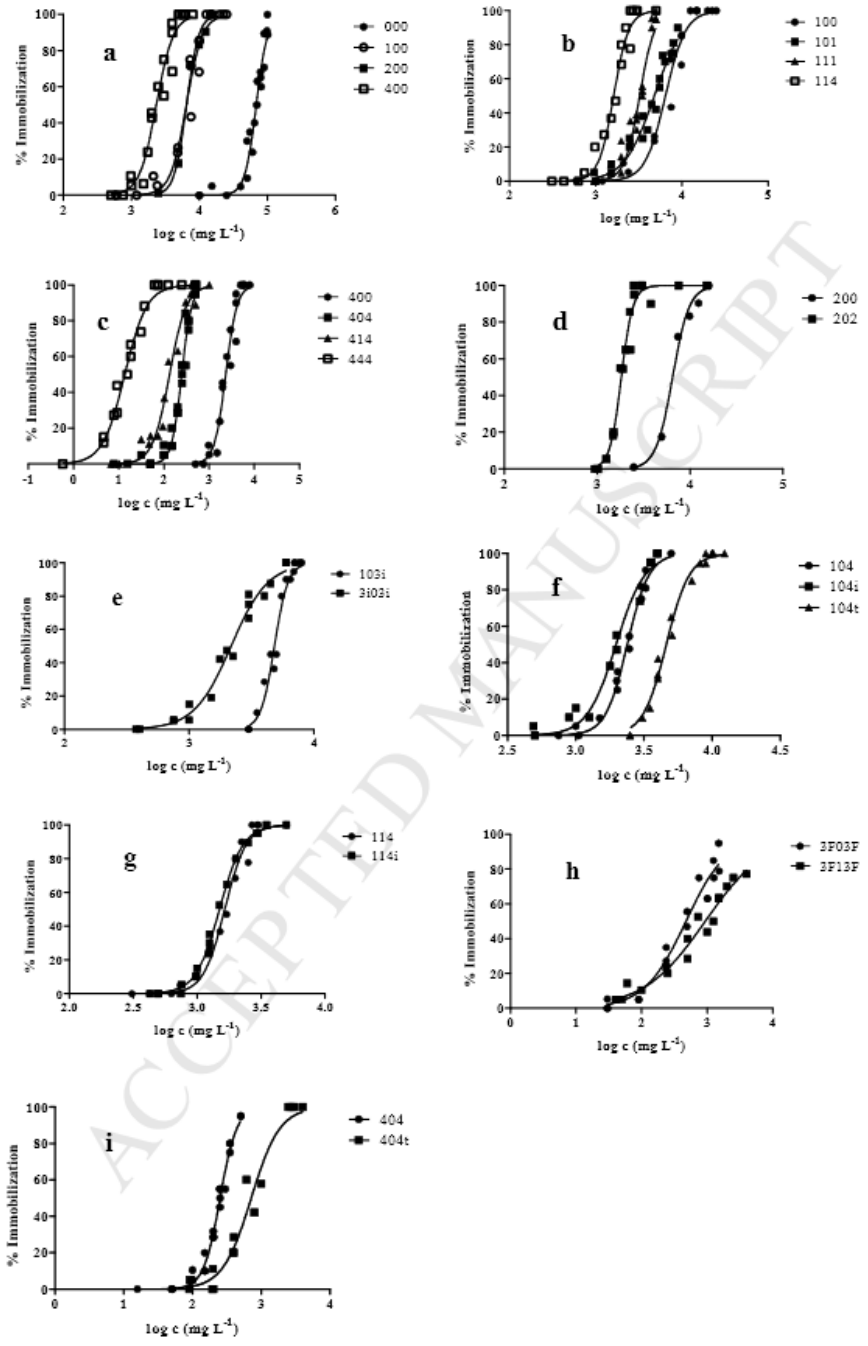

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Fig. 2. Dose-response curves for the studied glycerol-derived solvents. 2a: 000, 100, 200, and 400; 2b: 100, 101, 111, and 114; 2c: 400, 404, 414, and 444; 2d: 200 and 202; 2e: 103i and 3i03i; 2f: 104, 104i, and 104t; 2g: 114 and 114i; 2h: 3F03F and 3F13F; and 2i: 404 and 404t.

According to the obtained results, the immobilization of the crustaceans and, thus, the ecotoxicity increase with the number, size and linearity of the alkyl groups on the glycerol moiety. As stated in our previous study (García et al., 2015), this relationship may be related to the hydrophobicity of the glycerol derivatives (Fig. 3a). The following regression equation (Eq. 2) was obtained:

$$\log EC_{50} = 3.604(\pm 0.065) - 0.607(\pm 0.050)\cdot \log P \qquad \text{(Eq. 2)}$$

$N = 20$, $R = 0.95$, $R_{CV} = 0.92$, $\sigma(y) = 0.254$

$F = 149.3$ ($F_{(1,18,\ 0.05)} = 4.4$)

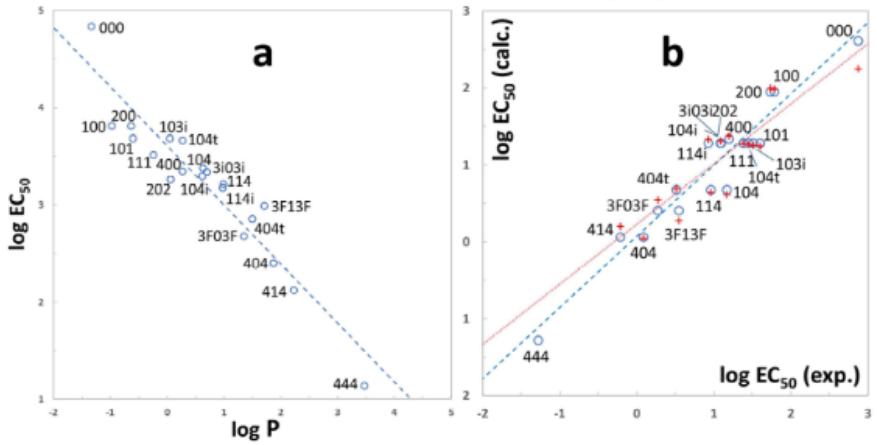


Fig. 3. 3a: Plot of the log EC_{50} (original values in mg L^{-1}) *vs.* log P and the linear regression fitting line; 3b: Plot of the predicted *vs.* experimental values of log EC_{50} as calculated by regression analysis using the DARC/PELCO model. Circles represent the predictions made with the plain regression equation, and the crosses represent the cross-validated predictions. Dashed and dotted lines represent the least squares fit between both sets of data, respectively.

The general trend is that the higher the log P of the compound, the lower the EC_{50} values. Nevertheless, there is a clear exception in this general behaviour with the fluorinated compounds (3F03F and 3F13F, Fig. 2h). In this case, there is an inversion of the observed trend, which means that the EC_{50} value of the least lipophilic compound (3F03F) is lower than the most lipophilic molecule (3F13F). Notably, the size of the branched substituents does not

explain the inversion of the trend, and in the molecules containing a "tert" substituent (104-104t and 404-404t, Fig. 2f and 2i), the lipophilicity correlates with the ecotoxicity.

3.2 QSAR analysis

3.2.1. DARC-PELCO analysis

Given the EC_{50} values for *D. magna* in the glycerol-derived compounds, QSAR analyses were performed. First, we used the DARC-PELCO approach, in which each molecular descriptor indicates only the presence or absence of a group of atoms at a given molecular position, the latter defined as a series of shells expanding from the common glycerol structure (detailed information about this model can be found in the Supplementary Information and in references (Dubois, 1974; García et al., 2013)). As occurred in our previous study, we verified that the QSAR models behaved slightly better using molar concentrations; hence, we used the EC_{50} values expressed in these units for the QSAR analyses. In any case, the differences in the developed equations are only marginal using either mg L^{-1} or mM units (see the Supplementary Information for comparison).

The regression equation found using the DARC-PELCO descriptors was as follows (Eq. 3):

$$\log EC_{50} = 2.613(\pm0.203) - 0.612(\pm0.095)\cdot D_2 - 0.665(\pm0.115)\cdot A_2 - 1.338(\pm0.0308)\cdot D_1 - 0.438(\pm0.104)\cdot {}^{F}B_2 \quad \text{(Eq. 3)}$$

$N = 20$, $R = 0.96$, $R_{CV} = 0.89$, $\sigma(y) = 0.266$

$F = 46.5$ ($F_{(4,15,\,0.05)} = 3.1$)

The fitting of the model with the experimental data is fairly good, as 92% of the experimental variance is explained by the model. To obtain better knowledge of the predictive ability of this equation, a cross-validation procedure was applied. In this procedure, the experimental response of each glycerol derivative was predicted by the regression equation

obtained using only the remaining 19 experimental data. It is worth mentioning that this cross-validation procedure cannot be applied in the case of compound 444. As this compound is the only one bearing an alkyl group longer than methyl at the central oxygen, it is also the only one for which coefficients beyond A_1 can be estimated. If this compound is leaved out in the regression analysis (as does the cross-validation procedure) no B_1–D_1 coefficients can be calculated, as all the corresponding descriptors in the data matrix become zero. In fact, the D_1 coefficient calculated in the normal regression analysis represents the sum of the contributions of carbon atoms at the B_1, C_1 and D_1, positions (see the Supplementary Information for a more detailed explanation), which explains its relatively high value. However, predictions of ecotoxicity for ethers showing an alkyl group longer than methyl at the central oxygen have to be taken with caution, and this issue should be fixed in further works by including additional ethers bearing ethyl, propyl or butyl groups at this position.

Using the remaining solvents (without 444) R_{CV} = 0.89 was obtained, still fairly reasonable. Fig. 3b plots the experimental values vs. those calculated with the regression models. As expected from the previous discussion, the cross-validated prediction for basic glycerol was far from the experimental value, while the predictions for the rest of the derivatives were much closer.

All the regression coefficient values were negative, which means that any substitution on the glycerol moiety could lead to an increase in the acute ecotoxicity of the resulting compound to *D. magna*. The highest coefficient in absolute terms is D_1, which contrasts with the previously found for *V. fischeri*, where the substitution at the central oxygen atom led to a decrease in toxicity (García et al., 2015).

3.2.2. Analysis of topological parameters

We also considered the use of topological parameters, which were derived from the molecular graph for each compound. Topological parameters are descriptors of molecular size, shape and flexibility. Opposite the DARC-PELCO descriptors, these parameters are global

because a whole molecular structure is reduced to a single numerical value (García et al., 2013). The following best-fitting equation (Eq. 4) was obtained with the topological atomic valence connectivity index, χ_1^v, (for a definition of this and other topological indices, see the Supplementary Information):

$$\log EC_{50} = 3.337(\pm 0.205) - 0.582(\pm 0.048)\cdot\chi_1^v \quad \text{(Eq. 4)}$$

$N = 20$, $R = 0.94$, $R_{CV} = 0.93$, $\sigma(y) = 0.294$

$F = 146.1$ ($F_{(1,18,\ 0.05)} = 4.4$)

A good fit was also obtained with this model (with 89% of the total experimental variance explained), which was mostly kept for the cross-validation analysis (with 86% of variance explained). As the χ_1^v was primarily related to the molecular size, the negative coefficient of the regression equation could be interpreted as an increase in toxicity with the size of the glycerol derivative. This result is in line with that using the DARC-PELCO descriptors. Consequently, we tested the use of plain, molecular-sized descriptors, such as the calculated molecular volume (V_{mol}). The regression equation identified using this descriptor (Eq. 5) as follows:

$$\log EC_{50} = 3.693(\pm 0.229) - 0.016(\pm 0.001)\cdot V_{mol} \quad \text{(Eq. 5)}$$

$N = 20$, $R = 0.95$, $R_{CV} = 0.93$, $\sigma(y) = 0.289$

$F = 152.2$ ($F_{(1,18,\ 0.05)} = 4.4$)

This equation corroborates with the previous interpretation given to the structure-activity relationship and points to the molecular size as the primary factor that determines the ecotoxicity in the case of *D. magna*. Nevertheless, a word of caution must be stated concerning the interpretation of these QSAR results given the (not unexpected) correlation between all the parameters used as descriptors in the above equations (Table 2). Because most of the

substituents are hydrocarbon chains, longer chains are associated with a larger size, greater molecular volume, and higher hydrophobicity.

	log P	D-P[a]	χ_1^v
log P	—		
D-P	0.942	—	
χ_1^v	0.975	0.985	—
V_{mol}	0.984	0.971	0.978

[a] Sum of all DARC-PELCO descriptors for a given compound.

Table 2. Correlation between the molecular descriptors used in this work.

In these conditions, the establishment of a clear cause-effect relationship was not that evident, although the abovementioned hypothesis of a non-specific membrane action driven by the lipophilicity of these compounds remains to be the most plausible one (Levet et al., 2016).

3.3. Glycerol derivatives vs. *other solvents*

It was also interesting to compare the ecotoxicity determined for the glycerol-derived ethers with a selection of individual conventional solvents (Bringmann and Kühn, 1982; Galassi et al., 1988; García et al., 2005; Lilius et al., 1995) and other ionic liquids (García et al., 2005). An overview of certain exemplary EC_{50} values for these substances is shown in Table 3. The ecotoxicity of most of the studied glycerol derivatives is lower than several widely used organic solvents, such as toluene, dichloromethane or chloroform, and higher than others such as ethanol or isopropanol. Only 444 could be considered toxic at the same level as toluene for *Daphnia magna*. Regarding ionic liquids, they are generally more toxic than the glycerol-derived compounds, except, once again, in the case of 444. This innocuous result is notable since the authors of previous studies (Mallakpour and Rafiee, 2011a,b; Zhao et al., 2005) remarked on the environmental harmlessness of ionic liquids. Therefore, according to their corresponding EC_{50} values in *D. magna* and *V. fischeri*, our glycerol-derived solvents are better green solvent options (García et al., 2015). In fact, our group has already described some of

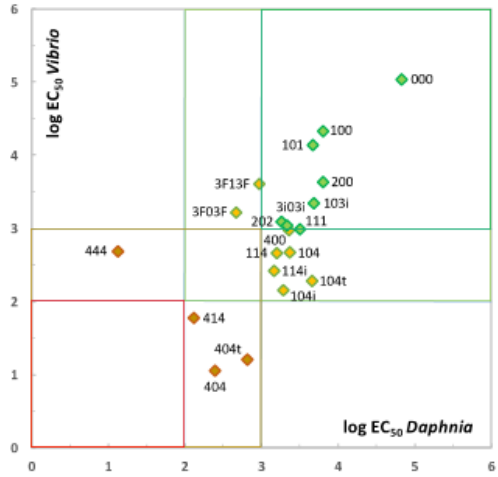
297 these solvents as being advantageously used in place of the ionic liquids as a reaction media for
298 catalytic reactions (Aldea et al., 2010, 2012).

Traditional solvents	EC_{50} (mg L^{-1})
Toluene	7[a]
Dichloromethane	223[b]
Chloroform	573[c]
1,4-Dioxane	8,450[d]
Ethanol	9,847[b]
Isopropanol	9,959[b]
Acetonitrile	10,076[b]
Acetone	13,615[b]
Methanol	22,682[b]
Ionic liquids	
1-Octyl-3-methylimidazolium chloride	0.8[b]
1-Octyl-3-methylimidazolium tetrafluoroborate	1.3[b]
1-Hexyl-methylimidazolium chloride	2.5[b]
1-Hexyl-3-methylimidazolium tetrafluoroborate	3.4[b]
1-Butyl-3-methylimidazolium chloride	12.4[b]
1-Butyl-3-methylimidazolium bromide	13.2[b]
1-Butyl-3-methylimidazolium tetrafluoroborate	13.9[b]
1-Butyl-3-methylimidazolium hexafluorophosphate	25.3[b]

299 [a] Galassi et al., 1988; [b] García et al., 2005; [c] Lilius et al., 1995; [d] Bringmann and Kühn, 1982.

300 **Table 3.** Several EC_{50} values for different traditional solvents and ionic liquids at 24 h of exposure.

301 With the aim of increasing the understanding of the environmental impact of these
302 solvents, we compared the obtained toxicity data from the *D. magna* bioindicators with those
303 previously determined for *V. fischeri*. When both sets of data were plotted against each other, a
304 rough correlation emerges between them (r=0.66) (Fig. 4). Nevertheless, there are clear
305 discrepancies in the correlated values for the "extreme" solvents, such as glycerol and, above
306 all, 444, as well as for other minorly substituted but still significantly fluorinated solvents and
307 for those derivatives bearing two linear butyl chains (414 and 404). Consequently, most of the
308 studied glycerol-derived solvents display a similar ecotoxicity pattern for both bioindicators,
309 although there are certainly noticeable exceptions that may be attributed to particular toxicity
310 mechanisms.



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312 **Fig. 4.** Plot of log EC_{50} (original values in mg L^{-1}) of glycerol derivatives on *Daphnia magna vs.* log EC_{50} of the same solvent set for *Vibrio fischeri.* The colour codes correspond to the Passino–Smith classification margins (red: moderately toxic, yellow: practically harmless, green: harmless).

313

314 To determine which of these glycerol-derived ethers are the safest according to the
315 utilized bioindicators, it is illustrative to consider the common Passino-Smith areas for both of
316 the bioindicators (Fig. 4). Fig. 4 shows that none of the tested derivatives falls in the joint area
317 for moderately toxic compounds (between 0 and 2 on the logarithmic scale), and only four of
318 them fall in the area between 0 and 3, which would include those compounds that are
319 moderately toxic or practically harmless for either biomodel. On the other hand, up to eight
320 compounds fall in the harmless area for both bioindicators (beyond three logarithmic units in
321 both axes), which points to a safe use of these glycerol derivatives from an acute
322 ecotoxicological perspective.

323 *3.4. Predictive ability of the model in ethers with longer substituents*

324 Due to their many industrial and health applications such as surfactants,
325 pharmaceuticals, cosmetics, ink or cleaning formulations, optimized syntheses of glycerol
326 monoethers with longer alkyl chains have been described (Sutter et.al., 2013b; Gaudin et.al.,
327 2011). Unfortunately, the environmental impact of most of these chemicals have not been

evaluated yet. With the double aim of validate the models described in this work and provide a first approximation of the toxicity of these important glycerol ethers in the biomodel *D. magna*, we have carried out the following calculations.

The predicted EC_{50} values for *D. magna*, 24 h of exposition, for the ethers 3-(pentyloxy)propane-1,2-diol, 3-(hexyloxy)propane-1,2-diol, 3-(2-ethylhexyloxy)propane-1,2-diol, 3-(octyloxy)propane-1,2-diol, 3-(decyloxy)propane-1,2-diol and 3-(dodecyloxy)propane-1,2-diol, codified as 500, 600, 6(2)00, 800, (10)00 and (12)00 respectively, have been calculated making use of the Eq. 2. The values are exposed in the Supplementary Information. As expected and according to the lipophilicity of these ethers and our model, the longer the alkyl substituent chain, the higher the toxicity; the ethers 6(2)00, 800, (10)00 can be considered as slightly harmful to *D. magna* since its EC_{50} is below 100 mg L^{-1}, according to the Passino-Smith classification (Passino and Smith, 1987), while 400 and 500 can be classified as practically harmless. Only (12)00 could be moderately toxic according to this classification.

On the other hand, ECOlogical Structure-Activity Relationship Model (ECOSAR) version 1.11 from the EPIweb 4.1, available from the Risk Assessment Division of the Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, has been used for predicting the EC_{50} values for *D. magna* after 48 h of exposition. The predicted values are shown in Supplementary Information, including these long glycerol ethers. A good correlation has been found between two set of values (from our model and ECOSAR) (Fig. 5). Thus, our method is able to predict the toxicity of glycerol alkyl ethers with the same trend that the well-stablished predicting method for evaluating the (eco)toxicity ECOSAR.

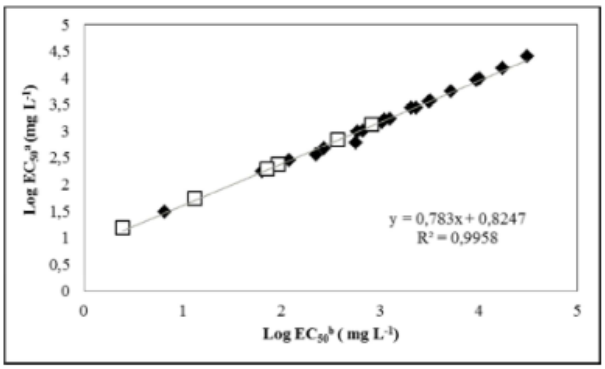

Fig. 5. Plot of the correlation between EC_{50} calculated form this work (Eq. 2.)[a] vs. ECOSAR[b]. Diamonds: studied chemicals in this work; squares: longer alkyl glycerol ethers.

4. Conclusions

The ecotoxicity of a series of glycerol ethers with potential utility as solvents (including basic glycerol) has been studied using the crustacean *D. magna* as a bioindicator. Sixteen out of the twenty studied compounds can be classified as harmless or practically harmless by the results with this bioindicator. According to the Passino-Smith classification, only one compound is clearly harmful. These results support the conclusions previously obtained using a different aquatic biomodel, *V. fischeri*, and provide stronger evidence for the harmlessness of these bio-based solvents.

The QSAR studies point to a monotonous dependence of the toxicity on molecular size and lipophilicity. Given the high correlation existing between these two molecular properties in the solvent set used in the study, getting a deeper insight into the mechanisms involved in the toxicity of these compounds is difficult.

A comparison of the ecotoxicity towards *D. magna* exhibited by several traditional solvents with that of the studied glycerol ethers indicates that the EC_{50} of many of the latter are at the same point on the Passino-Smith classification as are short-chain alcohols. When

compared with widely used ionic liquids, the ecotoxicity of the studied compounds is generally lower, supporting better environmental performance.

The predictive ability of the models developed in this work has been validated by comparison with the well-established ECOSAR model.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Highlights

1. Ecotoxicity of 20 solvents using *Daphnia magna* as bioindicator has been explored.
2. EC_{50} values have been used to discuss structure-toxicity relationships QSAR.
3. Ecotoxicity of these compounds increases with lipophilicity.

Electronic Supplementary Information for

Ecotoxicity and QSAR studies of glycerol ethers in *Daphnia magna*

Eduardo Perales[a], Jose Ignacio García[b], Elisabet Pires[b,c], Luis Aldea[a], Laura Lomba[a] and Beatriz Giner[a*]

[a] *Facultad de Ciencias de la Salud. Universidad San Jorge. Villanueva de Gállego.* E-50830. Zaragoza. Spain; E-mail: bginer@usj.es.

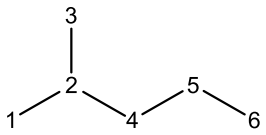
[b] *Instituto de Síntesis Química y Catálisis Homogénea (ISQCH). Facultad de Ciencias. CSIC-Univ. de Zaragoza. Pedro Cerbuna. 12. E-50009 Zaragoza. Spain. Tel: +34 976762271; E-mail: jig@unizar.es.*

[c] *Departamento de Química Orgánica. Facultad de Ciencias. Univ. de Zaragoza. Pedro Cerbuna. 12. E-50009 Zaragoza. Spain; E-mail: epires@unizar.es.*

Content

Definition of the topological parameters

Topological indices are usually obtained from two-dimensional molecular structures (molecular graphs, G), mostly through the connectivity adjacency (A(G)) and topological distance matrices (D(G)), and the vertex degree vector (δ (G)):



A(G)

	1	2	3	4	5	6
1	0	1	0	0	0	0
2	1	0	1	1	0	0
3	0	1	0	0	0	0
4	0	1	0	0	1	0
5	0	0	0	1	0	1
6	0	0	0	0	1	0

δ (G)

	1	2	3	4	5	6
□	1	3	1	2	2	1

D(G)

	1	2	3	4	5	6
1	0	1	2	2	3	4
2	1	0	1	1	2	3
3	2	1	0	2	3	4
4	2	1	2	0	1	2
5	3	2	2	1	0	1
6	4	3	3	2	1	0

Topological indices are calculated from different invariant features of the molecular graph, and contain information about molecular size, molecular shape, branching, molecular flexibility, etc. The exact definition of the indices used in this work are given below.

Balaban indices (JX, JY):

Balaban index is defined as:

$$J = \frac{M}{M - N + 2} \sum \frac{1}{\sqrt{s_i^a s_{ij}^a}}$$

where M is the number of bonds, N is the number of atoms in the molecule, and s_i is calculated as the sum of terms from a modified topological distance matrix. In this modified distance matrix, each bond contributes with 1/b to the total connectivity, with b=1 for single bonds, b=2 for double bonds, b=3 for triple bonds, and b=1.5 for aromatic bonds:

$$s_i = \sum_{i=1}^{N} d_{ij}$$

Corrections for heteroatoms have been introduced through contributions for the modification of the electronegativity (X) and the atomic radii (Y):

$$X = 0.4196 - 0.0078i + 1.1567G_i$$
$$Y = 1.1191 - 0.0160i + 0.0537G_i$$

where i is the atomic number and G_i is the group number in the Periodic Table of the elements. From these corrections, the s_i^a values are defined as:

$$s_i^a = X \cdot s_i \text{ (for JX index)}$$

$$s_i^a = Y \cdot s_i \text{ (for JY index)}$$

Wiener index (W):

The Wiener index is defined as the sum of the lengths of the shortest paths between all pairs of vertices in the chemical graph representing the non-hydrogen atoms in the molecule. It is easily computed from the topological distance matrix:

$$W = \frac{1}{2} \sum_i \sum_j d_{ij}$$

This index is a measure of the centrality of the graph, and hence it is related with the molecular compactness.

Zagreb index:

It is defined as the sum of squares of the difference between the number of electrons participating in covalent bonds and the number of hydrogen atoms bonded to the same atom. This is equivalent to the sum of the squares of the vertices degrees, δ_i:

$$Zagreb = \sum_i \delta_i^2 = \sum_i (\sigma_i - h_i)^2$$

Randic and Kier & Hall connectivity indices (χ):

χ indices were first proposed by Randic from the vertices degrees, as:

$\chi = \sum_B \frac{1}{\delta_i \cdot \delta_j}$, extended to all bonds in the molecule (B).

Kier and Hall extended the definition by including the number of edges of a given sub-graph (h), and different kinds of sub-graphs (r):

$$^n\chi_r(G) = \sum_{i=1}^{\sigma_n} \left(\prod_{j=1}^{h+1} \sqrt{\frac{1}{\delta_i}} \right)$$

where σ_n is the number of sub-graph of length h and δ_i is the vertex degree.

There are four kinds of sub-graphs, known as *path* (linear chains), *cluster* (branched chains), *path/cluster*, and *chain* (cycles), each one emphasizing a particular aspect of the molecular connectivity. The n superindex refers to the number of bonds considered to calculate the topological index. Thus, n=0 refers to individual atoms, n=1 refers to directly connected atoms, n=2 refers to three atoms connected through two consecutive bonds, and so on.

$$^nP_{(sub)} = \frac{1}{\sqrt{(\delta_i \times \delta_j \times \cdots \times \delta_n)}} = \prod_{i=1}^{n} \frac{1}{\sqrt{\delta_i}}$$

, and hence

$$^n\chi_s \equiv Chi(n)(sub) = \sum {}^nP_{(sub)}$$

A further refinement can be included to the χ indices by considering the atom valences, thus allowing distinguishing the presence of heteroatoms in the structure. This is accomplished by calculating s "corrected" d value, using the atomic number and the number of valence electrons of the vertex atoms:

$$\delta^v = \frac{(Z^v - h)}{(Z - Z^v - h)}$$

Where Z_v is the number of valence electrons, Z is the atomic number and h is the number of hydrogen atoms bonded to the vertex atom. The resulting "valence-corrected" indices are named as χ_v.

Kier & Hall count indices (SC):

SC is the count of sub-graphs of a given length present in the molecules. Thus, SC=0 is the number of atoms, SC=1, the number of chemical bonds, SC=2, the number of pair bonds, and so on. For longer sub-graphs, *path*, *cluster*, *path/cluster* and *chain* types can be also considered.

Kier shape indices (κ_n):

All the prededent topological indices are heavily influenced by the size of the molecular graph. Kier developed the κ indices to best discriminate between different shapes of the molecules. They are defined from sub-graphs of a given length, taking into account also the maximum and minimum connectivity of the molecule for the same length (a way to "normalize" the κ values, making them independent of the molecular size):

$$\kappa_n = K \cdot \frac{{}^mP_{min}\,{}^mP_{max}}{\left({}^mP_i\right)^2}$$

Where m is the length chosen of the sub-graph, ^mP_i the number of sub-graphs of length m contained in the total

graph, and ${}^{m}P_{max}$ and ${}^{m}P_{min}$ is the maximum and minimum number possible of sub-graphs of length m that can contain the total graph. Some examples are given below.

κ_1, K =2:

$$ {}^{1}P_{min} = N - 1 $$

$$ {}^{1}P_{max} = \frac{N(N+1)}{2} $$

$$ {}^{1}P_{i} = number\_of\_edges $$

κ_2, K =2:

$$ {}^{2}P_{min} = N - 2 $$

$$ {}^{2}P_{max} = \frac{(N-1)(N-2)}{2} $$

$$ {}^{2}P_{i} = number\_of\_adjacent\_edges $$

κ_3, K =4:

$$ {}^{3}P_{min} = N - 1 $$

$$ {}^{3}P_{max} = \frac{(N-2)^2}{4} \quad (N\ even) $$

$$ {}^{3}P_{max} = \frac{(N-1)(N-3)}{4} \quad (N\ \ odd) $$

$$ {}^{3}P_{i} = trios\_of\_adjacent\_edges $$

Similarly to the χ indices, a modification has been sugested for κ indices to account for the presence of heteroatoms in the molecular graph. In this modification, both the covalent radii and the hybridizations are considered. The κ_n^{α} indices are defined as the κ_n ones, but substituting N by N+α, where a is defined as:

$$ \alpha = \sum_i \left(\frac{r_i}{r_{Csp^3}} - 1 \right) $$

Where r_i is the covalent radium of atom i and r_{Csp^3} is taken as 0.77 Å (the covalent radius of a carbon atom with sp^3 hybridization).

Molecular flexibility index (φ)

The starting hypothesis to define f is that an infinitely long linear saturated hydrocarbon molecule (i.e. all-sp^3 C−C bonds) is infinitely flexible. Flexibility is reduced by the presence of a limited number of atoms, rings, branched chains, and the presence of atoms with covalent radii shorter than that of C_{sp^3}:

$$ \varphi = \frac{\kappa_1^{\alpha} \kappa_2^{\alpha}}{N} $$

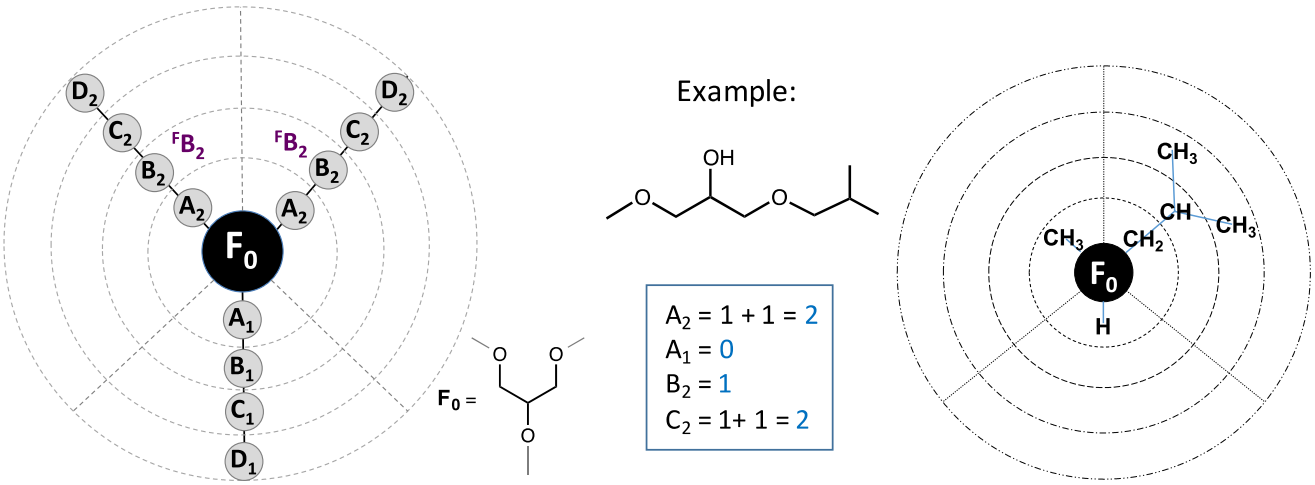
Table S1. Topological parameters of 20 glycerol-derived solvents.

Code	HBA	HBD	RB	φ	Bal^{JX}	Bal^{JY}	W	Z	κ_1^{am}	κ_2^{am}	κ_3^{am}	SC_p^0	SC_p^1	SC_p^2	SC_p^3	SC_c^3	$^0\chi$	$^1\chi$	$^2\chi$	$^3\chi_p$	$^3\chi_{cl}$	$^0\chi^{vm}$	$^1\chi^{vm}$	$^2\chi^{vm}$	$^3\chi_p^{vm}$	$^3\chi_{cl}^{vm}$
000	3	3	5	3.02	2.572	2.814	31	20	5.88	3.08	2.88	6	5	5	4	1	4.99	2.81	1.92	1.39	0.29	3.33	1.71	1.02	0.42	0.13
100	3	2	5	3.98	2.620	2.901	50	24	6.88	4.05	3.72	7	6	6	5	1	5.70	3.31	2.30	1.48	0.29	4.29	2.09	1.29	0.57	0.13
200	3	2	6	4.95	2.665	2.926	76	28	7.88	5.03	4.88	8	7	7	6	1	6.41	3.81	2.66	1.75	0.29	5.00	2.68	1.50	0.73	0.13
400	3	2	8	6.91	2.723	2.939	153	36	9.88	6.99	6.88	10	9	9	8	1	7.82	4.81	3.36	2.25	0.29	6.42	3.68	2.26	1.16	0.13
101	3	1	5	4.95	2.686	2.996	75	28	7.88	5.03	4.88	8	7	7	6	1	6.41	3.81	2.68	1.56	0.29	5.26	2.47	1.56	0.72	0.13
103i	3	1	6	5.58	2.915	3.193	143	38	9.88	5.65	6.88	10	9	10	8	2	7.98	4.66	3.87	2.02	0.70	6.83	3.45	2.49	0.98	0.37
104	3	1	8	7.89	2.788	3.033	202	40	10.88	7.98	7.78	11	10	10	9	1	8.53	5.31	3.74	2.33	0.29	7.38	4.06	2.54	1.31	0.13
104i	3	1	7	6.51	2.909	3.162	194	42	10.88	6.58	7.78	11	10	11	9	2	8.69	5.16	4.22	2.26	0.70	7.54	3.91	3.04	1.12	0.54
104t	3	1	6	4.65	3.173	3.444	180	46	10.88	4.70	7.78	11	10	13	9	5	8.91	4.96	4.99	2.17	1.85	7.76	3.76	3.53	1.07	1.24
202	3	1	7	6.91	2.792	3.066	149	36	9.88	6.99	6.88	9.88	9	9	8	1	7.82	4.81	3.39	2.10	0.29	6.67	3.64	1.97	1.03	0.13
3i03i	3	1	7	6.34	3.079	3.334	243	48	11.88	6.40	8.88	12	11	13	10	3	9.56	5.52	5.05	2.47	1.11	8.41	4.43	3.42	1.24	0.60
404	3	1	11	10.86	2.907	3.121	419	52	13.88	10.96	10.88	14	13	13	12	1	10.65	6.81	4.80	3.10	0.29	9.50	5.64	3.51	1.91	0.13
404t	3	1	9	7.15	3.152	3.380	388	58	13.88	7.21	10.88	14	13	16	12	5	11.03	6.46	6.05	2.94	1.85	9.88	5.35	4.51	1.66	1.24
111	3	0	5	5.93	2.907	3.263	102	32	8.88	6.01	4.39	9	8	8	8	1	7.11	4.35	2.85	1.97	0.20	6.22	2.85	1.77	1.04	0.12
114	3	0	8	8.88	2.974	3.260	250	44	11.88	8.97	7.32	12	11	11	11	1	9.23	5.85	3.91	2.74	0.20	8.34	4.44	2.74	1.63	0.12
114i	3	0	7	7.46	3.089	3.383	241	46	11.88	7.53	7.32	12	11	12	11	2	9.40	5.70	4.39	2.67	0.61	8.50	4.30	3.24	1.44	0.53
414	3	0	11	11.86	3.105	3.357	488	56	14.88	11.95	10.17	15	14	14	14	1	11.36	7.35	4.97	3.51	0.20	10.46	6.03	3.72	2.23	0.12
444	3	0	14	14.84	3.443	3.683	789	68	17.88	14.94	13.17	18	17	17	17	1	13.48	8.85	6.06	4.15	0.20	12.58	7.62	4.70	2.74	0.12
3F03F	9	1	7	6.05	3.136	3.615	557	72	15.46	6.26	12.46	16	15	21	14	9	12.82	7.10	8.01	3.25	3.41	7.94	4.07	2.91	1.15	0.49
3F13F	9	0	7	6.80	3.325	3.835	638	76	16.46	7.02	11.72	17	16	22	16	9	13.53	7.64	8.18	3.66	3.33	8.90	4.46	3.12	1.47	0.48

Definition of the DARC-PELCO model

The DARC-PELCO method is particularly suitable for studying families of compounds with a common chemical substructure, and is based on the exhaustive generation of all possible substitution sites around the reference structure (F_0) —corresponding to the glycerol skeleton common to all ethers— and the evaluation of their contribution to the property (Figure S1). The presence or absence of an atom grouping at a given position in the shell is coded by means of indicator variables (1 or 0). In the case of branched chains, in which two atom groupings are present at the same position, the indicator variables are added (for instance, 2 in the case of the methyl groups of an isopropyl group). Special variables have been used in the case of fluorinated derivatives, since different influence of trifluoromethyl and methyl groups at the same molecular position on the global toxicity of the compound is to be expected.

Different ways to define the shields around the core (F_0) are possible, and in some cases symmetry simplifications could reduce considerably presence–absence matrixes. In our structural definition we have incorporated the symmetry of the glycerol derivatives used, by assuming that the contributions of groups occupying equivalent positions (*i.e.* those linked to carbons 1 and 3 of the glycerol moiety) will display the same influence on the property under study.



The independent variable vector (Fo,A1,A2,B1,B2,FB2,C1,C2,D1,D2) for 104i would then be (1,0,2,0,1,0,0,2,0,0)

Figure S1.DARC-PELCO scheme used to describe the molecular structures of the glycerol-derived solvents.

Table S2.DARC-PELCO parameters of 20 glycerol-derived solvents.

Code	A_1	A_2	B_1	B_2	$^{F}B_2$	C_1	C_2	D_1	D_2
000	0	0	0	0	0	0	0	0	0
100	0	1	0	0	0	0	0	0	0
200	0	1	0	1	0	0	0	0	0
400	0	1	0	1	0	0	1	0	1
101	0	2	0	0	0	0	0	0	0
103i	0	2	0	2	0	0	0	0	0
104	0	2	0	1	0	0	1	0	1
104i	0	2	0	1	0	0	2	0	0
104t	0	2	0	3	0	0	0	0	0
202	0	2	0	2	0	0	0	0	0
3i03i	0	2	0	4	0	0	0	0	0
404	0	2	0	2	0	0	2	0	2
404t	0	2	0	4	0	0	1	0	1
111	1	2	0	0	0	0	0	0	0
114	1	2	0	1	0	0	1	0	1
114i	1	2	0	1	0	0	2	0	0
414	1	2	0	2	0	0	2	0	2
444	1	2	**1**	2	0	**1**	2	**1**	2
3F03F	0	2	0	0	2	0	0	0	0
3F13F	1	2	0	0	2	0	0	0	0

[a]Note that B_1. C_1 and D_1 columns are identical (and thus the data matrix is singular), because there is a single compound with a substituent at 2-position longer than methyl (444). As a consequence. the coefficient for D_1 in any regression model will account for the contribution of the whole butyl group. and not only for that of a single carbon at that position. This is not the case for D_2, since B_2 and C_2 columns are different from D_2, and thus the variables are linearly independent.

Table S3. Experimental EC_{50} values for *D. magna* expressed in mM units and calculated log P for the solvent set used.

Code	EC_{50} (mM)	logEC_{50}	log P[a]
000	746.921	2.873	–1.33
100	60.856	1.784	–0.97
200	53.916	1.732	–0.63
400	15.735	1.197	0.28
101	39.867	1.601	–0.60
103i	32.578	1.513	0.05
104	14.720	1.168	0.64
104i	12.174	1.085	0.62
104t	28.158	1.450	0.27
202	12.274	1.089	0.07
3i03i	12.311	1.090	0.71
404	1.214	0.084	1.88
404t	3.268	0.514	1.51
111	24.147	1.383	–0.24
114	9.174	0.963	1.00
114i	8.487	0.929	0.98
414	0.611	–0.214	2.24
444	0.052	–1.280	3.48
3F03F	1.874	0.273	1.35
3F13F	3.493	0.543	1.71

[a] Ghose A.K., Crippen, G.M., 1987. Atomic physicochemical parameters for three-dimensional-structure-directed quantitative structure-activity relationships. 2. Modeling dispersive and hydrophobic interactions. J. Chem. Inf. Comput. Sci. 27, 21-35.

5

Multiple Linear Regression (MLR)

It is often assumed that the relationship between structural parameters and experimental properties is well represented by a linear model:

$y = b_0 + b_1 x_1 + b_2 x_2 + ... + b_n x_n$ or

$\mathbf{Y} = \mathbf{X} \cdot \mathbf{B}$ (in matrix form)

In this regression equation the b_i are unknown coefficients, and the objective of regression analysis is to estimate their values. When X is of full rank the least squares solution is: $\mathbf{B} = (\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T\mathbf{Y}$, where **B** is the estimator vector for the regression coefficients. However, very often, not all these coefficients have statistical significance, so the final QSAR model should only keep those descriptors really contributing to the variation in the property observed. To this end we used a stepwise method for variable selection. In this way, independent variables x_i are entering and leaving in the regression equation, and only those having statistically significant coefficients ($p<0.05$) are finally kept in the final model fitting.

To establish the goodness of the model fitting, the determination coefficient (R^2) or, more often, its square root, the multiple correlation coefficient (R), are commonly used. The formula for the latter is the following:

$$R = \sqrt{\frac{\sum_i (\hat{y}_i - \bar{y})^2}{\sum_i (y_i - \bar{y})^2}} = \sqrt{\frac{SS_R}{SS_T}} = \sqrt{1 - \frac{SS_E}{SS_T}}$$

where SS_R is the Sum of Squares due to the Regression (i.e., the sum of the squares of the difference between the calculated y values, $\hat{y}_i$, and the mean y value, $\bar{y}$), SS_T is the Total Sum of Squares (i.e., the sum of the squares of the difference between the experimental y values, y_i, and the mean $\bar{y}$ value), and SS_E is the Sum of Squares due to the Error or Residual Sum of Squares (i.e., the sum of the squares of the difference between the experimental y values and the calculated y values).

However, for a model to be useful, not only has to fit well the experimental data, but also predict values for unknown data. To estimate the predictive ability of a regression equation the cross-validated R coefficients (R_{CV}) are often used. In our case, these values are determined by predicting the toxicity of a given derivative using the regression equation fitted with the remaining compounds, i.e., using the predicted residual sum of squares (PRESS) instead of the fitted residual sum of squares, SS_E:

$$R_{CV} = \sqrt{1 - \frac{PRESS}{SS_T}}$$

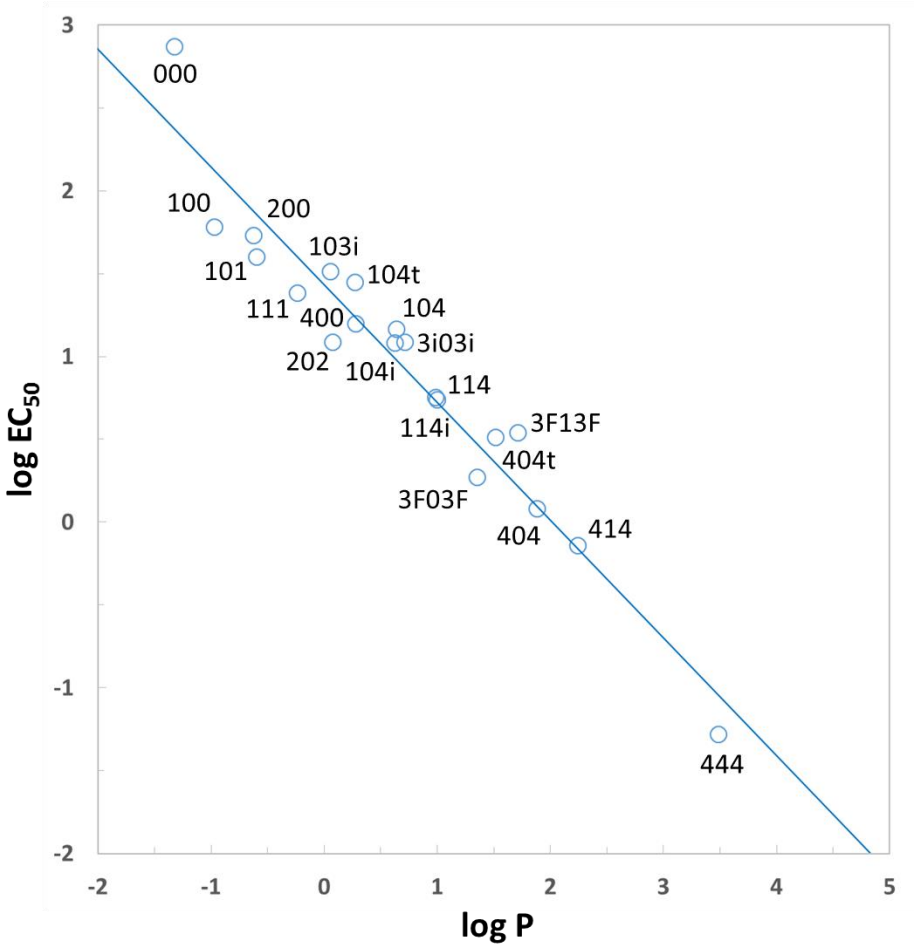


Figure S2. Plot of $\log EC_{50}$ (EC_{50} expressed in mM units) *vs.* log P.

$\log EC_{50} = 1.451\ (\pm 0.0062) - 0.710(\pm 0.0047) \cdot \log P$

N = 20. R = 0.96. R_{CV} = 0.95. σ(y) = 0.239
F = 230.0 ($F_{(1,18,\ 0,05)}$ = 4.4)

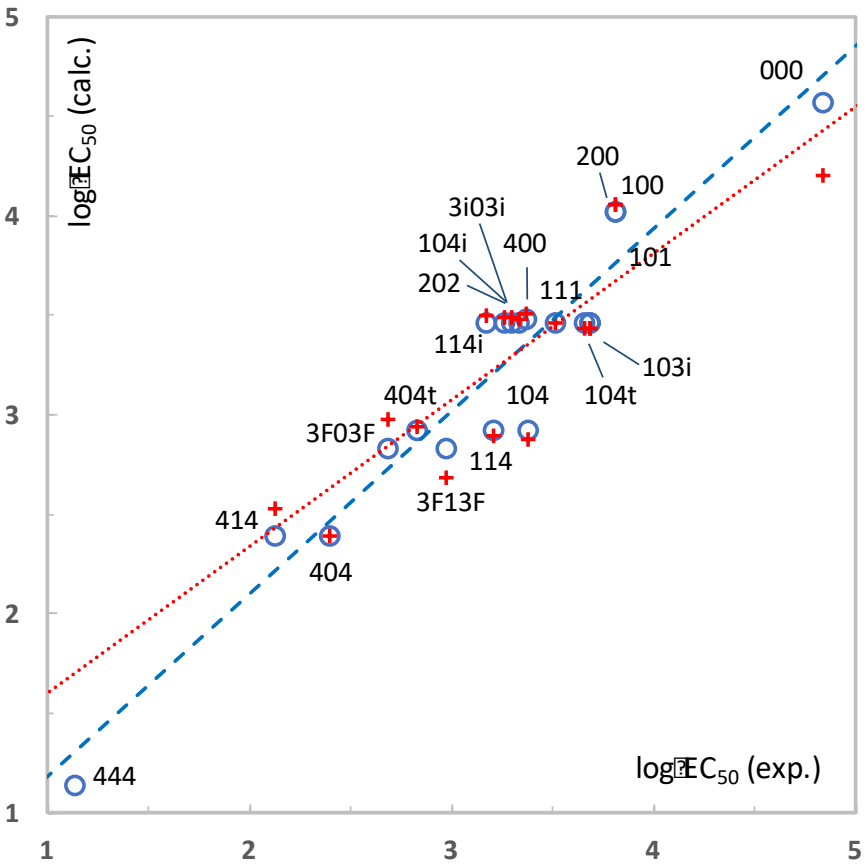


Figure S3. Plot of predicted *vs.* experimental values of $\log EC_{50}$ (EC_{50} expressed in mg L^{-1} units) as calculated through MLR analysis using the DARC-PELCO model. Circles represent the predictions made with the plain regression equation and crosses are the cross-validated predictions. Blue and red lines represent the least squares fit between both sets of data, respectively.

$\log EC_{50} = 4.573(\pm 0.185) - 0.537(\pm 0.086)\cdot D_2 - 0.555(\pm 0.105)\cdot A_2 - 1.253(\pm 0.0281)\cdot B_1 - 0.318(\pm 0.0095)\cdot {}^{F}B_2$

$\log EC_{50} = -0.542\cdot D_2 - 0.404\cdot A_2 - 0.371\cdot B_1 - 0.259\cdot {}^{F}B_2$ (Standardized coefficients)

$N = 20$, $R = 0.96$, $R_{CV} = 0.86$, $\sigma(y) = 0.242$

$F = 42.3$ ($F_{(4,15,\ 0,05)} = 3.1$)

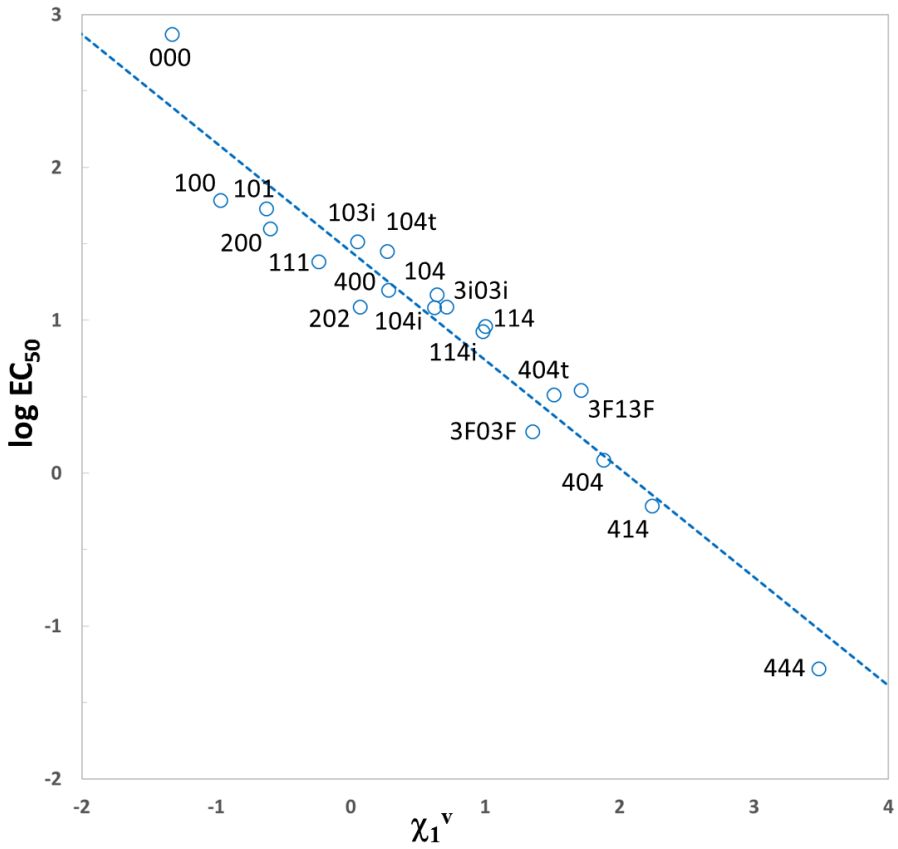


Figure S4. Plot of $logEC_{50}$ (EC_{50} expressed in mM units) *vs.* the topological index χ_1^{vm}

$logEC_{50} = 3.337(\pm0.205) - 0.582(\pm0.048)\cdot\chi_1^{vm}$

$logEC_{50} = -0.944\cdot\chi_1^{vm}$ Standardized coefficient

N = 20. R = 0.94. R_{CV} = 0.93. (y) = 0.294

F = 146.1 ($F_{(1,18,\ 0,05)}$ = 4.4)

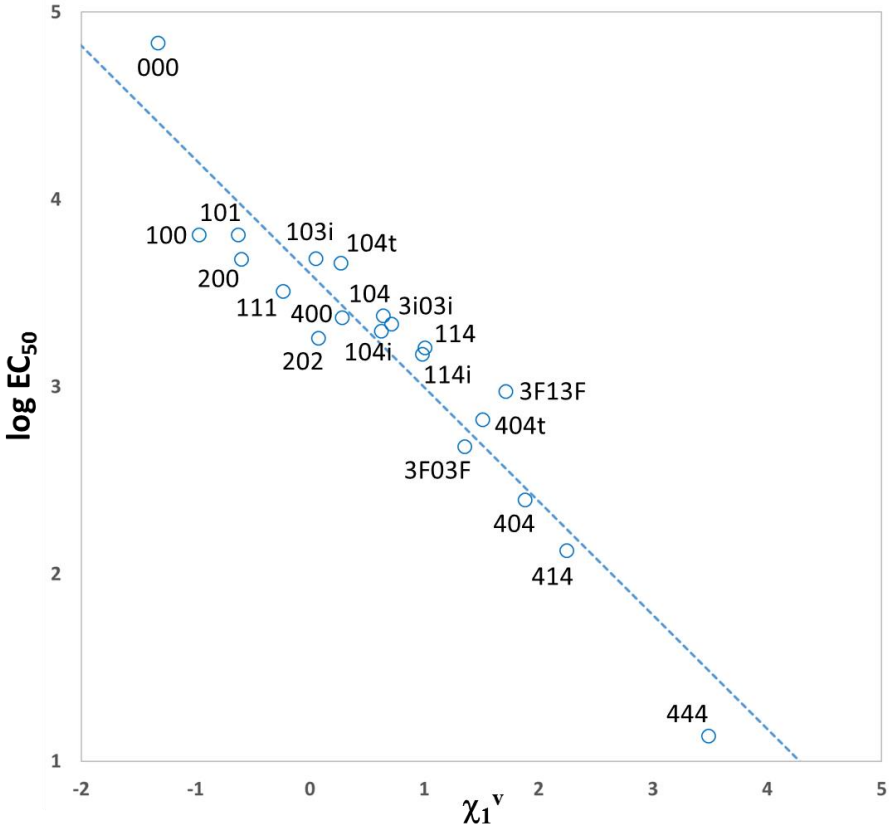


Figure S5. Plot of $logEC_{50}$ (EC_{50} expressed in mg L^{-1} units) *vs.* the topological index χ_1^{vm}

$logEC_{50} = 5.244(\pm 0.188) - 0.505(\pm 0.044\ \chi_1^{vm}$
$logEC_{50} = -0.938\ \chi_1^{vm}$ Standardized coefficient

N = 20. R = 0.94. R_{CV} = 0.85. σ(y) = 0.269
F = 131.1 ($F_{(1,18,\ 0,05)}$ = 4.4)

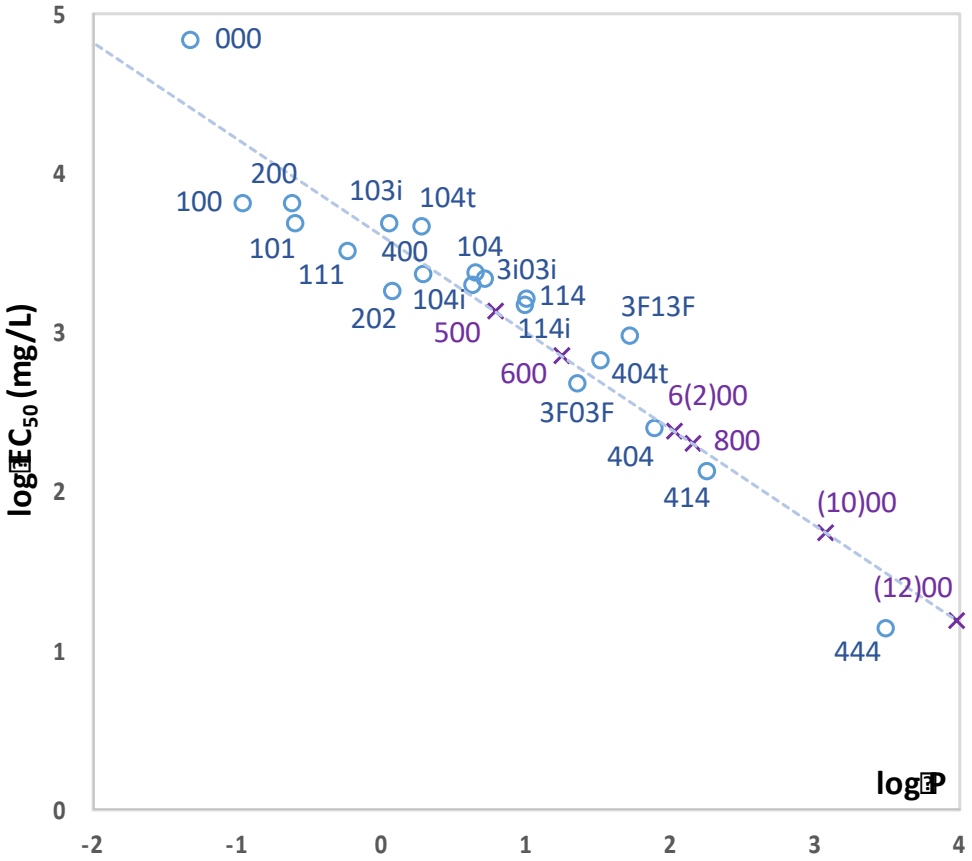


Figure S6. Plot of the log EC_{50} (original values in mg L^{-1}) *vs.* log P and the linear regression fitting line, including long alkyl ethers (×)

Solvent code	Log EC_{50}[a]	Log EC_{50}[b]
0	4.41	4.49
100	4.19	4.24
200	3.99	4.01
400	3.43	3.32
101	3.97	3.98
103i	3.57	3.51
104	3.22	3.05
104i	3.23	3.10
104t	3.44	3.36
202	3.56	3.50
3i03i	3.17	3.02
404	2.46	2.08
404t	2.69	2.43
111	3.75	3.72
114	3.00	2.77
114i	3.01	2.82
414	2.24	1.80
444	1.49	0.81
3F03F	2.78	2.76
3F13F	2.57	2.35
500	3.13	2.92
600	2.85	2.57
6(2)00	2.38	1.96
800	2.30	1.85
(10)00	1.74	1.12
(12)00	1.19	0.39

Table S4. Predicted log EC_{50} values of the longer alkyl chain glycerol ethers for *D. magna* using Eq. 2[a] and ECOSAR[b].

Artículo 4: Comparative ecotoxicology study of two neoteric solvents: Imidazolium ionic liquid vs. glycerol derivative.

Autores: Eduardo Perales[A], Cristina Belén García[A], Laura Lomba[A], Luis Aldea[A], José Ignacio García[B], Beatriz Giner[A].

[A]*Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, 50830, Zaragoza, España.*

[B]*Instituto de Síntesis Química y Catálisis Homogénea (ISQCH) y Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Zaragoza– Consejo Superior de Investigaciones Científicas-(CSIC), Calle Pedro Cerbuna 12, 50009 Zaragoza, España.*

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Áreas temáticas: Salud, toxicología y mutagénesis, contaminación; salud pública, medioambiental y ocupacional.

En este artículo se comparó la bondad medioambiental de dos disolventes considerados como verdes mediante metodología EHSA: el líquido iónico 1-butil-3-metilimidazolio hexafluorofosfato ([BMIM][PF_6]), y el derivado del glicerol 1,3-bis(2,2,2-trifluoro-etoxi)propan-2-ol (3F03F). Para ello, se obtuvieron los valores de EC/LC_{50} en *V. fischeri*, *D. magna* y en individuo adultos de *D. rerio*, y se utilizaron datos previos y estimados mediante el software Biowin y TEST.

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Comparative ecotoxicology study of two neoteric solvents: Imidazolium ionic liquid vs. glycerol derivative

Eduardo Perales [a], Cristina Belén García [a], Laura Lomba [a], Luis Aldea [a], José Ignacio García [b], Beatriz Giner [a,*]

[a] Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, 50830 Zaragoza, Spain
[b] Instituto de Síntesis Química y Catálisis Homogénea (ISQCH), Universidad de Zaragoza–CSIC, Calle Pedro Cerbuna, 12, 50009 Zaragoza, Spain

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ABSTRACT

In this study we have compared the acute ecotoxicity of two solvents, with very different structure and origin, but sharing many physical-chemical properties, so they can be used for similar purposes; a well-known ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) and a solvent partially derived from biomass, 3-bis(2,2,2-trifluoroethoxy)propan-2-ol (BTFIP). We have used three biomodels (*Vibrio fischeri*, *Daphnia magna* and *Danio rerio*) and performed the comparison applying the Environmental, Health and Safety (EHS) hazard assessment. According to the results, ecotoxicity of [BMIM][PF_6] and BTFIP is quite similar in the simplest model *Vibrio fischeri*, while in *Daphnia magna* [BMIM][PF_6] is clearly more toxic. However, in *Danio rerio*, toxicity of these chemicals is again quite similar and both can be classified as "nontoxic". The higher index value of [BMIM][PF_6] in water mediate effect in the EHS assessment indicates that this ionic liquid is more dangerous than BTFIP, although accumulation and degradation properties have not been taken into account. Further studies will be necessary to ascertain these conclusions.

1. Introduction

The search for new solvents, coming from new sources and/or able to provide special features (often known as neoteric solvents), is a field of growing interest, especially in connection with the possibility of using renewable raw materials to produce harmless solvents, more respectful with the environment than those derived from petroleum (the so-called green solvents). For many years, ionic liquids (IL) have been considered as the "solvents of the future" (Earle and Seddon, 2000), due to their very particular combination of physical-chemical features: high polarity, almost null volatility, immiscibility with low-polar organic solvents, and, in some cases, with water as well. As a consequence, there is a huge amount of studies describing the use of IL for numerous different applications, and many IL are nowadays available from commercial sources. 1-Butyl-3-methylimidazolium hexafluorophosphate (henceforth [BMIM][PF_6]) is one of the most prominent examples of successful IL. However, as more knowledge has being gained on the toxicological profiles of this family of compounds, it has become clearer that the label of "green solvents" is not deserved in many cases (Bubalo et al., 2014; Deetlefs and Seddon, 2010; Petkovic et al., 2011; Romero et al., 2008). For instance, in the case of [BMIM][PF_6] it has been reported that the hexafluorophosphate anion can decompose in aqueous acidic medium to lead to 1-butyl-3-methylimidazolium fluoride hydrate, and hence to the toxic product HF (Holbrey et al., 2003; Swatloski et al., 2003).

On the other hand, biomass-derived chemicals are attracting a great interest in the last years, in connection with the development of the biorefinery concept. Agricultural and some industrial activities are able to generate huge amounts of raw materials, capable of being used to produce commodity and fine chemicals. In this sense, glycerol is one of the platform molecules that has received much attention in the last years (Katryniok et al., 2011; Pagliaro et al., 2007; Zhou et al., 2008). Glycerol appears as a concomitant product in the production of biodiesel, amounting ca. 10% weight of the total output. At present, the world production of glycerol coming from vegetable oil transformations surpasses 2 million metric tons, so it constitutes a valuable starting point to obtain bio-based chemicals, useful as, for instance, solvents (Diaz-Álvarez et al., 2011; Diaz-Álvarez and Cadierno, 2013; García et al., 2014; Gu and Jerome, 2010). In this context, our research group has described the synthesis and application as solvents of a family of glycerol ethers (García et al., 2010). Some of these glycerol derivatives, namely those bearing fluoroalkyl chains, exhibited especial physical-chemical features, in some way similar to those displayed by some IL: high polarity, low vapour pressure at room

* Corresponding author.
E-mail address: bginer@usj.es (B. Giner).

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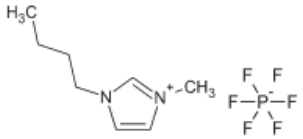
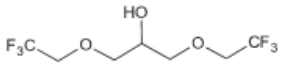
0147-6513/

Table 1.
Studied solvents and some of their relevant physical-chemical properties.

Property	Solvent	
Name	1-butyl-3-methylimidazolium hexafluorophosphate	1,3-bis(2,2,2-trifluoro-ethoxy)propan-2-ol
Code	[BMIM][PF_6]	BTFIP
Structure		
Molecular mass (g mol^{-1})	284.18	256.14
Density (g cm^{-3})	1.365[a]	1.384[b]
Refraction index	1.411[a]	1.352[b]
m.p. (°C)	−8 °C[a]	−5 °C
b.p. (°C)	> 350 °C	197 °C[b]
Vap. P at r.t. (mm Hg)	~0	0.4[b]
Viscosity at r.t. (cP)	312[a]	8.14[b]
Water solubility (wt./wt.)	0.0230[c]	0.0284[b]
Solvatochromic polarity parameters:		
E_T^N	0.64–0.69[d]	0.70[b]
π^*	0.89–1.04[d]	0.38[b]
α	0.63–0.68[d]	0.82[b]

[a] Carda-Broch et al. (2003).
[b] García et al. (2010).
[c] Chapeaux et al. (2007).
[d] Jessop et al. (2012).

temperature, an immiscibility both with hydrocarbons and with water. The most prominent example of these compounds is 1,3-bis (2,2,2-trifluoroethoxy)propan-2-ol, henceforth BTFIP, which can be efficiently prepared from trifluoroethanol and epichlorohydrin (a commodity produced from glycerol using the Solvay procedure). Table 1 gathers the comparison of some physical-chemical properties of [BMIM][PF_6] and BTFIP.

Both [BMIM][PF_6] and BTFIP have been used as solvents for biphasic enantioselective catalysis in two comparative studies carried out by our group. In the first one, the use of BTFIP in an enantioselective conjugate reduction catalysed by chiral azabis (oxazoline)–cobalt complexes showed to superior to that of [BMIM][PF_6], allowing better recovery of the catalytic phase and better enantioselectivities (90–96% ee vs. 40–85% ee with the IL (Aldea et al., 2010). The same situation arose in the second study, where the biphasic enantioselective Kharasch–Sosnovsky allylic oxidation, based on neoteric solvents and copper complexes of ditopic ligands, was studied (Aldea et al., 2012).

The question arises as to whether BTFIP can be considered an environmentally benign solvent or not, given the total lack of experimental evidences on its toxicity and ecotoxicity. We hypothesize that the ecotoxicity of this solvent, partially originating from biomass, is lower than the abovementioned ionic liquid. With the aim of verifying our hypothesis, the ecotoxicity of BTFIP and [BMIM][PF_6] has been obtained through the evaluation of the toxic effect in three bioindicators (bacteria, crustacean and fish) corresponding to several trophic levels.

In order to perform a comparative study, the studied solvents have been evaluated making use of Environmental, Health and Safety (EHS) hazard assessment. This method was firstly proposed by Koller et al. (2000) as an intermediate attempted to account for the problems of early design phases. Environmental, Health and Safety (EHS) aspects are assessed in several categories corresponding to environmental, health or safety related properties.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

1-Butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) was provided by Sigma-Aldrich (purity ≥ 97%). In order to minimizing the water content, [BMIM][PF_6] was periodically dried for 24 h under a vacuum of ca. 0.05 kPa with stirring and stored before use in a desiccator.

1,3-Bis(2,2,2-trifluoroethoxy)propan-2-ol (BTFIP) was synthesised by the following procedure:

In a round bottom flask were placed 1 mol of trifluoroethanol (100 g, aprox. 75 mL) and then 1 mol (140 g) of potassium carbonate. The flask was heated up at 70 °C, and 0.5 mol of epichlorohydrin (47 g) were then dropped into the flask. After 2 h the reaction was complete. Cooling down the flask, the mixture was filtered to remove the carbonate salt. The unreacted fluorinated alcohol was removed by heating under vacuum in a rotary evaporator. The remaining product was purified by vacuum distillation to yield 108 g of BTFIP (84%, GC purity > 99.5%).

Trend analysis and quantitative structure–activity relationship (QSAR) models were evaluated previously using the QSAR Toolbox, 2.3 (2009) which helped to select the concentrations to be tested. QSAR is based on the correlation between structural molecular characteristics of series of molecules and their chemical reactivity or biological activity. Additionally, a previous study was carried out to refine the range of concentrations and make sure the tested concentrations within EC_{50}/LC_{50}.

2.2. Ecotoxicological tests

2.2.1. Vibrio fischeri (V. fischeri) Inhibition of bioluminescence test

The lyophilized *V. fischeri* (strain NRRL-B-11177) used for Inhibition of bioluminescence test were purchased from Macherey-Nagel (ref. 945 006). This experiment was carried out according

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with the test conditions and the operating protocol of the *V. fischeri* acute toxicity test (UNE-EN-ISO 11348-3, 2007). Prior to testing, bacteria were rehydrated using the corresponding reactivation solution provided by the manufacturer. Afterwards, bacteria were stored at a temperature between 2 and 8 °C for 5 min

Several dilutions for each of the studied solvents were prepared using a 2% NaCl stock solution. The different concentrations range for these compounds have been between 500 and 5000 mg L^{-1} (500, 1000, 1250, 2250, 2500, 3000, 3750, 4000, 4500, 5000) for BMIM][PF_6] and 300–2500 mg L^{-1} (300, 475, 625, 950, 1250, 1900, 2500, 5000) for BTFIP. Additionally, negative and positive controls with zinc sulfate (2.2 mg/L) and phenol (42.5 mg/L) were tested (Jennings et al., 2001). The pH of the solutions was adjusted to 7–7.5 using either 0.1 M HCl or 0.1 M NaOH solutions.

Next, the initial luminescence of the bacteria was measured after transferring 0.5 mL of the reactivated bacterial suspension at 15 °C to cuvettes; then 0.5 mL of each dilution to be tested was added to the cuvette. The toxicity is reflected in the ratio of the decrease in bacterial light production to the remaining light. The luminescence was measured again after 30 min. The test was repeated twice.

Luminescence was measured with a Biofix® Lumi-10 luminometer (Macherey-Nagel) using the acute mode (Biotox B) with an ultra-fast single-photon counter detector covering the 380–660 nm spectral range. The sensitivity is 10 fmol ATP when using ATP bioluminescence assays CLS II (Roche Diagnostics GmbH, Mannheim Germany).

The percentage of bioluminescence inhibition (%I) is calculated from the initial and final bacterial light intensity. Details of the specific Biofix® method used can be found elsewhere (Lomba et al., 2014).

2.2.2. *Daphnia magna (D. magna) acute immobilization test*

The *D. magna* used in the acute immobilization test were purchased from Vidrafoc (ref. DM090812) and were stored at 4 °C. This experiment was carried out following the guidelines of the OECD 202 test conditions and operating protocol (OECD 202, 1984; OC SE TG 202, 2004).

Firstly, the medium for the eggs was prepared according to the specifications of the supplier. Then, the eggs were incubated for 72 h at 20–22 °C with 6000 lx in a TOXKIT model CH-0120D-AC/DC incubator (supplied by ECOTEST) and fed with Spirulina 2 h prior to starting the bioassay.

Several dilutions for the studied chemicals were prepared in aqueous medium solution. The different concentrations range for these compounds have been between 3 and 100 mg/L (3, 6, 10, 20, 25, 42.5, 75, 100) for [BMIM][PF_6] and between 30 and 1500 mg/L (30, 90, 250, 500, 750, 1000, 1250, 1500) for BTFIP. Furthermore, negative and positive controls with $K_2Cr_2O_7$ (0.6–2.1 mg/L) were also tested (OECD 202, 1984; OC SE TG 202, 2004). The pH of the solutions was adjusted to be between 7 and 7.5 using 0.1 M NaOH or 0.1 M HCl solutions.

A total of 20 daphnids aged < 24 h, were exposed to the studied chemicals in complete darkness for 24 h at 20–22 °C for each concentration tested. The organisms were divided into four groups of five organisms per group. Once again, the test was repeated twice.

The immobilization of the daphnids was measured taking into account that the organisms that were unable to swim for 15 s after gentle stirring were considered immobile.

2.2.3. *Danio rerio (D. rerio) acute toxicity test*

Fish acute toxicity experiments were performed in a laboratory (The Centre de Recerca i Innovació en Toxicología from the Universitat Politècnica de Catalunya in Spain) fulfilling the criteria of Good Laboratory Practice. They were conducted in accordance with specifications of OECD 203 (1992).

All of the toxicity tests were carried out at a temperature of 22 ± 2 °C in 1.5 L aquaria with dechlorinated drinking water. The number of fishes in each experimental and control group was 7. The light regimen was 16 h light/8 h diffuse light, oxygen concentration > 60% and pH = 8.3–8.5.

The acute toxicity tests of 96 h duration were run in a static exposure system (without renewing the test solution). Fish were exposed to eight and seven different concentrations of BTFIP and [BMIM][PF_6], respectively, according to OECD indications. They were not fed during the test period and their mortality and behavioral changes were recorded at 3, 24, 48, 72 and 96 h.

Results were validated and repetitions were not necessary. Validation criteria included the maintenance of constant assay conditions, mortality of control under 10% and diluted oxygen concentration at least 60% of air saturation value.

2.3. *Statistics and graphical representation*

Experimental results obtained have been fitted using the least squares method to the following function to obtain the corresponding EC_{50}/LC_{50} values and standard deviations (SD):

$$\%I = 100/(1 + 10^{(\log EC_{50} - \log c)a})$$

where %*I* denotes % bioluminescence inhibition for *V. fischeri*, % immobilization for *D. magna* and % death for *D. rerio*, *log* EC_{50} and *a* are the adjustable parameters.

The statistical analysis was performed using the SPSS 18.00 software (IBM® SPSS software). A threshold of p = 0.05 has been set to accept or reject the null hypothesis.

2.4. *EHS assessment*

EHS method includes a total of 11 effects corresponding to three categories: environment, health and safety. In this case, according to the nature of the study which aims to conduct an environmental assessment of the risks associated with the use of the two solvents under study, it has been decided to assess only the Environment category.

The assessment of the EHS aspects is divided into different effects. If these effects can be analysed in a similar way, they are combined to the so called dangerous properties. There are two values defined for each dangerous property: an index ($IndVal_{ij}$) and a physical value ($PhysVal_{m,j}$).

We have compared the environmental risk taking into account the effective dangerous property (EDP_{ij}) of each solvent (Eq. (1)).

$$EDP_{ij} = IndVal_{ij} + F_{ij} \quad (1)$$

where $IndVal_{ij}$ is defined as:

$$IndVal_{ij} = \max\ (IndVal_{ij,m}) \quad (2)$$

being $IndVal_{ij,m}$ the index value of the substance *j* defined for each the *i* dangerous properties of each of the *m* categories.

In this case, the selected dangerous properties for assessing the Environment category are water-mediated effects (LC_{50}/EC_{50} acute), degradation (half-life in environment) and accumulation (log k_{ow}).

F_{ij} is set to 0 for dangerous property accumulation and degradation while for water-mediated effects for organic substances is defined as follows:

$$F_{ij} = 0.2 x \log (PhysVal_{degradation,j} x PhysVal_{accumulation,j}) \quad (3)$$

Being $PhysVal_{m,j}$ the physical value.

From the original EHS approach (Koller et al., 2000), several

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Table 2.
Substance data used for ESH assessment and results.

	Water mediated effects			Accumulation		Degradation	
	Mean value EC_{50} (mg/L)	$IndVal_{ij}$	EDP_{ij}	Log k_{ow}	EDP_{ij}–$IndVal_{ij}$	Halflife (days)	EDP_{ij}–$IndVal_{ij}$
BTFIP	809	0.02	−0.44	1.424[a]	−0.79	21[b]	0.66
[BMIM][PF_6]	685	0.04	−1.39	−1.66[c]	−2.33	4[b]	0.30

[a] Garcia et al. (2010).
[b] Using Ultimate and Primary Biodegradation Models (Biowin 3 and 4) from EPIWEB 4.1 (US EPA, 2012) $IndVal_{ij}$ = − 0.109* ln(*Mean valueEC*$_{50}$)+0.75 for water mediate effects. $IndVal_{ij}$=0.5*(Log kow)−1.5 and $PhysVal$ = 0.001*$e^{(2.3026*Log\,kow)}$ for accumulation. $IndVal_{ij}$=0.2171* ln(*Halflife*) and $PhysVal$ = 0.01**Halflife* − 0.037 for degradation.
[c] Ropel et al. (2005).

Table 3.
Effect concentrations and lethal concentration in mg/L and their corresponding standard deviations.

	V. fischeri		*D. magna*		*D. rerio*	
	EC_{50}	SD	EC_{50}	SD	LC_{50}	SD
BTFIP	1597	2.375	477	6.978	353	0.773
[BMIM][PF_6]	1473	3.293	31	4.538	550	6.348

correlations between the experimental or calculated properties used for the evaluation and the index ($IndVal_{ij}$) and physical value $PhysVal_{m,j}$. The information needed to carry out the method is gathered in Table 2.

3. Results and discussion

3.1. Ecotoxicology tests

EC_{50}/LC_{50} values obtained in *V. fischeri*, *D. magna* and *D. rerio* with their respective standard deviations are gathered in Table 3. Furthermore, results are graphically represented in Figs. 1–3.

The proposed hypothesis has been partially verified; ecotoxicity of the [BMIM][PF_6] is higher compared to BTFIP except for *D. rerio*.

In the case of *V. fischeri*, EC_{50} obtained values are quite similar for both chemicals and, in general, none of them can be considered as toxic for the environment using this bioindicator (United Nations, 2011). However, the ionic liquid [BMIM][PF_6] is slightly more ecotoxic. Although the action mechanism is still unidentified, it is known that the bacterial bioluminescence reactions are coupled to the electron transport system in cellular respiration and are indicative of cellular metabolism (Onorati and Mecozzi, 2004). In that sense, lower bioluminescence implies decreased cellular respiration. Thus, [BMIM][PF_6] causes a slightly higher effect in the cellular respiration than BTFIP.

Both solvents affected the mobility of *D. magna*. Although, once again, the action mechanisms are not known yet, several authors have suggested that solvents could cause enzyme inhibition, disruption of membrane permeability, structural damage and oxidative stress (Bernot et al., 2005). In this case, the results obtained for *D. magna* indicate that the glycerol-derived solvent BTFIP is much less toxic than the ionic liquid [BMIM][PF_6]. Thus, it is possible to categorize [BMIM][PF_6] as belonging to the Category: Acute III and as "moderately toxic" whereas BTFIP can be classified as nontoxic or "practically harmless", according to the United Nations classification (United Nations, 2011) or Passino and Smith (Passino and Smith, 1987) classification respectively.

According to the lethal concentrations in *D. rerio*, both solvents are quite similar and would be classified as Category: Acute III by the United Nations classification (United Nations, 2011) or as "practically harmless" by Passino and Smith (Passino and Smith, 1987), although LC_{50} is higher in [BMIM][PF_6] than in BTFIP.

Nevertheless, fishes died during the first hour of exposition to concentrations of BTFIP higher than 2000 mg/L. Additionally, behavioral alterations of fish were observed during the assay, including immobility and periods of swimming on their back followed by erratic movements and death, when exposed to higher concentrations of BTFIP (at 500 mg/L and 750 mg/L concentrations). This result suggested some kind of alteration of the central nervous system. Normal behavior was only observed when fishes were exposed to concentrations of BTFIP lower than 250 mg/L.

No alterations of behavior of zebrafish were observed in expositions to [BMIM][PF_6].

Based on the EC_{50} and LC_{50} values calculated, toxicity of BTFIP is higher in *D. rerio* than in the other two biomodels. In contrast,

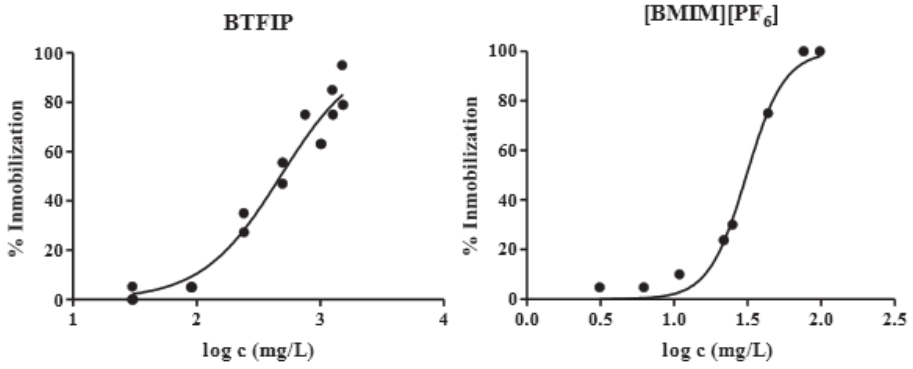

Fig. 1. Results for *D. magna*.

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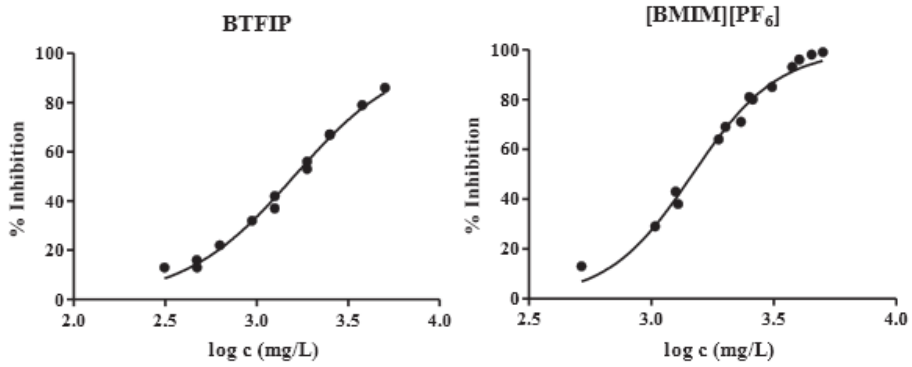

Fig. 2. Results for *V. fischeri*.

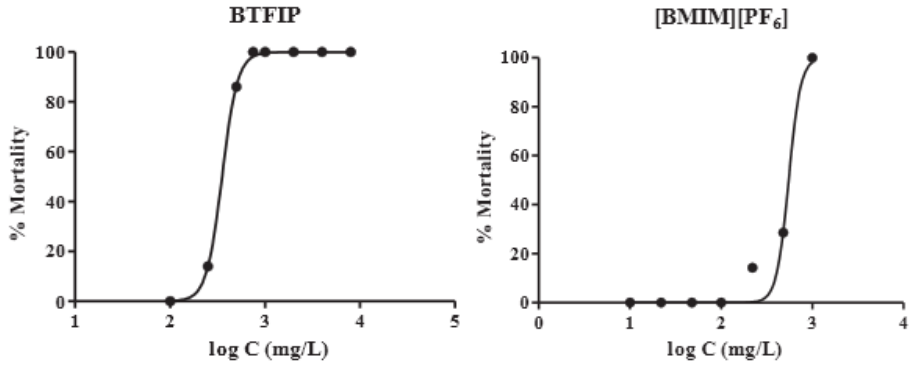

Fig. 3. Results for *D. rerio*.

the crustacean *D. magna* is the most sensitive organism to [BMIM][PF_6], followed by the fish *D. rerio* and finally by the bacteria *V. fischeri*. This clearly shows the substantial difference in the sensitivities of the different organisms studied.

It should be emphasized that single bioassays have limitations, so transfer or prediction of ecotoxicological data obtained with different biomodels is not always valid. It is hard to make predictions from a lower level of organization to higher ones (Lange et al., 2010). For this reason, it is necessary to include in the study organisms from different levels to allow a better understanding of possible effects of the studied chemicals in ecosystems.

3.2. ESH assessment

It is important to note that ecotoxicity data are not enough to completely assess the environmental risk of the studied compounds. The evaluation of other properties, important from the environmental point of view, such as bioavailability or biodegradability, provide a more accurate view of the associated risk, when combined with ecotoxicity data. ESH assessment takes into account these parameters.

According to this approach, an index value of value of 0 represents harmless substances whereas a value of 1 indicates dangerous substances regarding the considered ESH effect. It is worth mentioning, that the calculated indexes for accumulation are negative values. This means that none of the studied compounds show a risk from the viewpoint of mobility and bioaccumulation in biological systems, since the threshold value of partition coefficient that corresponds to the minimum index value is set to 3. In the case of the dangerous property water mediate effect, the higher index value is obtained for [BMIM][PF_6], indicating a more dangerous substance than BTFIP. However, taking into account accumulation and degradation dangerous properties, the ionic liquid [BMIM][PF_6] would be less dangerous.

When the index value water mediated effect is modified with the relevant fate index to obtain the effective dangerous property, BTFIP results more dangerous than the ionic liquid. The effective dangerous property is reduced if the substance is degradable or increased if the substance has an accumulation potential. In this case, although index value for water mediated effect is higher for [BMIM][PF_6], degradation rate of BTFIP modifies the index value significantly. However, it should be pointed out that experimental degradation data of the studied solvents are not available, so the information needed to perform the approach has been obtained from Biowin models (US EPA, 2012).

In addition to the physicochemical and ecotoxicological properties of the solvents, there are also other important factors affecting their greenness: uses and applications of the solvents, their lifecycle, production processes or removal rate in depuration processes.

4. Conclusions

This work provides experimental data on ecotoxicity potentials of BTFIP and [BMIM][PF_6], two solvents with very different

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structure and origin, but sharing many physical-chemical properties, so they can be used for similar purposes. Although raw data based only in the calculations of effect and lethal concentrations suggests that BTFIP (a solvent partially derived from biomass) is less harmful than [BMIM][PF_6] (an ionic liquid), specially for *D. magna* (since for the rest of biomodels, none of studied solvents can be considered toxic). This could justify the substitution of the ionic liquid by the glycerol derivative. However, the alteration of behavior of the vertebrate biomodel and the ESH assessment points to the fact that BTFIP could be more harmful than suspected for the environment, so further studies will be necessary to ascertain this point.

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DISCUSIÓN POR ARTÍCULOS

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Artículo 1: Comparative ecotoxicity study of glycerol biobased solvents.

Ecotoxicidad en biomodelos acuáticos

Todos los derivados del glicerol de este estudio presentaron toxicidad dependiente de la concentración. Los valores se encuentran expuestos en la Tabla 3 del capítulo de Resultados de esta tesis.

En *D. magna* y *C. reinhardtii*, sólo 444 fue considerado como "ligeramente tóxico", según la clasificación de Passino y Smith (Passino & Smith 1987), mientras que el resto de compuestos fueron considerados como "no tóxicos" o "prácticamente no tóxicos". En cambio, en *V. fischeri* y *D. rerio,* el compuesto 404 fue clasificado como "ligeramente tóxico", mientras que el resto se incluyeron en las categorías "prácticamente no tóxico" o "no tóxico", por la misma clasificación.

La tendencia general vista en estos compuestos es que su toxicidad está relacionada con la lipofilia. Ésta, a su vez, también tiene relación con la presencia y el mayor tamaño de cadenas de alquilo unidas a los grupos hidroxilo de la molécula base. De esta manera, la estructura de los derivados del glicerol se relacionó con los valores de log EC_{50} de los biomodelos utilizados. Fundamentalmente se ha visto esta correlación en los biomodelos *D. magna* y *C. reinhardtii*. Este tipo de mecanismo de ecotoxicidad en bioindicadores acuáticos se denomina "narcosis". Involucra interacciones inespecíficas no covalentes entre un compuesto orgánico sin grupos funcionales activos con las paredes celulares lipófilas del organismo. Como consecuencia, se alteran la estructura y la función de las membranas, causando el efecto tóxico (Levet et al. 2016; Gao et al. 2016; Zhang et al. 2010).

En cambio, en *D. rerio* y *V. fischeri* la relación entre ecotoxicidad y lipofilia no es tan evidente, principalmente en el pez cebra. En el biomodelo vertebrado, esta falta de correlación podría explicarse por el ser un organismo más complejo que los otros biomodelos. En el biomodelo bacteriano, una posible explicación sería por la presencia de un medio salino donde vive la bacteria bioluminiscente. Moléculas orgánicas neutrales, tales como los derivados del glicerol, podrían verse excluidas debido a las interacciones entre las moléculas de agua y los iones salinos. Este efecto se conoce como *salting out,* y provocaría una reducción de la solubilidad en los derivados del glicerol en agua y, por tanto, una disminución de la actividad química en los compuestos más lipofílicos (Wheeler et al. 2002).

Cabría destacar dos observaciones sobre la estructura y la ecotoxicidad en función del biomodelo utilizado. La primera sería en *D. magna* y *D. rerio*, ya que la toxicidad de los compuestos con dos sustituyentes etilo en las posiciones 1 y 3 es más alta que cuando sólo hay un sustituyente butilo. La segunda sería en *V. fischeri* y *D. rerio*, donde destaca la relevancia dela presencia de una cadena butilo en posición 2. En este caso, la toxicidad disminuye de manera destacable según aumenta el tamaño de la cadena de alquilo, invirtiendo la tendencia de las posiciones 1 y 3. No obstante, cuando sólo se añade un grupo metilo en esta posición, la toxicidad resulta ligeramente afectada.

Medio ambiente, salud y seguridad

Para poder estimar el daño potencial que podrían causar estos compuestos por emisiones al medio acuático y a la salud humana, se han usado las bases de la metodología EHSA (Koller et al. 2000). Este método se dirige al análisis de disolventes en reacciones industriales, valorando los posibles riesgos asociados a su uso a una

temperatura determinada. Para ello, se emplearon los valores de EC_{50} mostrados en este artículo y otros datos fisicoquímicos necesarios (García et al. 2010; US EPA 2016a; Khadzhibekov et al. 1985; Sambou 2005; US EPA 2016b).

- Movilidad del solvente durante su uso: Las propiedades fisicoquímicas seleccionadas fueron la volatilidad y el punto de ebullición, ya que informan sobre la probabilidad de generar nuevas fases y de ser liberadas a la atmósfera. Ninguno de estos compuestos se considerarían potencialmente peligrosos basándose en los datos de su presión de vapor y/o su punto de ebullición (US EPA 2016a).
- Riesgo de combustión o explosión: El riesgo de combustión o explosión fue analizado según el correspondiente punto de inflamabilidad. Según la metodología EHSA, todos estos solventes serían potencialmente peligrosos. Sin embargo, en comparación con otros disolventes orgánicos más utilizados, tales como dietiléter, etanol o 1-butanol, los disolventes derivados del glicerol tienen en general un punto de inflamabilidad mucho más bajo (Smallwood 1996).
- Toxicidad en humanos: Se seleccionó la LC_{50} en toxicidad oral como propiedad para valorar la posible toxicidad en humanos. Para estimar estos valores, se utilizó el software TEST (US EPA 2016b). Los bajos valores de LC_{50} indicaron el bajo riesgo que poseen estos compuestos derivados del glicerol por lo que serían poco tóxicos para el ser humano.

- Toxicidad medioambiental: Para establecer la ecotoxicidad en medio acuático, se realizó una media de las EC/LC_{50} de cada biomodelo estudiado. Según los valores obtenidos, sólo los compuestos 404 y 444 tendrían que ser objeto de tratamiento específico de depuración (US EPA 2016b).
- Degradación y bioconcentración: Se establecieron predicciones de la degradación medioambiental a través del software Biowin (US EPA 2016a). Según este simulador, sólo el compuesto 444 podría ser persistente en el medio ambiente, en comparación con los otros disolventes. Además, se evaluó su potencial de bioconcentración a través del suelo o de la cadena alimentaria, utilizando el modelo de Meylan (Meylan et al. 1999), incluido en el software Biowin (US EPA 2016a). De nuevo, sólo el compuesto 444 presentó cierta peligrosidad.

Recopilando la información obtenida, se puede concretar que aquellos gliceroles con cadenas de alquilo cortas (200 o 202), o con una sola cadena larga (400) parecen ser mejores candidatos como disolventes industriales verdes, según la metodología EHSA. Por otro lado, el compuesto 444 tiene varios índices negativos, tales como alta persistencia en el medio ambiente, toxicidad moderada en dos de los cuatro biomodelos utilizados (*D. magna* y *C. reinhardtii*), y un punto de inflamación relativamente bajo. De igual manera, el compuesto 404 mostró toxicidad moderada en los otros dos biomodelos empleados (*V. fischeri* y *D. rerio*), influyendo de manera negativa en la puntuación de toxicidad medioambiental.

Todas estas estimaciones necesitan ser confirmadas mediante más ensayos toxicológicos en diferentes compuestos, para poder verificar las relaciones entre

ecotoxicidad y lipofilia en los biomodelos *D. magna* y *C. reinhardtii*, y ver cuál podría ser la propiedad o factor que más influye en *V. fischeri* y *D. rerio*.

Artículo 2: Ecotoxicity studies of glycerol ethers in *Vibrio fischeri*: checking the environmental impact of glycerol-derived solvents.

Ecotoxicidad en *V. fischeri*

En este trabajo se evaluó la ecotoxicidad acuática en el biomodelo *V. fischeri* de veinte compuestos derivados del glicerol (éste inclusive) durante un periodo de exposición agudo (30 minutos). Los valores se muestran en la Tabla 3 del capítulo Resultados. Los ensayos realizados mostraron que sólo tres compuestos, 404, 404t y 414 serían considerados como "ligeramente tóxicos" según la clasificación de Passino y Smith (Passino & Smith 1987); el resto se encontraban dentro de los rangos "prácticamente no tóxicos" o "no tóxicos".

Este ensayo de ecotoxicidad se basa en relacionar la inhibición de la emisión de luz con las concentraciones de tóxico a las que se expone la bacteria, para hallar así su EC_{50}. Aunque se desconoce el mecanismo de toxicidad de este tipo de compuestos en *V. fischeri*, es sabido que las reacciones de bioluminiscencia bacteriana tienen relación con el sistema de transporte de electrones en la respiración celular. No obstante, cualquier efecto dañino que modifique la utilización de oxígeno produciría una disminución en la bioluminiscencia (Onorati & Mecozzi 2004).

En general, esta inhibición y, por tanto, la ecotoxicidad en este biomodelo, aumentó a mayor longitud de las cadenas de alquilo en las posiciones 1 y 3 del glicerol. Por el contrario, la presencia de una cadena de alquilo en la posición 2 de la estructura molecular hizo disminuir la ecotoxicidad, y este descenso es más pronunciado cuánto más grande sea la cadena. Finalmente, las cadenas de alquilo ramificadas (*iso* o *tert*) mostraron un aumento de la toxicidad, en comparación con las cadenas de alquilo lineales.

Para valorar la toxicidad global de los derivados del glicerol, se realizó una comparación de los valores de EC_{50} en exposición a 30 minutos con otros disolventes orgánicos y líquidos iónicos. Como se puede ver en la Tabla 5, la ecotoxicidad de la mayoría de los compuestos estudiados es menor que algunos disolventes más tradicionales, tales como el benzeno o tolueno. Sólo los valores de EC_{50} de 404, 404t y 414 son similares a los compuestos más tóxicos, tales como o-xileno y fenol. Por otro lado, se puede ver en la comparación con líquidos iónicos como los derivados del glicerol son menos tóxicos para este biomodelo de manera general.

Disolventes convencionales	EC_{50} (mg L^{-1}) *V. fischeri*
Metanol	101068
Propan-2-ol	35383
Etanol	23089
Acetonitrilo	21172
Acetona	19311
Diclorometano	2532
Cloroformo	1199
Etilenglicol	621
Benzeno	108
Tolueno	32
Fenol	31
O-xileno	9
Líquidos iónicos	
1-Metil-3-propilimidazolio tetrafluoroborato	1850
1-Pentil-3-etilimidazolio tetrafluoroborato	350
1-Butil-3-etilimidazolio tetrafluoroborato	151
1-Butilpiridinio dicinamida	98
1-Heptil-3-metilimidazolio tetrafluoroborato	74
1-Etil-3-hexilimidazolio tetrafluoroborato	38
1-Metil-3-octilimidazolio tetrafluoroborato	7
1-Hexil-3-metilpiridinio bromidio	2

Tabla 5: Valores de EC_{50} para diferentes disolventes tradicionales en el ensayo de *V. fischeri* durante 30 minutos de exposición.

Relaciones estructura-actividad cuantiativas (QSAR)

Se aplicaron varios análisis QSAR según la estructura molecular y la lipofilia de los compuestos derivados del glicerol, para relacionarlos con sus valores de EC_{50} obtenidos experimentalmente.

En el análisis estructural, se usaron dos metodologías diferentes: parámetros DARC-PELCO y parámetros topológicos. Ambos se basan en establecer una matriz con las propiedades estructurales de cada compuesto, y así poder determinar una relación entre ellas y sus efectos en el medio ambiente acuático mediante ecuaciones.

Análisis con parámetros DARC-PELCO

En el caso del análisis DARC-PELCO, la función obtenida fue la siguiente:

$$Log\ EC_{50} = 4.828\ (\pm 0.320) + 1.064\ (\pm 0.320) B_1 - 0.614\ (\pm 0.192)\ A_2 - 0.310\ (\pm 0.0082) B_2 - 0.685\ (\pm 0.123) C_2 \quad (1)$$

N= 20; R= 0.93; R_{cv} =0.83; σ(y) = 0.418; F=22.6. ($F_{(4,15,\ 0.05)}$ = 3.1)

El modelo se ajusta a los datos experimentales de toxicidad (86% según el coeficiente de determinación R^2), siendo el ratio de observaciones/parámetros ajustables =5. El error estándar del modelo (0.42) es cercano al valor experimental (0.54), lo que verifica la idoneidad del QSAR.

Esta función se comprobó mediante un método de validación cruzada, siendo el compuesto 444 el único que no podía ser predicho adecuadamente por la Función1. Esto es debido a que el coeficiente B_1 no puede ser calculado sin su correspondiente valor de EC_{50} del compuesto 444. No obstante, con los restantes diecinueve derivados de glicerol se comprobó que la R_{CV} fue de 0.83, siendo ésta una respuesta válida para explicar una posible relación entre la estructura y la ecotoxicidad.

Los coeficientes tipificados de cada parámetro fueron, en orden de relevancia, C_2 (-0.600), B_2 (-0.399), A_2 (-0.342) y B_1 (0.240). El signo negativo indica que el log EC_{50} disminuye cuando hay presencia de átomos en la cadena de alquilo; en este caso, en las posiciones C_2, B_2 y A_2, que se pueden apreciar en la Figura 16. Por tanto, el aumento de las cadenas de alquilo en las posiciones 1 y 3 de la estructura base del glicerol implica un aumento de la toxicidad en el bioindicador *V. fischeri*. De la misma manera, al ser positivo el coeficiente de variación indica que la presencia de una cadena de metilo en la posición 2 disminuye los valores de log EC_{50}.

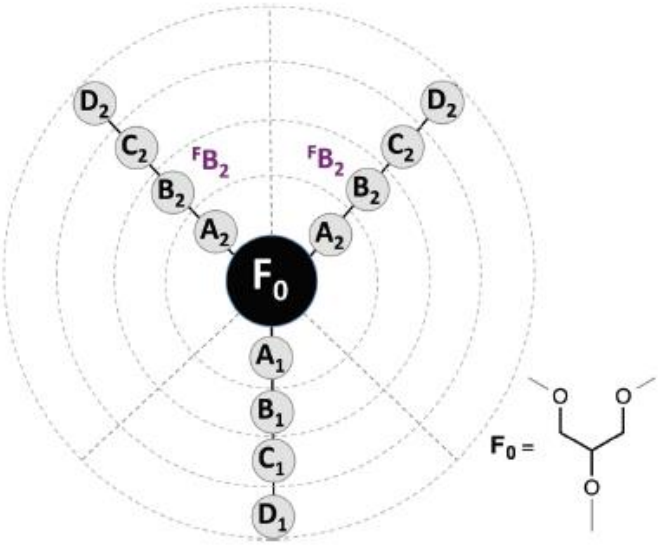

Figura 16. : Esquema DARC-PELCO usado para describir la estructura molecular de los disolventes derivados del glicerol.

Análisis con parámetros topológicos

Se aplicó un análisis de los parámetros topológicos usándolos como variables estructurales. La función obtenida fue la siguiente:

$$\text{Log } EC_{50} = \text{-7.715 } (\pm 2.710) \text{-1.455 } (\pm 0.200)\chi_2^{vm} + 4.779\ (\pm 1.023)\text{Bal}^{JX}$$

$$+0.553\ (\pm 0.180)\text{HBD} \qquad (2)$$

N= 20, r, 0.91, R_{CV} =0.85, σ(y) = 0.436, F = 27.0 ($F_{(3.16,\ 0.05)}$=3.2)

Se obtuvo un buen ajuste (84 % de la varianza experimental), el cual se mantiene en el análisis de validación cruzada (R_{cv}=0.85). Como en el anterior modelo QSAR, el compuesto 444 muestra una elevada desviación, lo cual posiblemente esté relacionado con ser el único compuesto con un grupo butilo en la posición 2.

La interpretación de los resultados en este QSAR es más compleja, debido a la naturaleza de los parámetros descriptores. Por un lado, el coeficiente positivo de dador de átomos de hidrógeno (HBD) refleja el hecho de que cuantos más grupos hidroxilo libres haya, la toxicidad es menor. Por otro lado, los otros dos índices topológicos, índice de Balaban (Bal^{JX}) e índice de conectividad (χ_2^{vm}) se relacionan principalmente con el tamaño molecular, los cuales tienen en cuenta correcciones a las electronegatividades atómicas y a la valencia. Estos ajustes afectan principalmente a los compuestos fluorinados, por lo que tienen que ser tenidos en cuenta a la hora de establecer el modelo. Según los coeficientes estandarizados de ambos parámetros (1.157) en Bal^{JX}, que indica la influencia de la distancia existente entre los átomos que forman una molécula en 2D y en χ_2^{vm}, que refleja la conectividad entre los átomos (-

1.513), puede verse que el incremento del tamaño molecular provoca un aumento de la toxicidad.

Análisis con propiedades fisicoquímicas: lipofilia

Se realizó una correlación entre los valores de EC_{50} en *V. fischeri* con la hidrofobicidad de los compuestos, estimada a partir del logaritmo del coeficiente de reparto octanol/agua (log P). Se comprobó que había una desviación de la progresión lineal en 3 compuestos, el 444, el 3F03F y el 3F13F, indicando que la hidrofobia no explicaba por si sola la toxicidad encontrada. No obstante, no es extraño que haya otros factores que influyan en la ecotoxicidad de un biomodelo, dando como resultado una relación no estrictamente lineal con la lipofilia. Otras propiedades que pueden afectar son la capacidad de adsorción y la tasa de biodregradación, que pueden influir a la biodisponibilidad de la sustancia y, consecuentemente, a la toxicidad (Buffle & Hermens 2009; Hendriks & Heikens 2001; Hendriks et al. 2001; McCarty 2012).

Dejando aparte estos compuestos, la función obtenida fue la siguiente:

$$Log\ EC_{50} = 3.243\ (\pm 0.112) - 0.992\ (\pm 0.110) log\ P \qquad (3)$$

N= 17; R = 0.92; R_{CV}= 0.88; σ(y) = 0.429; F = 81.0 ($F_{(1.16,\ 0.05)}$= 4.5)

A pesar de la buena relación lineal que hay entre log P y el log EC_{50} en *V. fischeri*, es complicado establecer una relación directa de causalidad, debido a las relaciones cruzadas que existen entre diferentes propiedades moleculares. Por ejemplo, la lipofilia tiene fuertes correlaciones con otras propiedades relacionadas con el tamaño de la molécula, tales como el volumen molar (0.984), o la superficie accesible al solvente (0.977).

Artículo 3: Ecotoxicity and QSAR studies of glycerol ethers in *Daphnia magna*

Ecotoxicidad en *D. magna*

En este artículo se valoró la toxicidad acuática del glicerol y de diecinueve derivados, modificados mediante la adición de cadenas de alquilo en sus extremos hidroxilo. También se incluyeron compuestos con cadenas de alquilo fluorinadas. La evaluación ecotoxicológica consistió en una exposición aguda (24 horas) del biomodelo *D. magna* a estos compuestos, considerando como *endpoint* la inmovilización ante la exposición al tóxico, para obtener el valor de EC_{50} correspondiente. En la Tabla 3 del capítulo de Resultados se pueden ver los valores de los compuestos estudiados.

De todos los derivados del glicerol, sólo el 444 puede ser considerado como "ligeramente tóxico" según la clasificación de Passino y Smith (Passino & Smith 1987), debido a que su valor de EC_{50} se encuentra entre 10 y 100 mg L^{-1}. Los diecinueve compuestos restantes podrían ser denominados como "prácticamente no tóxicos" o "no tóxicos", según la misma clasificación. En la Tabla 6 se puede ver la comparación con otros disolventes tradicionales y con líquidos iónicos, pensados para ser usados como posibles disolventes verdes.

Disolventes tradicionales	EC_{50} (mg L^{-1})
Tolueno	7
Diclorometano	223
Cloroformo	573
1,4-Dioxano	8450
Etanol	9847
Isopropanol	9959
Acetonitrilo	10076
Acetona	13615
Metanol	22682
Líquidos iónicos	
1-Octil-3-metilimidazolio cloruro	0.8
1-Octil-3-metilimidazolio tetrafluoroborato	1.3
1-Hexil- metilimidazolio cloruro	2.5
1-Hexil-3- metilimidazolio tetrafluoroborato	3.4
1-Butil-3- metilimidazolio cloruro	12.4
1-Butil-3- metilimidazolio bromuro	13.2
1-Butil-3- metilimidazolio tetrafluoroborato	13.9
1-Butil-3 metilimidazolio hexafluorofosfato	25.3

Tabla 6: Valores de EC_{50} para diferentes disolventes tradicionales en el ensayo de *D. magna* durante 24 horas de exposición.

En comparación con otros disolventes más utilizados, sólo el 444 estaría a un nivel de toxicidad comparable a la toxicidad del tolueno en *D. magna*. En líquidos iónicos, los disolventes derivados del glicerol muestran menor toxicidad en el mismo biomodelo, salvo de nuevo para el compuesto 444. Aun así, el valor de EC_{50} de este disolvente es mayor que, por ejemplo, 1-octil-3-metilimidazolio cloruro o tetrafluoroborato.

Por otro lado, también se estableció una relación entre los valores de EC_{50} en *V. fischeri* y *D. magna*. No hay correlación entre ellos (r = 0.66), ya que existen diferencias entre valores en los compuestos "extremos", tales como el 000 (glicerol) y el 444. Además, existen también diferencias en los disolventes fluorinados y los que llevan 2 cadenas de butilos en los extremos exteriores de la molécula. No obstante, la mayoría de los disolventes siguen un patrón similar para ambos bioindicadores.

Relaciones estructura-actividad cuantitativas (QSAR)

Análisis con propiedades fisicoquímicas: Lipofilia

Según los resultados obtenidos, la ecotoxicidad de estos compuestos en *D. magna* aumenta con el número y tamaño de los grupos alquilo unidos a través de los grupos hidroxilo. Como consecuencia el log P, indicativo de la lipofilia, se incrementa. Para establecer una correlación entre los valores de EC_{50} obtenidos en *D. magna* con el log P, se realizó una regresión lineal. La función de la recta se presenta a continuación:

$$LogEC_{50} = 3.604\ (\pm 0.065) - 0.607(\pm 0.050) logP \qquad (4)$$

N = 20; R = 0.95; R_{CV} = 0.92; σ(y) = 0.254; F = 149.3 ($F_{(1,18,\ 0.05)}$ = 4.4)

Según los coeficientes de la Función 4 se confirma la tendencia de que, a mayor lipofilia, mayor ecotoxicidad (menor log EC_{50}) en este biomodelo. No obstante, existe una excepción en los compuestos fluorinados, donde se observa una inversión en esta tendencia, siendo el valor de EC_{50} del compuesto 3F03F más bajo que el de 3F13F. También se destaca que el tamaño de las cadenas ramificadas no explica la leve inversión de la tendencia; no obstante, la modificación en el log P es mínima. Por último, en las moléculas que contienen un sustituyente *tert*, la lipofilia se correlaciona con la ecotoxicidad, ya que el cambio en el log P es más pronunciado.

Análisis con parámetros DARC-PELCO

Al igual que se hizo con el tratamiento en el estudio de *V. fischeri*, se estableció un análisis QSAR mediante los parámetros DARC-PELCO, para determinar la influencia de los diferentes sustituyentes alquilos añadidos a la molécula de glicerol sobre la ecotoxicidad aguda en el biomodelo *D. magna.*

La función obtenida considerando los veinte compuestos fue la siguiente:

$$Log\ EC_{50} = 2.613\ (\pm 0.203) - 0.612\ (\pm 0.095) D_2 - 0.665\ (\pm 0.115) A_2 - 1.338(\pm 0.0308) D_1 - 0.438(\pm 0.104)\ {}^{F}B_2 \quad (5)$$

N = 20; R = 0.96; R_{cv} = 0.89; σ(y) = 0.266; F = 46.5 ($F_{(4.15,\ 0.05)}$=3.1)

Este modelo representa bastante bien la relación entre las propiedades estructurales de los derivados del glicerol en *D. magna* con su ecotoxicidad. Como comprobación adicional, se hizo un estudio de validación cruzada. Sin embargo, hay que tener en cuenta que uno de los disolventes, el 444, no puede ser incluido en este procedimiento, ya que es el único de todo el conjunto que tiene un grupo butilo unido al oxígeno central de la molécula. Si este compuesto se deja fuera del análisis de regresión, ningún coeficiente B_1-D_1 puede ser calculado, ya que todos los correspondientesvalores en la matriz DARC-PELCO son igual a 0. De hecho, si nos fijamos en la Función 5, se puede ver la relevancia que tiene el coeficiente D_1. Esto es debido a que representa la suma de los hipotéticos átomos de carbono en las posiciones B_1, C_1 y D_1, lo cual explicaría su alto valor. Teniendo en cuenta esta información, en futuras estimaciones con esta metodología se tendría que valorar la relevancia de esta posición con este biomodelo, incluyendo derivados del glicerol con cadenas mayores que metilos unidos al oxígeno central. A pesar de que la función no fuera del todo representativa para

derivados del glicerol con grupos alquilos de mayor tamaño en la posición 2, se obtuvo un valor de R_{cv} = 0.89 en la validación cruzada, siendo un resultado bastante ajustado para determinar una relación entre la estructura y la ecotoxicidad.

Todos los coeficientes fueron negativos en la función, indicado que cualquier adición de grupos alquilo, incluyendo fluoruros, derivaría en una disminución del log EC_{50}. Dicha adición coincide con el aumento en log P, lo cual también coincide con el signo del coeficiente de la lipofilia en la Función 4.

Análisis con parámetros topológicos

Se realizó una comparación de los valores de log EC_{50} en *D. magna* con los valores topológicos de cada compuesto, los cuales describen el tamaño molecular, la forma y la flexibilidad de la molécula mediante índices matriciales. Se puede ver en la siguiente función.

$$Log\ EC_{50} = 3.337(\pm 0.205) - 0.582(\pm 0.048)\chi_1^{vm} \quad (6)$$

N= 20; R = 0.94; R_{cv} = 0.93; σ(y) =0.294; F =146.1 ($F_{(1.18,\ 0.05)}$= 4.4)

Se destaca que, a pesar de la variedad de parámetros topológicos utilizados, sólo el índice χ_1^{vm} está fuertemente relacionado con el log EC_{50}. Se obtuvo un buen ajuste, validado con el análisis de validación cruzada (varianza del 86 %). Debido a que el parámetro χ_1^{vm} tiene una concordancia con el tamaño molecular, el coeficiente negativo puede ser interpretado como un incremento en la toxicidad con el aumento de tamaño del derivado del glicerol, lo cual se confirma con los resultados obtenidos en la función QSAR con parámetros DARC-PELCO.

Finalmente, se incluyó un ensayo QSAR adicional con otro descriptor relacionado con el tamaño que ocupan los derivados del glicerol, el volumen molecular (V_{mol}):

$$Log\ EC_{50} = 3.693\ (\pm 0.229) - 0.016\ (\pm 0.001) V_{mol} \quad (7)$$

N = 20; R =0.95; R_{CV} = 0.93; σ(y) = 0.289; F = 152.2 ($F_{(1.18,\ 0.05)}$= 4.4)

Esta función confirma que existe una relación entre el tamaño molecular y la ecotoxicidad en *D. magna* en estos compuesos. No obstante, hay que tener en cuenta que se descubrieron correlaciones entre la lipofilia, la suma de los valores de los descriptores DARC-PELCO (Par. DARC-PELCO), y el tamaño y volumen molecular, lo cual se muestra en la Tabla 7.

	Log P	Par. DARC-PELCO	χ_1^v
Log P	-		
Par. DARC-PELCO	0.942	-	
χ_1^{vm}	0.975	0.985	-
V_{mol}	0.984	0.971	0.978

Tabla 7: Correlación entre los descriptores moleculares.

Por ello, establecer una relación directa entre la ecotoxicidad y cualquier parámetro no es tan evidente, aunque la lipofilia se establece como la más plausible (Levet et al. 2016).

Validez predictiva del modelo en éteres con mayores sustituyentes.

En este estudio se ha comprobado que un aumento de la longitud de las cadenas de alquilo deriva a su vez en un aumento de la lipofilia de los compuestos, relacionándolo con un incremento de su ecotoxicidad, según sus correspondientes valores de EC_{50} hallados en *D. magna*.

Para estimar el valor de otros derivados no evaluados en laboratorio con mayores cadenas de alquilo, se utilizó la Función 4. Para ello, se incluyeron los siguientes derivados del glicerol: 3-pentiloxipropano-1,2-diol (500), 3-hexiloxipropano-1,2-diol (600), 3-(2-etil)hexiloxiproano-1,2-diol 6(2)00, 3-octiloxipropano-1,2-diol (800), 3-deciloxipropano-1,2-diol (10)00, y 3-dodeciloxipropano-1,2-diol (12)00.

En función de los resultados, tal como se esperaba, a mayor longitud de la cadena, mayor toxicidad, manteniendo la relación con la lipofilia. El compuesto (12)00 sería considerado como "moderadamente tóxico", según la clasificación de Passino y Smith (Passino & Smith 1987), mientras que 6(2)00, 800 y (10)00 serían clasificados como "ligeramente tóxicos". Sólo 500 y 600 serían incluidos dentro del rango de "prácticamente no tóxicos".

Para verificar estos resultados, se comparó el modelo establecido en este trabajo con el modelo *ECOlogical Structure-Activity Relationship Model* (ECOSAR, versión 1.11), perteneciente al *software* EPI Suite (versión 4.1) (US EPA 2016a). Éste se utiliza para estimar valores de EC_{50} en diversos biomodelos acuáticos, incluyendo *D. magna*, en exposición aguda. La correlación entre ambos modelos de estimación se muestra en la Figura 17, considerándose muy buena (r=0.99). Por tanto, se verificó la calidad de nuestro modelo para predecir la toxicidad de los disolventes derivados del glicerol.

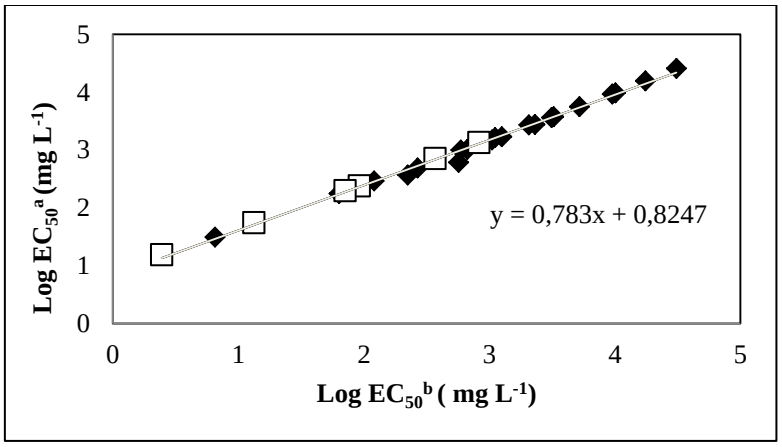

Figura 17: Gráfica de correlación de las EC50 calculadas en este manuscrito (Función 4)[a] y ECOSAR[b]. Rombos: Valores EC_{50} calculados de disolventes experimentales. Cuadrados: Valores EC_{50} estimados.

Artículo 4: Comparative ecotoxicity study of two neoteric solvents: Imidazolium ionic liquid vs. glycerol derivative

Resultados en ecotoxicidad

Se compararon los valores de EC_{50} de *V. fischeri* y de *D. magna,* y los de LC_{50} en *D. rerio*, durante exposición aguda en el compuesto derivado del glicerol 3F03F (mostrado en el artículo como BTFIP) y en el líquido iónico [BMIM][PF_6]. En la Tabla 3 del capítulo Resultados se pueden ver estos valores. En ellos se puede ver como los valores de EC_{50} en *D. magna* y *V. fischeri* son superiores en el compuesto 3F03F, principalmente en el primer biomodelo. No obstante, en *D. rerio* ocurre el efecto contrario.

Según los resultados obtenidos, la ecotoxicidad del compuesto [BMIM][PF_6] es mayor en los biomodelos estudiados, a excepción del pez cebra. En este bioindicador, se detectaron alteraciones en los comportamientos de los peces expuestos en el compuesto 3F03F, mientras que no se registraron este tipo de alteraciones en el comportamiento de los biomodeos vertebrados expuestos al líquido iónico. Concretamente, en los peces expuestos al derivado de glicerol hubo periodos de inactividad y de movimientos erráticos, a partir de concentraciones de 500 mg L^{-1} y 750 mg L^{-1}. Este resultado sugiere alguna clase de efecto tóxico en el sistema nervioso central. Sin embargo, estas concentraciones no son halladas habitualmente en un medio acuático por ser excesivas.

En general, estos compuestos se podrían considerar como "prácticamente no tóxicos" o "no tóxicos" para todos los biomodelos según la clasificación de Passino y Smith (Passino & Smith 1987), o fuera del rango evaluable según la clasificación del SGA (Naciones Unidas 2015), con la excepción del compuesto [BMIM][PF_6] en *D.*

magna, donde se clasificaría como "ligeramente tóxico" según Passino y Smith, o dentro de la categoría de Agudo 3 según las Naciones Unidas.

Evaluación en medio ambiente, salud y seguridad (EHSA)

Para complementar la información proporcionada por los valores de EC/LC_{50}, se realizó una evaluación en medio ambiente, salud y seguridad según la metodología EHSA (Koller et al. 2000). Se pueden ver los valores utilizados y obtenidos en la Tabla 8.

	Efectos en medio acuático			Acumulación		Degradación	
	Promedio EC/LC_{50}	$IndVal_{ij}$	EDP_{ij}	Log P	EDP_{ij}= $IndVal_{ij}$	Vida media	EDP_{ij}= $IndVal_{ij}$
3F03F	809	0.02	-0.44	1.424	-0.79	21[a]	0.66
[BMIM][PF_6]	685	0.04	-1.39	-1.66	-2.33	4[a]	0.3

.

Tabla 8: Datos usados para la evaluación EHSA. [a]Usando modelos de biodegradación Biowin 3 y 4 (EPIWeb 4.1). $IndVal_{ij}$ = Propiedad peligrosa; EDP_{ij} = Propiedad peligrosa efectiva.

Se aplicó la metodología en función del riesgo que pueden causar estos disolventes en el medio acuático, junto con la bioconcentración y la degradación. Los efectos en el medio acuático fueron calculados mediante la media de los valores (EC/LC_{50}) disponibles. La bioconcentración se estimó a través de los valores de log P (García et al. 2010; Ropel et al. 2005), y la degradación a través del software Ultimate and Primary Biodegradation Models (Biowin 3 y 4) de EPIWeb 4.1, obteniendo la vida media en el medio.

De acuerdo a este sistema de evaluación, un valor indexado en las propiedades $IndVal_{ij}$ y EDP_{ij} de 0 indica sustancias no peligrosas, mientras que valores de 1 indican un nivel de máximo peligro. Mientras que el derivado del glicerol 3F03F muestra un mejor índice en su evaluación ecotoxicológica, el líquido iónico [BMIM][PF_6] muestra mejores valores de acumulación y degradación, sobre todo de éste último.

DISCUSIÓN DE LOS RESULTADOS

DISCUSIÓN DE LOS RESULTADOS

Ecotoxicidad de los compuestos derivados del glicerol

En esta tesis se ha realizado un estudio ecotoxicológico sistemático del glicerol y de diecinueve compuestos derivados, mediante bioindicadores acuáticos durante un tiempo de exposición agudo. Con el uso estos datos iniciales se pretendió hacer una primera estimación sobre la ecotoxicidad en medio acuático de los disolventes derivados del glicerol. Con la excepción de esta sustancia (Bringmann & Kühn 1977; Bridié et al. 1979) y del compuesto 111 (trimetoxipropano) (Sutter et al. 2013), estos compuestos no habían sido previamente evaluados en un ámbito ecotoxicológico, por lo que era necesario caracterizarlos antes de considerar su salida al mercado (Reglamento 1907/2006/CE). Destacar que los rangos de los valores de EC_{50} aportados por Sutter en *V. fischeri* y *D. magna* se encuentran dentro de los valores obtenidos durante el transcurso de esta tesis.

De todos los compuestos evaluados en los cuatro biomodelos utilizados (*D. magna*, *V. fischeri*, *C. reinhardtii* y *D. rerio*) (Tabla 3 del capítulo Resultados), sólo cuatro derivados del glicerol se pueden considerar como "ligeramente tóxicos" en alguno de estos ensayos, según la clasificación de Passino y Smith (Passino & Smith 1987). Estos disolventes son 444, 404, 404t, y 414. En *V. fischeri*, han sido los compuestos 404, 404t y 414. En *D. magna*, sólo el compuesto 444. Por otro lado, de un conjunto de cinco compuestos (200, 202, 400, 404 y 444), en *C. reinhardtii* fue clasificado como "ligeramente tóxico" el compuesto 444 y, finalmente, en el mismo grupo de disolventes, en el embrión de *D. rerio*, el 404 fue el más tóxico ante una

exposición aguda. Los dieciséis compuestos restantes, incluyendo el glicerol, están dentro de los rangos “prácticamente no tóxicos” o “no tóxicos”.

Es importante hacer notar que los cuatro derivados del glicerol que pueden presentar mayor riesgo para el medio acuático tienen cadenas de butilo en ambos extremos de la molécula de glicerol (posiciones 1 y 3), y su lipofilia, estimada mediante el log P, se encuentra desde 1.5 a 3.5, aproximadamente, lo cual es un relativo amplio rango. Sin embargo, mientras que esta propiedad fisicoquímica parece ser muy relevante en los valores de EC_{50} de los biomodelos *D. magna* y *C. reinhartdtii*, en *V. fischeri* y embrión de *D. rerio* esta relación está tan establecida a la hora de poder determinar la ecotoxicidad.

Relación entre diferentes normativas de clasificación

En la determinación de los valores de EC/LC_{50}, las metodologías empleadas en esta tesis provienen principalmente de protocolos establecidos por la OCDE (*D. magna*, *D. rerio*) (OCDE 1984;OCDE 1992a; OCDE 2004; OCDE 2013), y la ISO (*V. fischeri*) (ISO 11348-3 2009). De esta manera, los datos generados pueden ser recopilados según la legislación REACH y utilizados para la clasificación y etiquetado de sustancias químicas. Como uno de sus objetivos establecidos, esta ley regula la normativa de protección de la salud humana y medioambiental en estos productos.

A la hora de establecer una comparación toxicológica entre valores de EC/LC_{50} con estos y otros disolventes industriales, se ha empleado la clasificación de Passino y Smith (Passino & Smith 1987). Consiste en una categorización de peligrosidad en escala logarítmica que permite clasificar sustancias químicas que se pueden encontraren medios acuáticos, en función de los valores de EC/LC_{50} obtenidos a partir de un periodo

de exposición agudo en diferentes organismos. En la Tabla 9 se puede ver la clasificación, junto con la correspondiente interpretación de los descriptores tóxicos.

Valores de EC/LC_{50} (mg L^{-1})	Descriptor tóxico
< 0.1	Extremadamente tóxico
0.1-1	Altamente tóxico
1-10	Moderadamente tóxico
10-100	Ligeramente tóxico
100-1000	Prácticamente no tóxico
> 1000	No tóxico ("Relatively harmless")

Tabla 9: Clasificación de Passino y Smith (Passino & Smith 1987)

Otra de las clasificaciones utilizadas a la hora de determinar la peligrosidad de compuestos químicos es la establecida por las Naciones Unidas, SGA (Naciones Unidas 2015), un sistema internacional que permite evaluar la toxicidad y peligrosidad de los productos químicos, empleado para unificar su clasificación y etiquetado. Dentro del ámbito medioambiental, esta categorización considera diferentes biomodelos y tiempos de exposición tanto a corto como a largo plazo. Aunque SGA indica un número mínimo de ensayos con unos protocolos determinados pertenecientes a la OCDE, este sistema deja libertad de elección a la hora de escoger los ensayos para evaluar los compuestos, siempre que provengan de métodos científicamente validados. Esta clasificación es muy similar a la de Passino y Smith, con la diferencia de que los puntos límites de toxicidad máxima y mínima son inferiores o iguales a 1 mg L^{-1} y hasta 100 mg L^{-1}, respectivamente, y sólo poseen 3 categorías de toxicidad aguda (menor o igual 1 mg L^{-1}, de 1 a 10 mg L^{-1}, y de 10 a 100 mg L^{-1}).Se puede apreciar esta clasificación en la Tabla 10.

Categoría Aguda 1	**Valores de ecotoxicidad**
LC_{50} 96 horas (peces)	≤ 1 mg L^{-1} y/o
EC_{50} 48 horas (crustáceos)	≤ 1 mg L^{-1} y/o
EC_{50} 72 o 96 horas (algas u otras plantas acuáticas)	≤ 1 mg L^{-1}
La categoría Aguda 1 puede subdividirse en algunos sistemas reguladores para incluir un rango inferior con una $EC/LC_{50} \leq 0.1$ mg L^{-1}.	
Categoría Aguda 2	**Valores de ecotoxicidad**
LC_{50} 96 horas (peces)	$> 1- \leq 10$ mg L^{-1} y/o
EC_{50} 48 horas (crustáceos)	$> 1- \leq 10$ mg L^{-1} y/o
EC_{50} 72 o 96 horas (algas u otras plantas acuáticas)	$> 1- \leq 10$ mg L^{-1}
Categoría Aguda 3	**Valores de ecotoxicidad**
LC_{50} 96 horas (peces)	$> 10- \leq 100$ mg L^{-1} y/o
EC_{50} 48 horas (crustáceos)	$> 10- \leq 100$ mg L^{-1} y/o
EC_{50} 72 o 96 horas (algas u otras plantas acuáticas)	$> 10- \leq 100$ mg L^{-1}
Algunos sistemas reguladores pueden ampliar este rango más allá de una EC/LC_{50} de 100 mg L^{-1} introduciendo otra categoría.	

Tabla 10: Categorías para las sustancias peligrosas para el medio ambiente acuático a corto plazo, según SGA (Naciones Unidas 2015).

En el caso de los compuestos derivados del glicerol, aquellos que se han mencionado previamente como ligeramente tóxicos en la escala de Passino y Smith serían clasificados dentro de la categoría Aguda 3. De la misma manera, el resto de compuestos no se encuentran en ninguna categoría. Según este sistema, ninguno de los

veinte compuestos analizados requeriría ningún símbolo ni palabra de advertencia a la hora de ser etiquetados para su distribución. Sólo los compuestos 404, 404t, 414 y 444 deberían llevar la indicación de peligro "Nocivo para los organismos acuáticos" (Tablas 11, 12 y 13).

Compuesto	*V. fischeri*			*D. magna*		
	Valor de EC_{50} (mg L^{-1})	Categoría (Passino & Smith 1987)	Categoría (Naciones Unidas 2015)	Valor de EC_{50} (mg L^{-1})	Categoría (Passino & Smith 1987)	Categoría (Naciones Unidas 2015)
000	108421	No tóxico	-	68784	No tóxico	-
100	21052	No tóxico	-	6478	No tóxico	-
200	4240	No tóxico	-	6458	No tóxico	-
400	941	Prácticamente no tóxico	-	2332	No tóxico	-
101	13702	No tóxico	-	4790	No tóxico	-
111	969	Prácticamente no tóxico	-	3240	No tóxico	-
202	1215	No tóxico	-	1819	No tóxico	-
404	11	Ligeramente tóxico	Agudo 3	248	Prácticamente no tóxico	-
114	453	Prácticamente no tóxico	-	1617	No tóxico	-
104	464	Prácticamente no tóxico	-	2388	No tóxico	-
414	58	Ligeramente tóxico	Agudo 3	133	Prácticamente no tóxico	-
444	473	Prácticamente no tóxico	-	14	Ligeramente tóxico	Agudo 3
103i	2188	No tóxico	-	4828	No tóxico	-
104i	142	Prácticamente no tóxico	-	1975	No tóxico	-
3i03i	1064	No tóxico	-	2170	No tóxico	-
114i	258	Prácticamente no tóxico	-	1496	No tóxico	-
104t	189	Prácticamente no tóxico	-	4568	No tóxico	-
404t	16	Ligeramente tóxico	-	668	Prácticamente no tóxico	-
3F03F	1597	No tóxico	-	480	Prácticamente no tóxico	-
3F13F	4033	No tóxico	-	944	Prácticamente no tóxico	-

Tabla 11: Valores de ecotoxicidad de *V. fischeri* y *D. magna* en función de las clasificaciones de Passino y Smith(Passino & Smith 1987), y de las Naciones Unidas (Naciones Unidas 2015).

Compuesto	*C. reinhardtii*			*D. rerio*		
	Valor de EC_{50} (mg L^{-1})	Categoría (Passino & Smith 1987)	Categoría (Naciones Unidas 2015)	Valor de EC_{50} (mg L^{-1})	Categoría (Passino & Smith 1987)	Categoría (Naciones Unidas 2015)
200	52811	No tóxico	-	36700	No tóxico	-
400	4445	No tóxico	-	4300	No tóxico	-
202	8613	No tóxico	-	2800	No tóxico	-
404	631	Prácticamente no tóxico	-	17	Ligeramente tóxico	Agudo 3
444	64	Ligeramente tóxico	Agudo 3	2700	No tóxico	-

Tabla 12: Valores de ecotoxicidad de *C. reinhardtii*y *D. rerio* en función de las clasificaciones de Passino y Smith(Passino y Smith 1987), y de las Naciones Unidas(Naciones Unidas 2015).

	Categoría 1	Categoría 2	Categoría 3
Símbolo	Medio ambiente	Sin símbolo	Sin símbolo
Palabra de advertencia	Atención	Sin palabra de advertencia	Sin palabra de advertencia
Indicación de peligro	Muy tóxico para los organismos acuáticos	Tóxico para los organismos acuáticos	Nocivo para los organismos acuáticos

Tabla 13: Elementos que deben de figurar en las etiquetas de peligro para sustancias y mezclas peligrosas para el medio ambiente acuático, según el documento SGA (Naciones Unidas 2015).

Relaciones en el número de cadena de alquilos, lipofilia y ecotoxicidad

El objetivo de esta tesis ha sido valorar la influencia de la presencia/ausencia y la longitud de las cadenas de alquilo de disolventes derivados del glicerol en la ecotoxicidad acuática, durante una exposición aguda. Al producirse un aumento de cadenas hidrocarbonadas en una molécula orgánica, la consecuencia directa es el incremento de la lipofilia (Dearden 1985). Por tanto, es lógico pensar que en los resultados obtenidos se podrían encontrar buenas correlaciones entre los coeficientes de partición, representados por log P, y otros parámetros que tuvieran relación con el

volumen molecular. Por otro lado, estas modificaciones a partir de la molécula base (glicerol) también quedarían reflejadas en descriptores de la estructura molecular, en función de sus propiedades topológicas. Estas características se plasman mediante índices globales que estiman la conectividad y la flexibilidad molecular.

De manera más concreta, en los ensayos experimentales realizados se ha encontrado relación entre el número y longitud de las cadenas de alquilo, y la ecotoxicidad en el biomodelo *D. magna*, junto con otros parámetros relacionados con el volumen molecular. Esta asociación también se ha visto reflejada en el biomodelo *C. reinhardti* .No obstante, tanto en *V. fischeri* como en *D. rerio* no tiene una relación tan directa con la lipofilia. No se han establecido claramente las razones definitivas por las cuales existen estas diferencias.

En *V. fischeri* se tiene en cuenta que el medio en el cual se hicieron los ensayos tiene concentraciones del 2 % de NaCl, simulando un medio acuático marino. Los medios con salinidad pueden excluir moléculas orgánicas neutras debido a las fuertes interacciones iónicas entre moléculas de agua y los iones salinos, produciendo el fenómeno conocido como *salting out* (Wheeler et al. 2002). Por tanto, puede influir en la solubilidad y en la actividad bioquímica de sustancias más lipófilas o con menos posibilidad de disolverse por ausencia de grupos dadores o donantes de hidrógeno.

Por otro lado, usando el biomodelo *D. rerio* en cinco compuestos (200, 202, 400, 404, 444) se pudo ver que la relación entre ecotoxicidad y longitud de las cadenas alquilo, y por tanto en la lipofilia, no fue directa. En el caso de *D. rerio* sólo se han expuesto cinco compuestos, por lo que en futuros ensayos sería recomendable aumentar el número de compuestos a evaluar. También sería de considerable relevancia incluir

compuestos que no hayan sido evaluados previamente, destacando aquellos que tienen cadenas más largas de metilos en la posición central de la molécula de glicerol.

A lo largo de la tesis, la lipofilia se ha considerado como una de las principales características a tener en cuenta en un disolvente respetuoso con el medio ambiente. Esta característica, representada como el log P, estima la afinidad de una determinada molécula o grupo funcional por un medio lipófilo, en función de su distribución en un sistema bifásico compuesto por una fase lipídica (generalmente 1-octanol) y una fase polar (agua) (Rutkowska et al. 2013). Según Dearden, es uno de los parámetros más importantes para la cuantificación de la respuesta biológica proporcionada por un biomodelo acuático, debido a la relación que puede establecerse con muchas de las propiedades de un determinado compuesto (Dearden 1985).

En su relación con el medio ambiente acuático, la lipofilia determina la solubilidad, la reactividad y la degradación de las sustancias, lo cual puede influir en la biodisponibilidad de este compuesto. Por otro lado, involucra diversos factores en relación con el biomodelo, tales como la absorción, la distribución y el metabolismo. Esto es debido a que esta propiedad facilita el transporte de compuestos químicos a través de membranas en un sistema biológico (Rutkowska et al. 2013).

Precisamente, el aspecto fundamental a tener en cuenta en relación a la lipofilia en biomodelos acuáticos sencillos (pequeños invertebrados, bacterias y algas) son las membranas celulares. Estas estructuras son barreras permeables selectivas que consisten fundamentalmente en una doble capa de fosfolípidos con estructuras proteicas insertadas y propiedades anfifílicas (Rutkowska et al. 2013). Desde hace tiempo, las investigaciones realizadas sobre las relaciones entre lipofilia y membranas celulares han establecido que existe una relación con la ecotoxicidad en diferentes modelos acuáticos

(Dearden 1985).La lipofilia es relevante para facilitar el paso a través de estas barreras para poder alcanzar el sitio de acción (Meyer 1899; Overton 1897), o para establecer interacciones, produciendo efecto tóxico (Escher & Schwarzenbach 2002).

A raíz de estas observaciones, numerosos investigadores realizaron estudios QSAR en relación a la lipofilia para enlazar las propiedades de un compuesto con su actividad toxicológica, ya sea para la salud humana (Schultz et al. 2003) o para el medio ambiente (Qin et al. 2010). La información que puede proporcionar este tipo de aproximación puede ser desde entender el tipo de interacciones entre grupos funcionales en las moléculas, hasta para predecir las propiedades de nuevos compuestos sin ser necesaria su síntesis previa (Connors et al. 2014).

En el desarrollo de esta tesis se evaluó la relación de la toxicidad con la lipofilia en los compuestos derivados del glicerol, así como con sus correspondientes parámetros topológicos y DARC-PELCO. De esta manera, como se ha indicado previamente, se estableció una relación con las diferencias existentes en la longitud y posición de las cadenas de alquilo de estos 20 sustancias, en los biomodelos *V. fischeri* y *D. magna*. Para ello, se realizaron estudios QSAR con el objetivo de ver las posibles diferencias en la ecotoxicidad de los compuestos derivados del glicerol en biomodelos acuáticos. Esto se establece ya que, generalmente, los compuestos con el mismo mecanismo de acción tienen estructuras y/o propiedades similares. No obstante, hay que tener en cuenta que las especies acuáticas pueden tener diferencias biológicas entre sí, por lo que su mecanismo de acción puede variar de una especie a otra, siendo la sensibilidad diferente frente a un mismo compuesto (Nendza & Wenzel 2006). Debido a que los derivados del glicerol comparten entre sí la misma estructura base, se pueden ver claramente las diferencias en la ecotoxicidad causadas por las variaciones estructurales, ya sea

mediante los valores de EC/LC_{50} obtenidos (Tabla 3 de Resultados), o a través de las ecuaciones QSAR.

Sobre los mecanismos de acción en diferentes biomodelos, hay un reconocimiento sobre la influencia de la lipofilia en la ecotoxicidad aguda, como en pequeños crustáceos (Barata et al. 2008), bacterias bioluminiscentes (Wang et al. 2016), algas (Latała et al. 2009), células (Ranke et al. 2007) y otros biomodelos (Russom et al. 1997). Este tipo de influencia involucra en general a todos los compuestos químicos orgánicos (Russom et al. 1997) y puede tener importancia en otros, tales como líquidos iónicos (Peric et al. 2013; Viboud et al. 2012). Fundamentalmente, ocurre en sustancias en los que no están presentes grupos funcionales que puedan causar uniones con receptores, tales como tetra y pentaclorofenoles, acil halidos y isocianatos, y aldehídos alifáticos (Zhang et al. 2013). Este modo de toxicidad se denomina "narcosis". Se aplica a compuestos que no poseen ningún mecanismo de acción concreto, ya que no interactúan con receptores específicos en ningún organismo. Como resultado, en la ausencia de cualquier otro mecanismo de toxicidad, un compuesto químico, dentro de ciertos límites, siempre será tan tóxico como se refleje en su lipofilia, representada por el log P, u otros valores relacionados (Verhaar et al. 1992).

El fenómeno de narcosis ocurre cuando un compuesto químico se acumula en las membranas celulares interfiriendo con la función normal de las membranas. En el caso de biomodelos acuáticos superiores, tales como *Pimephales promelas*, se pueden apreciar con más claridad la exposición a un agente tóxico en el organismo, siendo las respuestas típicas en la narcosis tales como descenso en la actividad, reacción reducida ante estímulos externos y pigmentación incrementada (Bradbury et al. 2003; Russom et al. 1997). Los efectos son reversibles, y los organismos moribundos usualmente vuelven

a la actividad normal una vez que el compuesto químico es eliminado del medio. Algunos ejemplos de compuestos que actúan mediante narcosis en organismos acuáticos son los hidrocarburos alifáticos (Chambers 1987).

El primer autor que realizó una clasificación que permitió distinguir entre los diferentes mecanismos de acción generalizados de toxicidad en compuestos orgánicos fue Verhaar (Verhaar et al. 1992). Años más adelante, Enoch (Enoch et al. 2008) la actualizó, invirtiendo el orden de aplicación de las reglas de clasificación (Figura 18). Realmente, estos tipos de estimaciones toxicológicas pretenden ofrecer una primera idea sobre la peligrosidad de compuestos orgánicos ante la ausencia de datos ecotoxicológicos. De esta manera, pueden darnos una idea inicial de cuál va ser su daño potencial en el medio ambiente o en la salud humana a partir de su posible mecanismo de acción. Estas determinaciones en toxicidad acuática aguda han sido inicialmente basadas en ensayos de toxicidad en peces (Kramer et al. 2009; Russom et al. 1997; Verhaar et al. 1992), teniendo en cuenta su comportamiento y su fisiología, así como en relaciones de estructura actividad. Más adelante, se incorporaron a estudios con otros biomodelos, como *D. magna* (Cleuvers 2003) y *V. fischeri* (Vighi et al. 2009).

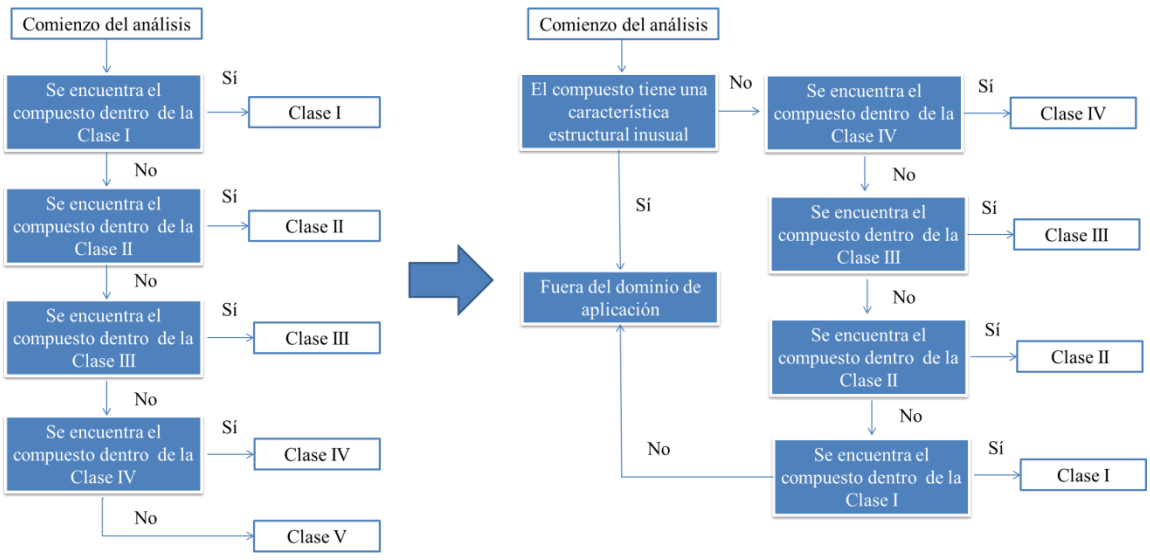

Figura 18: Clasificación de Verhaar (Verhaar et al. 1992) (izquierda) y clasificación modificada de Enoch (Enoch et al. 2008) (derecha).

Verhaar usó datos de toxicidad en peces para establecer unas reglas estructurales, basadas en la disposición 2-D de los átomos de la molécula, para asociar estructuras con una variedad de modos de acción (Ellison et al. 2016). Estableció que, aparte de la narcosis, existían otros mecanismos de acción en biomodelos acuáticos que podían explicar la variabilidad de resultados según la estructura de los compuestos estudiados. Los cuatro principales modos de acción de acuerdo con la clasificación son los siguientes (Enoch et al. 2008) :

Clase I: narcosis o toxicidad basal. Estos compuestos químicos no son reactivos en sus efectos agudos, ya que no interactúan con receptores específicos en un organismo, siguiendo un modo de acción denominado narcosis. Este modo de acción se caracteriza por depender en gran parte de la hidrofobicidad del compuesto. Por lo tanto, la ecotoxicidad de este tipo de compuestos puede predecirse con bastante aproximación mediante su lipofilia (tal como el log P).

Clase II: compuestos menos inertes. Estos compuestos son ligeramente más tóxicos que los de clase I, aunque no se considera que tengan un mecanismo de acción específico que incida en la toxicidad. Éste se denomina “de narcosis polar”, para diferenciarlo del anterior apartado.

Clase III: reactividad inespecífica. Son compuestos químicos que tienen una toxicidad claramente más elevada que la predicha sólo por su hidrofobicidad, tales como sustancias que forman enlaces irreversibles covalentes con residuos de aminoácidos.

Clase IV: compuestos químicos especialmente activos. Sustancias que reaccionan de una manera específica mediante enlaces no covalentes.

Clase V/Fuera del dominio de aplicación: Sustancias que no pueden ser clasificadas de acuerdo a estas reglas.

Esta clasificación tiene gran relevancia, y ha sido adoptada por diversas entidades reguladores, como la ECHA, donde se ha incluido dentro del software Toxtree (Ellison et al. 2015; Enoch et al. 2008; Lahl & Gundert-Remy 2008), junto con otras clasificaciones relacionadas con toxicidad humana y ambiental. Este software codifica diferentes árboles de decisión y esquemas de clasificación útiles para analizar los peligros potenciales de compuestos, siendo continuamente actualizado para mejorar las predicciones realizadas (Ellison et al. 2015).

Los compuestos derivados del glicerol fueron evaluados según el software Toxtree para determinar *a priori* cual sería la toxicidad en medio acuático en función de su estructura. Las principales normas para introducir a los compuestos derivados del glicerol en las categorías son las siguientes (Verhaar et al. 1992):

- Compuestos formados por átomos de carbono, hidrógeno, oxígeno, azufre, o halógenos (exceptuando yodo).
    - Éteres lineales o éteres monociclos, pero no epóxidos o peróxidos.
    - En el caso de contener halógenos, que no estén situados en posiciones alfa o beta.
- Tener un log P entre 0 y 6.
- Tener una masa molecular inferior a 600 Daltons.
- No contener grupos iónicos.

Los compuestos estudiados fueron clasificados dentro de la Clase I (narcosis o toxicidad base), ya que cumplen todos los condicionantes. De hecho, la principal

distinción que tienen estos derivados entre sí es la variación en su log P por la modificación de la longitud de las cadenas de alquilo.

No obstante, los resultados obtenidos en los compuestos 3F03F, 3F13F y 444 en *V. fischeri* se quedan fuera de la relación con su log P, presentando valores de EC_{50} más elevados de lo que correspondería. Se comprobó que, si no se incluyen en la función, su coeficiente de correlación mejora claramente. Realmente es interesante destacarlo, ya que en la clasificación de Verhaar se establece que las diferencias en la relación de ecotoxicidad con lipofilia tienden a ser más tóxicos de lo esperado, y no lo contrario.

Los compuestos fluorados son los que tienen más diferencia en su composición atómica con los otros derivados del glicerol. Éstos son considerados como inocuos (*V. fischeri*) o prácticamente inocuos (*D. magna* y *D. rerio*) en exposición aguda, según la clasificación de Passino y Smith (Passino & Smith 1987), así como fuera de rango de toxicidad según la clasificación de las Naciones Unidas (Naciones Unidas 2015). No obstante, en su comparación con el líquido iónico [BMIM][PF_6] se estimó que su índice de degradación era bastante elevado, no siendo tan adecuado para un disolvente verde. Por ello, se considera que son necesarios más ensayos de ecotoxicidad en estos compuestos, y en otras sustancias fluoradas similares, para complementar la información ya disponible de los derivados 3F03F y 3F13F.

Sobre el compuesto 444, la característica más notable que se ha presentado ha sido la diferencia de ecotoxicidad entre los diferentes biomodelos. Mientras que para *D. magna y C. reinhardtii* es el compuesto potencialmente más dañino para el medio ambiente acuático, para *V. fischeri* y *D. rerio* la toxicidad no es destacable según las clasificaciones ecotoxicológicas empleadas. Este compuesto es el único de todo el grupo de disolventes analizados que incluye una cadena de butilo en la posición 2. Como se ha

indicado con los compuestos fluorados, sería recomendable realizar análisis con una mayor variedad de compuestos que incluyan una cadena de hidrocarburos de mayor tamaño en esta posición, y poder comprobar cómo sigue la relación ecotoxicidad-lipofilia en función de los diferentes biomodelos empleados.

Análisis y tratamiento matemático de los resultados: EHSA y QSAR

Se han utilizado las metodologías EHSA y QSAR a partir de los datos experimentales obtenidos en los correspondientes biomodelos de evaluación ecotoxicológica. No sólo han servido para poder obtener información adicional que permita valorar su riesgo, sino también para realizar futuras estimaciones sobre la toxicidad de compuestos similares, determinando cuáles son los factores que más influyen.

EHSA

En el caso de los compuestos derivados del glicerol, este método es muy adecuado para su valoración, ya que, se trata de compuestos diseñados para actuar como disolventes verdes industriales. Además, en el aspecto de la disponibilidad de datos, se contaba previamente con medidas fisicoquímicas de estos compuestos (García et al. 2010; Khadzhibekov et al. 1985; Sambou 2005), y aquellos valores que no estaban disponibles experimentalmente, han podido ser estimados mediante herramientas informáticas, aplicando QSAR (US EPA 2016a; US EPA 2016b). Finalmente, los resultados pertenecientes a la categoría medioambiental de ecotoxicidad fueron obtenidos experimentalmente y fueron suficientes para valorarla mediante EHSA.

QSAR: propiedades topológicas y DARC-PELCO

De la misma manera, también se ha relacionado la toxicidad aguda de los compuestos estudiados, cuantificada mediante los valores de $EC/LC_{50,}$ con sus propiedades estructurales mediante metodologías QSAR. Estas herramientas pueden determinar cómo influye la disposición de átomos y grupos moleculares en la toxicidad de una molécula (Schultz et al. 2003).

Se han aplicado ambas metodologías QSAR con los valores de EC_{50} en *V. fischeri* (García et al. 2015)y en *D. magna*. Las propiedades estructurales describen cada compuesto como vectores numéricos, escogiendo descriptores basados en conectividad molecular. En la presente tesis, se plantearon dos desarrollos, sin contar la relación con la lipofilia: QSAR basado en parámetros topológicos y el método DARC-PELCO (García et al. 2013).

Parámetros topológicos

Los parámetros topológicos se establecen a partir de la estructura molecular en dos dimensiones de cada compuesto, en función de la conectividad y colindancia que tienen los átomos entre sí. Esta información se compacta en matrices, aplicadas para determinar descriptores de tamaño, forma y flexibilidad de la molécula total. Estos factores influyen de manera directa en la idoneidad de un disolvente. Los valores obtenidos se consideran como globales ya que la estructura molecular puede ser identificada en un solo número (característica invariante) para una propiedad o característica determinada (García et al. 2013). De esta manera, pueden ser utilizados para cálculos de sustancias que aún no han sido sintetizadas (Mamy et al. 2015).

DARC-PELCO

El método DARC-PELCO es un sistema especialmente adecuado para el estudio de compuestos que comparten una subestructura común en una molécula, lo cual se corresponde con el grupo de compuestos derivados del glicerol. Esta metodología está basada en la generación exhaustiva de espacios topocromáticos alrededor de la parte común del compuesto, denominada F_0. En el caso de los compuestos estudiados, sería la molécula de glicerol sin hidrógenos en los extremos alcoholes. De este modo, se puede determinar la influencia de cada átomo de la molécula en una determinada propiedad (como el valor de log EC_{50}). Los descriptores en el modelo DARC-PELCO son considerados como locales, ya cada uno de ellos indica la presencia o ausencia de un grupo de átomos en una posición de la molécula concreta dentro de una matriz estructural (García et al. 2013)

Cabe destacar que este tipo de procedimientos QSAR se usan para complementar la información obtenida mediante ensayos experimentales, utilizando regresiones lineales múltiples (*Multiple Lineal Regression*, MLR) para establecer correlaciones. Estas fórmulas matemáticas pueden aplicarse a un gran número de compuestos para determinar sus correspondientes valores de EC_{50}, disponiendo solamente de los datos de propiedades estructurales. Por lo tanto, estos modelos proporcionaron datos muy útiles para determinar cuáles son las características estructurales que influyen en la toxicidad de una molécula. Sólo analizando una muestra de las posibles opciones de derivados del glicerol (5 o 20 compuestos), se pudieron obtener ecuaciones con buenos coeficientes de correlación.

Summary

Ecotoxicity of compounds derived from glycerol

In this PhD thesis a systematic ecotoxicological study of glycerol (1,2,3-propanetriol) and nineteen derivative compounds have been carried out by aquatic bioindicators during an acute exposure time. With the exception of glycerol (Bridie et al. 1979; Bringmann & Kühn 1977) and 111 (trimethoxypropane) (Sutter et al. 2013), these compounds had not previously been evaluated in an ecotoxicological environment.

Only four glycerol derivatives have been considered "slightly toxic" in all of the ecotoxicity trials, according to the Passino and Smith classification (Passino & Smith 1987). These solvents are 444, 404, 404t, and 414. In *V. fischeri*, they were 404, 404t and 414. In *D. magna*, only 444 was considered as “slighty toxic”. On the other hand, in a set of five compounds (200, 202, 400, 404 and 444), only 444 was classified as "slightly toxic" for *C. reinhardtii*. In the same group of solvents, 404 was the most toxic compound on acute exposure in embryo in *D. rerio*. The remaining sixteen compounds, including glycerol, are within the "practically non-toxic" or "non-toxic" categories.

It should be noted that the four slightly toxic derivatives have butyl chains at both ends of the glycerol molecule (positions 1 and 3), and their log P is among 1.5 to 3.5.

Relationship between different classification regulations

The used methodologies in this thesis come from OECD and ISO protocols (OECD 1984, OECD 1992a, OECD 2004, OECD 2013, ISO 11348-3 2009). The data generated can be compiled according to REACH legislation.

In order to establish a toxicological comparison, the Passino and Smith classification (Passino & Smith 1987) and the GHS classification have been used (Naciones Unidas 2015).

All the compounds classified as slightly toxic by the Passino and Smith classification would be categorized within the Acute category 3 in GHS classification. In the same way, the rest of these compounds are not in any category. According to the GHS system, none of the twenty compounds analyzed would require any symbol or warning advice. Only 404, 404t, 414 and 444 should carry the hazard indication "Harmful to aquatic organisms".

Relationships in the alkyl chain number, lipophilicity and ecotoxicity

The larger an alkyl chain is added to the basic structure, the more lipophilic the molecule is (Dearden 1985). Therefore, good correlations could be found between the partition coefficients, represented by log P, and other parameters, i.e. molecular volume.

The number and length of the alkyl chains and the ecotoxicity in the *D. magna* biomodel were well correlated in *D. magna* and *C. reinhardtii*, and, in the same way, with the molecular volume. However, both *V. fischeri* and *D. rerio* EC/LC_{50} values are not as directly related to lipophilicity as the other bioindicators. The reasons for these differences have not been clearly established.

In *V. fischeri*, the assay medium had concentrations of 2% of NaCl, simulating a marine aquatic environment. Salinity media can exclude neutral organic molecules due to strong ionic interactions between water molecules and saline ions, producing a phenomenon called salting out (Wheeler et al. 2002). It may influence the solubility and

biochemical activity of more lipophilic or less dissolvable substances in the absence of donor or hydrogen donor groups.

On the other hand, using the *D. rerio* biomodel in five compounds (200, 202, 400, 404, 444), it was possible to see that the relationship between ecotoxicity and lipophilicity was not direct. In future trials, it would be better if increasing the number of compounds to be evaluated. It would also be an improvement to include compounds that have not been previously evaluated, highlighting those having longer methyl chains at the central position of the glycerol molecule.

In the development of this PhD thesis, the relationships between the ecotoxicity and the structure and lipophilicity parameters in these compounds were evaluated. The influence of lipophilicity in acute ecotoxicity assays has been referenced in several bioindicators, as small crustaceans (Barata et al., 2008), bioluminescent bacteria (Wang et al., 2016), algae (Latała et al. 2009) and other biomodels (Russom et al., 1997). Basically, it occurs to substances without functional groups that can cause linkages with receptors, such as tetra and pentachlorophenols, acyl halides and isocyanates, and aliphatic aldehydes (Zhang et al. 2013). This mode of toxicity is called "narcosis". It is applied to compounds that do not have any specific mechanism of action, since they do not interact with specific receptors. As a result, in the absence of any other mechanism of toxicity, a chemical compound, within certain limits, will always be as toxic as reflected in its lipophilicity (Verhaar et al. 1992). The phenomenon of narcosis occurs when a chemical accumulates in the cell membranes interfering with the normal function of the membranes.

Verhaar and Enoch (Enoch et al. 2008; Verhaar et al. 1992) created and optimized a classification system based in ecotoxicity data in fish, for establishing

several structural rules to associate with different modes of action (Ellison et al. 2016). Apart from narcosis, there were other mechanisms of action in aquatic biomodels that could explain the variability of results according to the structure of the studied compounds. The four main modes of action according to the classification are the following (Enoch et al. 2008):

Class I: narcosis or basal toxicity. These chemicals are not reactive in their acute effects, as they do not interact with specific receptors in an organism, following a mode of action called narcosis. This mode of action is characterized in the hydrophobicity of the compound. Therefore, the ecotoxicity of such compounds can be fairly well predicted by their lipophilicity (such as log P).

Class II: less inert compounds. These compounds are slightly more toxic than those of class I, although they are not considered to have a specific mechanism of action that affects the toxicity. This is called "polar narcosis", to differentiate it from the previous section.

Class III: non-specific reactivity. They are compounds which have a markedly higher toxicity than predicted by their hydrophobicity, such as irreversible covalent linking substances with amino acid residues.

Class IV: Particularly active chemicals. Substances that react in a specific way through non-covalent bonds.

Class V/Outside the application domain: Substances that cannot be classified according to these rules.

The compounds derived from glycerol were evaluated according to the software Toxtree to determine a priori what would be the toxicity in aquatic environment depending on its structure.

All the compounds were classified within Class I (narcosis or base toxicity), since they fulfill all the conditions. In fact, the main distinction these derivatives have with each other is the variation in their log P by the modification of the length of the alkyl chains.

However, the results obtained in compounds 3F03F, 3F13F and 444 in *V. fischeri* are out of the relationship with their log P, presenting higher EC_{50} values than would correspond. It is really interesting to note this, since the Verhaar classification establishes that the differences in the relationship of ecotoxicity with lipophilicity tend to be more toxic than expected, and not the opposite.

The fluorinated compounds have differences in their atomic composition, compared with the other derivatives of glycerol. These are considered as non-toxic (*V. fischeri*) or practically non-toxic (*D. magna and D. rerio*) in acute exposure, according to the classification of Passino and Smith (Passino & Smith 1987), as well as outside of classification according to classification of the United Nations (Naciones Unidas 2015). However, in comparison with the ionic liquid [BMIM][PF6], it was estimated that its degradation rate was quite high, not being suitable for a green solvent. Therefore, it is considered that it is necessary more ecotoxicity tests in these compounds, and in other similar fluorinated substances, to complement the available information on 3F03F and 3F13F.

About 444, the most notable feature has been the difference in ecotoxicity between different biomodels. For *D. magna* and *C. reinhardtii* it is the most harmful compound for the aquatic environment. However, for *V. fischeri* and *D. rerio* the toxicity is not remarkable according to the ecotoxicological classifications used. This compound is the only one of the entire group of the analyzed solvents which includes a butyl chain in the 2-position. As indicated with the fluorinated compounds, it would be advisable to carry out more assays with a greater variety of compounds. These new compounds should have a chain of hydrocarbons of greater size in this position. In this manner, it would be easier to verify how the ecotoxicity-lipophilic relation works for different bioindicators.

Mathematical analysis and treatment of results: EHSA and QSAR

The EHSA and QSAR methodologies have been used from the experimental data obtained in the corresponding ecotoxicological biomodels. They have served to obtain additional information to assess their risk, and also to make future estimations on the toxicity of similar compounds.

EHSA

In the case of compounds derived from glycerol, this method is very suitable for their evaluation, since they are industrial green solvents. The results were good enough to evaluate it.

QSAR: topological properties and DARC-PELCO

Similarly, the acute toxicity of the studied compounds, quantified by the EC/LC_{50} values, has also been related to their structural properties using QSAR methodologies. These tools can determine how the arrangement of atoms and molecular groups influences the toxicity of a molecule (Schultz et al., 2003).

Both QSAR methodologies have been applied with EC_{50} values in *V. fischeri* (García et al., 2005) and *D. magna*. The structural properties describe each compound as numerical vectors, choosing descriptors based on molecular connectivity.

Topological parameters

The topological parameters are established from the molecular structure in two dimensions of each compound, as a function of the connectivity and closeness of their atoms. This information is compacted in matrices, and it is applied to determine descriptors of size, shape and flexibility of the entire molecule. These factors directly influence the suitability of a solvent. The obtained values are global since the molecular structure can be identified in a single number (invariant characteristic) for a given property or characteristic (García et al., 2013). In this way, they can be used for substances that have not been synthesized yet (Mamy et al., 2015).

DARC-PELCO

The DARC-PELCO method is a particularly suitable system for the study of compounds that share a common substructure in a molecule. This methodology is based on the exhaustive generation of topochromatic spaces around the common part of the compound, called F_0. In the case of the studied compounds, it would be the glycerol molecule without hydrogens at the alcohols ends. In this way, the influence of each atom of the molecule can be determined about a property (such as the log EC_{50} value). The descriptors in the DARC-PELCO model are considered as local, and each one indicates the presence or absence of a group of atoms in a position of the particular molecule within a structural matrix (García et al., 2013)

It should be noted that this type of QSAR procedures are used to supplement the information obtained through experimental tests using multiple linear regressions

(MLR) to establish correlations. These mathematical formulas can be applied to a large number of compounds to determine their corresponding EC_{50} values, having only the structural properties data. Therefore, these models provided very useful data for determining the structural characteristics that influence the toxicity of a molecule. Analyzing a sample of the possible options of glycerol derivatives (5 or 20 compounds), it was possible to obtain equations with good correlation coefficients.

CONCLUSIONES

CONCLUSIONES

Tras los resultados y las discusiones obtenidas, las conclusiones a las que se ha llegado son las siguientes, según los objetivos previamente establecidos:

1. De los diecinueve derivados del glicerol estudiados (incluyendo el propio glicerol), sólo los compuestos 1,3-di-*n*-butoxi-2-propanol (404), 3-*n*-butoxi-1-*terc*-butoxi-2-propanol (404t), 1,3-di-*n*-butoxi-2-metoxipropano (414), y 1,2,3-tri-*n*-butoxipropano (444) podrían ser considerados como "ligeramente tóxicos", según la clasificación de Passino & Smith, o como toxicidad de categoría Aguda 3 según la clasificación de Naciones Unidas, ante una exposición aguda en los organismos *D. magna*, *V. fischeri*, *C. reinhardtii* y *D. rerio*. El resto de disolventes no son ecotóxicos para los biomodelos utilizados. Por tanto, los derivados del glicerol más peligrosos en exposición aguda son aquellos que tienen dos cadenas butilo en las posiciones 1 y 3 de la molécula.
2. En los biomodelos *D. magna* y *C. reinhardtii*, se ha visto una fuerte correlación entre la hidrofobicidad de los compuestos derivados del glicerol, representada por el log P, y de sus valores de EC_{50}. En los otros modelos estudiados, esta relación no es tan evidente, por lo que sería necesario aumentar el número de compuestos para analizar con estos biomodelos para obtener resultados más concluyentes.
3. Tanto en el biomodelo *D. magna* como en *V. fischeri* se han determinado funciones QSAR que permiten estimar con buena correlación sus valores de EC_{50}, tanto a través de parámetros topológicos como a través de la metodología DARC-PELCO.

4. En el modelo DARC-PELCO aplicado tanto en los resultados en *V. fischeri* y *D. magna*, se determinó la influencia de la longitud de las cadenas de alquilo en las posiciones 1 y 3 en la ecotoxicidad de los derivados del glicerol. No obstante, la influencia en la posición 2 difiere según el biomodelo.
5. En el modelo QSAR de parámetros topológicos, en ambos organismos mediante diferentes coeficientes, se muestra la relación entre el aumento del tamaño de la molécula con el incremento de la ecotoxicidad.
6. En la comparación realizada entre el líquido iónico 1-butil-3-metilimidazolio hexafluorofosfato [BMIM][PF_6] y el derivado del glicerol 3-bis(2,2,2-trifluoroetoxi)propan-2-ol (BTFIP o 3F03F), no se ha demostrado que el derivado del glicerol sea un compuesto menos ecotóxico que el líquido iónico, principalmente por presentar una menor tasa de biodegradación que este último compuesto.

Summary

Following the results and discussions obtained, the conclusions reached are the following ones:

1. Nineteen glycerol derivatives were studied (including glycerol itself). Only 1,3-di-n-butoxy-2-propanol (404), 3-n-butoxy-1-tert-butoxy- propanol (404t), 1,3-di-n-butoxy-2-methoxypropane (414) and 1,2,3-tri-n-butoxypropane (444) have been classified as "slightly toxic", or as Acute Category 3 toxicity according to Passino & Smith and the United Nations classifications, respectively. The remaining solvents are not ecotoxic, according to these biomodels. Thus, the most dangerous derivatives of glycerol in acute exposure are those having two butyl chains at positions 1 and 3 of the molecule.
2. About the *D. magna* and *C. reinhardtii* biomodels, there has been a strong correlation between the log P and their EC_{50} values. In other models, this relationship is not so obvious, so it would be necessary to increase the number of compounds to be analyzed with these biomodels to obtain more conclusive results.
3. QSAR functions in *D. magna* and *V. fischeri* biomodels showed a good correlation of their EC_{50} values.
4. In the DARC-PELCO model, the relevance of the length of the alkyl chains in positions 1 and 3 on the ecotoxicity of the glycerol derivatives was determined. However, the influence on position 2 differs according to the biomodel.
5. In the QSAR model of the topological parameters, the relationship between the increase of the size of the molecule with the increase of the ecotoxicity is shown.

6. In the comparison between the 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF6] ionic liquid and the glycerol derivative 3-bis (2,2,2-trifluoroethoxy) propan-2-ol (BTFIP or 3F03F), it has not been demonstrated that the glycerol derivative is a less ecotoxic compound than the ionic liquid, mainly because it presented a lower rate of biodegradation than the latter compound.

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ANEXO: AUTORIZACIONES DE COAUTORES PARA USO DE PUBLICACIONES

ANEXO: AUTORIZACIONES DE COAUTORES PARA USO DE PUBLICACIONES

A continuación, se incluyen las autorizaciones de los coautores para uso de publicaciones en esta tesis doctoral:

Artículo 1: Comparative ecotoxicity study of glycerol biobased solvents

Autores: Eduardo Perales, Cristina Belén García, Laura Lomba, José Ignacio García, Elísabet Pires, Mari Carmen Sancho, Enrique Navarro, Beatriz Giner.

Artículo 2: Ecotoxicity studies of glycerol ethers in *Vibrio fischeri*: checking the environmental impact of glycerol-derived solvents.

Autores: Jose Ignacio García, Elisabet Pires, Luis Aldea, Laura Lomba,Eduardo Perales, Beatriz Giner.

Artículo 3: Ecotoxicity studies of glycerol ethers in *Daphnia magna.*

Autores: Eduardo Perales, José Ignacio García, Elisabet Pires, Luis Aldea, Laura Lomba, Beatriz Giner.

Artículo 4: Comparative ecotoxicology study of two neoteric solvents: Imidazolium ionic liquid vs. glycerol derivative.

Autores: Eduardo Perales, Cristina Belén García, Laura Lomba, Luis Aldea, José Ignacio García, Beatriz Giner.

Datos del coautor	
Apellidos	Nombre
GINER PARACHE	BEATRIZ
Universidad o Centro de Investigación	Departamento
UNIVERSIDAD SAN JORGE	FACULTAD DE CIENCIAS DE LA SALUD

Por el presente documento informo que:

Tomo conocimiento de que las siguientes publicaciones en las que he participado como coautor,

"Ecotoxicity studies of glycerol ethers in *Vibrio fischeri*: checking the greenness of glycerol-derived solvents", Green Chem. 2015, 17, 4326–4333;

"Comparative Ecotoxicology Study of Two Neoteric Solvents: Imidazolium Ionic Liquid vs. Glycerol Derivative", Ecotox. Environ. Saf. 2016, 132, 429–434;

"Ecotoxicity and QSAR studies of glycerol ethers in *Daphnia magna*", Chemosphere, 2017, 183, 277–285.

"Comparative ecotoxicity study of glycerol- biobased solvents", Environmental Chemistry (CSIRO) 2017, in press.

Declaro que dichas publicaciones no han sido previamente utilizadas como parte de otra Tesis Doctoral.

Renuncio al derecho que como coautor pudiera corresponderme para el uso de las publicaciones en Tesis Doctorales propias y simultáneas a la indicada. (Nota: solo si procede y se desea el uso simultaneo y delimitado de las aportaciones de los coautores en sus respectivas tesis doctorales, deberá completarse con acuerdo por escrito concretando el presente punto).

Por tanto, **AUTORIZO** el uso de estas publicaciones para esta Tesis Doctoral.

En Villanueva de Gállego, a 09 de octubre de 2017

Firmado: BEATRIZ GINER PARACHE

Datos del coautor	
Apellidos	Nombre
GARCÍA GARCÍA	CRISTINA BELÉN
Universidad o Centro de Investigación	Departamento
UNIVERSIDAD SAN JORGE	

Por el presente documento informo que:
Tomo conocimiento de que las siguientes publicaciones en las que he participado como coautor, "**Comparative ecotoxicity study of glycerol biobased solvents**", "**Comparative ecotoxicology study of two neoteric solvents: Imidazolium ionic liquid vs. glycerol derivative**", van a ser utilizadas como parte de la Tesis Doctoral "CARACTERIZACIÓN ECOTOXICOLÓGICA DE NUEVOS DISOLVENTES DERIVADOS DEL GLICEROL",, escrita por Eduardo Pablo Perales Sarría, la cual se presenta por compendio de publicaciones.
Declaro que dichas publicaciones no han sido previamente utilizadas como parte de otra Tesis Doctoral.
Renuncio al derecho que como coautor pudiera corresponderme para el uso de las publicaciones en Tesis Doctorales propias y simultáneas a la indicada. (Nota: solo si procede y se desea el uso simultaneo y delimitado de las aportaciones de los coautores en sus respectivas tesis doctorales, deberá completarse con acuerdo por escrito concretando el presente punto).

Por tanto, **AUTORIZO** el uso de estas publicaciones para esta Tesis

Doctoral. En Zaragoza, a 26 de Septiembre de 2017

Firmado: CRISTINA B. GARCÍA GARCÍA

Datos del coautor	
Apellidos	Nombre
Lomba Eraso	Laura
Universidad o Centro de Investigación	Departamento
Universidad San Jorge	Facultad de ciencias de la salud

Por el presente documento informo que:

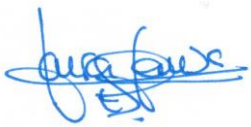
Tomo conocimiento de que las siguientes publicaciones en las que he participado como coautor, *Comparative ecotoxicity study of glicerol biobased solvents, Ecotoxicity and QSAR studies of glycerol ethers in Daphnia magna, Ecotoxicity studies of glicerol ethers in Vibrio fischeri: checking the greeness of glicerol-derived solvents, Comparative Ecotoxicology Study of Two Neoteric Solvents: Imidazolium Ionic Liquid vs. Glycerol Derivative* van a ser utilizadas como parte de la Tesis Doctoral "CARACTERIZACIÓN ECOTOXICOLÓGICA DE NUEVOS DISOLVENTES DERIVADOS DEL GLICEROL", escrita por Eduardo Pablo Perales Sarría, la cual se presenta por compendio de publicaciones.

Declaro que dichas publicaciones no han sido previamente utilizadas como parte de otra Tesis Doctoral.

Renuncio al derecho que como coautor pudiera corresponderme para el uso de las publicaciones en Tesis Doctorales propias y simultáneas a la indicada. (Nota: solo si procede y se desea el uso simultaneo y delimitado de las aportaciones de los coautores en sus respectivas tesis doctorales, deberá completarse con acuerdo por escrito concretando el presente punto).

Por tanto, **AUTORIZO** el uso de estas publicaciones para esta Tesis Doctoral.

En Zaragoza, a 12 de Septiembre de 2017



Firmado: Laura Lomba

Datos del coautor	
Apellidos	Nombre
GARCÍA LAUREIRO	JOSÉ IGNACIO
Universidad o Centro de Investigación	Departamento
INSTITUTO DE SÍNTESIS QUÍMICA Y CATÁLISIS HOMOGÉNEA (CSIC-UNIV. ZARAGOZA)	CATÁLISIS Y PROCESOS CATALÍTICOS

Por el presente documento informo que:

Tomo conocimiento de que las siguientes publicaciones en las que he participado como coautor, "Ecotoxicity studies of glycerol ethers in Vibrio fischeri: checking the greenness of glycerol-derived solvents", *Green Chem.* **2015**, *17*, 4326–4333, A., "Comparative Ecotoxicology Study of Two Neoteric Solvents: Imidazolium Ionic Liquid vs. Glycerol Derivative", *Ecotox. Environ. Saf.* **2016**, *132*, 429–434, "Ecotoxicity and QSAR studies of glycerol ethers in *Daphnia magna", Chemosphere,* **2017**, *183*, 277–285, y "Comparative ecotoxicity study of glycerol biobased solvents", *Environ. Chem.,* **2017**, *14*, en prensa, van a ser utilizadas como parte de la Tesis Doctoral "CARACTERIZACIÓN ECOTOXICOLÓGICA DE NUEVOS DISOLVENTES DERIVADOS DEL GLICEROL", escrita por Eduardo Pablo Perales Sarría, la cual se presenta por compendio de publicaciones.

Declaro que dichas publicaciones no han sido previamente utilizadas como parte de otra Tesis Doctoral.

Renuncio al derecho que como coautor pudiera corresponderme para el uso de las publicaciones en Tesis Doctorales propias y simultáneas a la indicada. (Nota: solo si procede y se desea el uso simultaneo y delimitado de las aportaciones de los coautores en sus respectivas tesis doctorales, deberá completarse con acuerdo por escrito concretando el presente punto).

Por tanto, **AUTORIZO** el uso de estas publicaciones para esta Tesis Doctoral.

En Zaragoza, a 11 de septiembre de 2017

GARCIA LAUREIRO JOSE IGNACIO - DNI 17150616E

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Fecha: 2017.09.11 13:10:08 +02'00'

Firmado: José I. García Laureiro

Datos del coautor	
Apellidos	Nombre
PIRES EZQUERRA	MARÍA ELÍSABET
Universidad o Centro de Investigación	Departamento
UNIVERSIDAD ZARAGOZA	QUÍMICA ORGÁNICA

Por el presente documento informo que:

Tomo conocimiento de que las siguientes publicaciones en las que he participado como coautor, "Ecotoxicity studies of glycerol ethers in Vibrio fischeri: checking the greenness of glycerol-derived solvents", *Green Chem.* **2015**, *17*, 4326–4333, A., "Ecotoxicity and QSAR studies of glycerol ethers in *Daphnia magna", Chemosphere,* **2017**, *183*, 277–285, y "Comparative ecotoxicity study of glycerol biobased solvents", *Environ. Chem.,* **2017**, *14*, en prensa, van a ser utilizadas como parte de la Tesis Doctoral "CARACTERIZACIÓN ECOTOXICOLÓGICA DE NUEVOS DISOLVENTES DERIVADOS DEL GLICEROL", escrita por Eduardo Pablo Perales Sarría, la cual se presenta por compendio de publicaciones.

Declaro que dichas publicaciones no han sido previamente utilizadas como parte de otra Tesis Doctoral.

Renuncio al derecho que como coautor pudiera corresponderme para el uso de las publicaciones en Tesis Doctorales propias y simultáneas a la indicada. (Nota: solo si procede y se desea el uso simultaneo y delimitado de las aportaciones de los coautores en sus respectivas tesis doctorales, deberá completarse con acuerdo por escrito concretando el presente punto).

Por tanto, **AUTORIZO** el uso de estas publicaciones para esta Tesis Doctoral. En Zaragoza, a 11 de septiembre de 2017

Firmado: Elísabet Pires

Datos del coautor	
Apellidos	Nombre
Navarro Rodríguez	Enrique
Universidad o Centro de Investigación	Departamento
Ins. Pirenaico de Ecología	Biodiversidad y Restauración

Por el presente documento informo que:

Tomo conocimiento de que las siguientes publicaciones en las que he participado como coautor:

Comparative ecotoxicity study of glycerol biobased solvents. Eduardo Perales, Cristina Belén García, Laura Lomba, José Ignacio García, Elísabet Pires, Mari Carmen Sancho, Enrique Navarro, Beatriz Giner. *In press in Environmental Chemistry (http://www.publish.csiro.au/EN/justaccepted/EN17082)*

va a ser utilizada como parte de la Tesis Doctoral "CARACTERIZACIÓN ECOTOXICOLÓGICA DE NUEVOS DISOLVENTES DERIVADOS DEL GLICEROL", escrita por Eduardo Pablo Perales Sarría, la cual se presenta por compendio de publicaciones.

Declaro que dichas publicaciones no han sido previamente utilizadas como parte de otra Tesis Doctoral.

Renuncio al derecho que como coautor pudiera corresponderme para el uso de las publicaciones en Tesis Doctorales propias y simultáneas a la indicada. (Nota: solo si procede y se desea el uso simultaneo y delimitado de las aportaciones de los coautores en sus respectivas tesis doctorales, deberá completarse con acuerdo por escrito concretando el presente punto).

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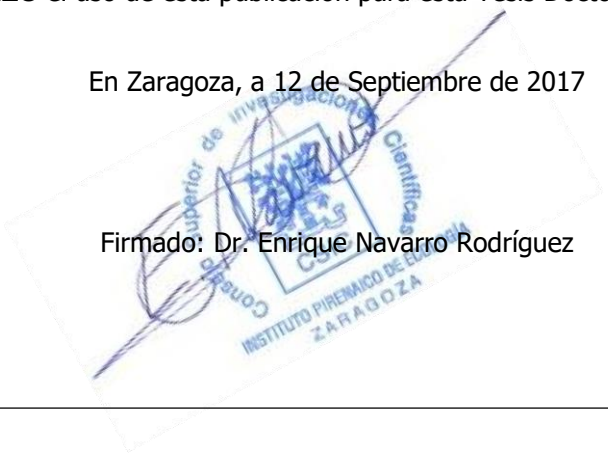
En Zaragoza, a 12 de Septiembre de 2017



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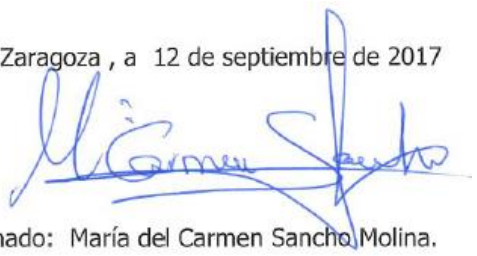

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