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FROM EFFECT-DIRECTED ANALYSIS TO METABOLOMIC ASSESSMENT:

HOW DO THE MAIN EMERGING CONTAMINANTS RELEASED INTO THE ADOUR ESTUARY AFFECT GLASS EELS (*Anguilla anguilla*)?

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ABSTRACT

Since countless xenobiotic compounds are being found in the environment, ecotoxicology faces an astounding challenge in identifying toxicants. The combination of high-throughput *in vivo/in vitro* bioassays with high-resolution chemical analysis is an effective way to elucidate the cause-effect relationship. However, these combined strategies imply an enormous workload that can hinder their implementation in routine analysis. The first aim of this thesis was to develop a new high throughput screening method to implement the sea urchin embryo test in effect-directed analysis. This way, we developed a novel predictive expert system, the SETApp, which can be used to automatically quantify the two endpoints of the sea urchin embryo test from a given image set. We demonstrated that chemometrics, and especially multivariate linear classification models, can be successfully implemented in bioassay automation to avoid the cumbersome measurement of embryo sizes and malformation levels. In addition, we have also shown the efficiency of this HTS in a very demanding scenario, the EDA of Bayonne's (Basque Country, France) Pont de l'aveugle WWTP effluent. This EDA study concluded that the SETApp is an efficient, fast, cost-effective, and reproducible tool that can approach EDA to routine analysis.

On the other hand, the presence of these contaminants of emerging concern (CECs) in the aquatic environment directly impacts water-living organisms and can alter their living functions. These compounds are often metabolized and excreted, but they can also be accumulated and spread through the food chain. The metabolized contaminants can also lead to the formation of new compounds with unknown toxicity and bioaccumulation potential. In the second study of this work, we studied the occurrence, bioconcentration, and biotransformation of CECs in glass eels (Anguilla anguilla) using UHPLC-HRMS. To select the target CECs, we first carried out an environmental risk assessment of the aforementioned WWTP effluent, which releases directly into the Adour estuary. The risk quotients of every detected contaminant were calculated and three ecotoxicologically relevant contaminants were chosen to perform the exposure experiment: propranolol, diazepam, and irbesartan. An experiment of 14 days consisting of 7 days of exposure and 7 days of depuration was carried out to measure the bioconcentration of the chosen compounds. The quantitative results of the concentrations in glass eel showed that diazepam and irbesartan reached a bioconcentration factor (BCF) ≈10 on day 7, but both compounds were eliminated after 7 days of depuration. On the other hand, propranolol's concentration remains constant all along with the experiment, and its presence can be detected even in the non-exposed control group, which might suggest environmental contamination. Two additional suspect screening strategies were used to identify metabolization products of the target compounds and other xenobiotics already present in wild glass eels. Only one metabolite was identified, nordiazepam, a well-known diazepam metabolite, probably due to the low metabolic rate of glass eels at this stage. The xenobiotic screening also confirmed the presence of more xenobiotics in wild glass eels, prominent among them, the pharmaceuticals exemestane, primidone, iloprost, and norethandrolone.

Since glass eels are continuously exposed to contamination throughout their migratory journey through the estuaries, to a certain extent the fall in the population of this endangered species may be due to this exposure, which is especially acute in estuaries under high urban pressure. Metabolomics was used in the third study of this thesis to assess the effects of the previously selected contaminants, diazepam, and irbesartan, on glass eels. An exposure experiment to diazepam, irbesartan, and their mixture was carried out over 7 days followed by 7 days of depuration phase. After exposure, glass eels were individually killed using a lethal bath of anaesthesia, and then an unbiased sample extraction method was used to extract separately the polar metabolome and the lipidome. The polar metabolome was submitted to targeted and non-targeted analysis, whereas for the lipidome only the non-targeted analysis was carried out. A combined strategy using partial least squares discriminant analysis and statistical analysis (ANOVA, t-t,est and fold-change analysis) was used to identify the metabolites altered in the exposed groups with respect to the control group. The results of the polar metabolome analysis revealed that glass eels exposed the to diazepam-irbesartan mixture were the most impacted ones, with altered levels for 11 metabolites, some of them belonging to the energetic metabolism, which was confirmed to be sensitive to these contaminants. Additionally, seven lipids were also found dysregulated after the exposure to the mixture. In the same vein, the same metabolomics approach was also used in the second part of the third study to find the differences in the metabolic profile of the behavioural phenotypes of glass eels. Non-migrant glass eels showed lower overall metabolic values than migrant glass eels, and the lipidome analysis also showed alterations in the levels of eight cholesterol esters. These two findings offer new research perspectives to explain this facultative migration of glass eels and confirm the suitability of metabolomics in attempting to explain the differences between migrant and non-migrant glass eels.

RESUMEN

Dada la presencia de innumerables compuestos xenobióticos en el medio ambiente, la ecotoxicología se enfrenta a un reto asombroso en la identificación de tóxicos. La combinación de bioensayos *in vivo/in vitro* de alto rendimiento con análisis químicos de alta resolución es una forma eficaz de dilucidar la relación causa-efecto. Sin embargo, estas estrategias combinadas implican una enorme carga de trabajo que puede dificultar su aplicación en el análisis de rutina. El primer objetivo de esta tesis fue desarrollar un nuevo método de alto rendimiento (*high-throughput screening,* HTS) para implementar el bioensayo con embriones de erizo de mar (SET) en estrategias de análisis dirigido a efectos. De este modo, desarrollamos un novedoso sistema predictivo, la SETApp, que puede utilizarse para cuantificar automáticamente los dos *endpoints* del SET a partir de un conjunto de imágenes. Demostramos que la quimiometría, y especialmente los modelos de clasificación lineal multivariante, pueden implementarse con éxito en la automatización de bioensayos para evitar la tediosa medición de los tamaños de los embriones y los niveles de malformación. Además, también hemos demostrado la eficacia de este HTS en un escenario muy exigente, el EDA del efluente de la EDAR de Pont de l'aveugle de Bayona (País Vasco, Francia). Este estudio concluyó que la SETApp es una herramienta eficiente, rápida, rentable y reproducible que puede acercar el EDA a los análisis de rutina.

Por otra parte, la presencia de estos contaminantes de interés emergente (CECs) en el medio acuático afecta directamente a los organismos acuáticos y puede alterar sus funciones vitales. Estos compuestos suelen ser metabolizados y excretados, pero también pueden acumularse y propagarse a través de la cadena alimentaria. Los contaminantes metabolizados también pueden dar lugar a la formación de nuevos compuestos con un potencial de toxicidad y bioacumulación desconocido. En el segundo capítulo de este trabajo, hemos estudiado la aparición, bioconcentración y biotransformación de algunos CECs en angulas (Anguilla anguilla) mediante UHPLC-HRMS. Para seleccionar los CECs objetivo, primero llevamos a cabo una evaluación del riesgo ambiental del mencionado efluente de la EDAR, que vierte directamente en el estuario del Adur. Se calcularon los cocientes de riesgo de cada contaminante detectado y se eligieron tres contaminantes ecotoxicológicamente relevantes para realizar el experimento de exposición: propranolol, diazepam e irbesartán. Se llevó a cabo un experimento de 14 días que consistió en 7 días de exposición y 7 días de depuración para medir la bioconcentración de los compuestos elegidos. Las concentraciones en angula mostraron que el diazepam y el irbesartán alcanzaron un factor de bioconcentración (BCF) ≈ 10 en el día 7, pero ambos compuestos se eliminaron tras 7 días de depuración. Por otro lado, la concentración de propranolol se mantiene constante a lo largo del experimento, y su presencia puede detectarse incluso en el grupo de control no expuesto, lo que podría sugerir la existencia de contaminación ambiental. Se utilizaron dos estrategias adicionales de análisis no dirigido para identificar productos de metabolización de los compuestos objetivo y otros xenobióticos ya presentes en las angulas salvajes. Sólo se identificó un metabolito, el nordiazepam, un conocido metabolito del diazepam, probablemente

debido a la baja tasa metabólica de las angulas en esta fase. El análisis de xenobióticos también confirmó la presencia de más xenobióticos en las angulas salvajes, destacando entre ellos los productos farmacéuticos exemestano, primidona, iloprost y noretandrolona.

Dado que las angulas están continuamente expuestas a la contaminación a lo largo de su viaje migratorio por los estuarios, en cierta medida el descenso de la población de esta especie en peligro de extinción puede deberse a esta exposición, que es especialmente aguda en los estuarios sometidos a una alta presión urbana. En el tercer y último capítulo de esta tesis se utilizó la metabolómica para evaluar los efectos de los contaminantes previamente seleccionados, el diazepam y el irbesartán, en las angulas. Se llevó a cabo un experimento de exposición al diazepam, al irbesartán y a su mezcla durante 7 días, seguido de 7 días de fase de depuración. Después de la exposición, las angulas se sacrificaron individualmente utilizando un baño letal de anestesia y luego se utilizó un método de extracción de muestras no sesgado para extraer por separado el metaboloma polar y el lipidoma. El metaboloma polar se sometió a un análisis dirigido y no dirigido, mientras que para el lipidoma sólo se llevó a cabo el análisis no dirigido. Para identificar los metabolitos alterados en los grupos expuestos con respecto al grupo de control se utilizó una estrategia combinada de análisis discriminante de mínimos cuadrados parciales y de análisis estadístico (ANOVA, prueba t y análisis de cambio o *fold change*). Los resultados del análisis del metaboloma polar revelaron que las angulas expuestas a la mezcla diazepam-irbesartán fueron las más impactadas, con niveles alterados para 11 metabolitos, algunos de ellos pertenecientes al metabolismo energético, por lo que se pudo confirmar que esta ruta metabólica es sensible a estos contaminantes. Además, también se encontraron siete lípidos desregulados tras la exposición a la mezcla. En la misma línea, el mismo enfoque metabolómico se utilizó también en la segunda parte del tercer capítulo para encontrar las diferencias en el perfil metabólico de los dos fenotipos de comportamiento de las angulas. Las angulas no migrantes mostraron valores metabólicos globales más bajos que las angulas migrantes, y el análisis del lipidoma también mostró alteraciones en los niveles de ocho ésteres de colesterol. Estos dos hallazgos ofrecen nuevas perspectivas de investigación para explicar esta migración facultativa de las angulas y confirman la idoneidad de la metabolómica para intentar explicar las diferencias entre las angulas migrantes y no migrantes.

LABURPENA

Ingurumenean konposatu xenobiotiko ugari daudenez, ekotoxikologiak erronka harrigarria du toxikoak identifikatzeko orduan. Kausa-efektu erlazioa argitzeko modu eraginkorra errendimendu handiko *in vivo/in vitro* biosaiakuntzak bereizmen handiko analisi kimikoekin konbinatzea da. Hala ere, estrategia konbinatu horiek lan handia eskatzen dute, eta horrek zaildu egin dezake estrategia horiek ohiko analisian aplikatzea. Tesi honen lehen helburua errendimendu handiko metodo berri bat garatzea izan zen (high-throughput screening, HTS), itsas trikuen enbrioiekin biosaiakuntza (SET) efektuei bideratutako estrategietan ezartzeko. Hala, SETApp izeneko aditu-sistema prediktibo berri bat garatu dugu, irudi-multzo batetik abiatuta SET-aren bi endpointak automatikoki kuantifikatzeko erabil daitekeena. Kimiometria, eta bereziki aldagai anitzeko sailkapen linealeko ereduak, biosaiakuntzen automatizazioan arrakastaz ezar daitezkeela frogatu dugu, enbrioien tamainak eta malformazio-mailak eskuz ez neurtzeko. Gainera, HTS horrek konplexutasun handiko egoera batean duen eraginkortasuna frogatu dugu, Baionako (Euskal Herria, Frantzia) Pont de l'aveugle ur araztegiaren efluentearen EDA. Azterketa horren arabera, SETApp tresna eraginkorra, azkarra, errentagarria eta erreproduzigarria da, eta EDA ohiko analisietara hurbil dezake.

Bestalde, uretan kutsatzaile emergente (CEC) horiek agertzeak zuzeneko eragina du uretako organismoetan, eta haien bizi-funtzioak alda ditzake. Konposatu horiek metabolizatu eta iraizten dira, baina elikadura-katearen bidez ere metatu eta heda daitezke. Metabolizatutako kutsatzaileek, halaber, toxikotasun eta biokontzentrazio potentzial ezezaguneko konposatu berriak sor ditzakete. Lan honen bigarren kapituluan, CEC batzuen agerpena, biokontzentrazioa eta bioeraldaketa aztertu ditugu anguletan (Anguilla anguilla), UHPLC-HRMS bidez. Helburuko CECak hautatzeko, lehenik eta behin, aurretik aipatutako ur araztegiko efluentearen ingurumen-arriskuaren ebaluazioa egin genuen, zuzenean Aturriko estuarioan isurtzen dena. Detektatutako kutsatzaile bakoitzaren arrisku-kozienteak kalkulatu ziren eta garrantzi ekotoxikologiko handieneko hiru kutsatzaile aukeratu ziren esposizio-esperimentua egiteko: propranolol, diazepam eta irbesartan. Aukeratutako konposatuen biokontzentrazioa neurtzeko, 14 eguneko esperimentua egin zen: esposizioko 7 egun eta garbiketako 7 egun. Anguletan topatutako kontzentrazioen arabera diazepam eta irbesartan kutsatzaileak 10 bider kontzentratu ziren (biokontzentrazio-faktorea, BCF = 10), baina bi konposatuak 7 eguneko arazketaren ondoren organismotik garbitzen ziren. Bestalde, propranolol kontzentrazioa konstante mantendu zen esperimentuan zehar, kontrol-taldea barne, eta horrek ingurumen-kutsadura dagoela iradoki lezake. Susmagarrien analisian oinarritutako beste bi estrategia erabili ziren helburu-konposatuen metabolizazio-produktuak eta angula basatietan jada agertzen diren beste xenobiotiko batzuk identifikatzeko. Metabolito bat bakarrik identifikatu zen, nordiazepama, diazepamaren metabolito ezaguna, fase horretan angulek duten tasa metaboliko txikiaren ondorioz ziurrenik. Xenobiotikoen analisiak angula basatietan xenobiotiko gehiago zeudela baieztatu zuen, eta horien artean nabarmentzekoak dira

produktu farmazeutiko batzuk, hala nola, exemestane, primidona, iloprost eta noretandrolona.

Estuarioetatik migrazio-bidaian zehar angulak etengabe kutsatuta daudenez, desagertzeko arriskuan dagoen espezie honen populazioaren beherakada, neurri batean, esposizio horren ondorio izan daiteke, bereziki larria dena hiri-presio handiaren eraginpean dauden estuarioetan. Tesi honen hirugarren eta azken kapituluan, metabolomika erabili zen aurrez aukeratutako kutsatzaileek, diazepamak eta irbesartanak, anguletan duten eragina aztertzeko. Esposizio esperimentu berri bat egin zen, angulak diazepam, irbesartan eta haien nahasketarekin 7 egunez esposatuz eta, ondoren, 7 eguneko arazketa-fasea utziz. Esposizioaren ondoren, angulak bakarka hil ziren anestesiazko bainu hilgarri bat erabiliz, eta, ondoren, laginak erauzteko zuzendu gabeko metodo bat erabili zen, metaboloma polarra fase urtsuan eta lipidoma fase organikoan bananduz. Metaboloma polarra analisi zuzendua eta zuzendu gabekoaren bidez aztertu zen; lipidomaren kasuan, aldiz, bideratu gabeko analisia bakarrik egin zen. Esposatutako taldeetan kontrol-taldearekiko desberdinak ziren metabolitoak identifikatzeko, estrategia konbinatu bat erabili zen: minimo karratu partzialen analisi bereizlea eta analisi estatistikoa (ANOVA, t proba eta aldaketa analisia). Metaboloma polarraren analisiaren emaitzen arabera, diazepam-irbesartan nahastearen eraginpean zeuden angulak izan ziren kaltetuenak, eta 11 metabolitorentzako maila aldatuak izan zituzten. Metabolito horietako batzuk energia-metabolismokoak ziren, bide metaboliko hau kutsatzaile horiekiko sentikorra dela egiaztatuz. Horrez gain, nahastearen eraginpean egon ondoren, zazpi lipido ere aurkitu ziren esposizio taldean maila aldatuekin. Ildo beretik, hirugarren kapituluaren bigarren zatian ere ikuspegi metabolomiko bera erabili zen angulen bi portaera-fenotipoen profil metabolikoen desberdintasunak aurkitzeko. Angula ez-migratzaileek, oro har, angula migratzaileek baino maila metaboliko txikiagoak izan zituzten, eta lipidomaren azterketak aldaketak erakutsi zituen zortzi kolesterol-esterren mailetan. Bi aurkikuntza horiek ikerketa-ikuspegi berriak eskaintzen dituzte angulen migrazio fakultatibo hori azaltzeko, eta angula migratzaileen eta ez-migratzaileen arteko desberdintasunak azaltzen saiatzeko metabolomikaren egokitasuna berresten dute.

RESUME

Étant donné que d'innombrables composés xénobiotiques se retrouvent dans l'environnement, l'écotoxicologie doit relever de nombreux défis pour identifier les substances toxiques. La combinaison de bioessais *in vivo/in vitro* à haut débit et d'analyses chimiques à haute résolution est un moyen efficace d'élucider la relation de cause à effet. Cependant, ces stratégies combinées impliquent souvent des procédures fastidieuses qui peuvent entraver leur mise en œuvre dans la cadre d'analyses de routine. Le premier objectif de cette thèse était de développer une nouvelle méthode de criblage à haut débit pour mettre en œuvre le test sur des embryons d'oursin dans le cadre d'analyses sur les effets directs (EDA). Nous avons ainsi développé un nouveau système expert prédictif, le SETApp, qui peut être utilisé pour quantifier automatiquement les deux paramètres du test sur les embryons d'oursin à partir d'un ensemble d'images. Nous avons démontré que la chimiométrie, et en particulier les modèles de classification linéaire multivariée, peuvent être mis en œuvre avec succès dans l'automatisation des essais biologiques pour éviter la mesure fastidieuse de la taille des embryons et des niveaux de malformation. En outre, nous avons également démontré l'efficacité de ce SETapp dans un scénario très exigeant, l'analyse EDA de l'effluent de la station d'épuration de Pont de l'aveugle à Bayonne (Pays Basque, France). Cette étude EDA a conclu que le SETApp est un outil efficace, rapide, rentable et reproductible qui peut approcher l'EDA à l'analyse de routine.

D'autre part, la présence de ces contaminants émergents (CECs) dans l'environnement aquatique a un impact direct sur les organismes vivant dans l'eau et peut altérer leurs fonctions vitales. Ces composés sont souvent métabolisés et excrétés, mais ils peuvent aussi s'accumuler et se bioamplifier dans la chaîne alimentaire. Les contaminants métabolisés peuvent également conduire à la formation de nouveaux composés dont la toxicité et le potentiel de bioaccumulation sont inconnus. Dans le deuxième chapitre de ce travail, nous avons étudié l'occurrence, la bioconcentration et la biotransformation des CECs dans les civelles (Anguilla anguilla) en utilisant la technique UHPLC-HRMS. Pour sélectionner les CECs cibles, nous avons d'abord effectué une évaluation des risques environnementaux de l'effluent de la station d'épuration susmentionnée, qui se déverse directement dans l'estuaire de l'Adour. Les quotients de risque de chaque contaminant détecté ont été calculés et trois contaminants pertinents du point de vue écotoxicologique ont été choisis pour réaliser l'expérience d'exposition : le propranolol, le diazépam et l'irbesartan. Une expérience de 14 jours consistant en 7 jours d'exposition et 7 jours de dépuration a été réalisée pour mesurer la bioconcentration des composés choisis. Les résultats quantitatifs des concentrations dans la civelle ont montré que le diazépam et l'irbésartan ont atteint un facteur de bioconcentration (BCF) proche de 10 au jour 7, mais les deux composés ont été éliminés après 7 jours de dépuration. En revanche, la concentration de propranolol reste constante tout au long de l'expérience, et sa présence peut être détectée même dans le groupe témoin non exposé, ce qui pourrait suggérer une contamination environnementale. Deux autres stratégies de screening ont été utilisées pour identifier les produits de métabolisation des composés cibles et d'autres xénobiotiques déjà

présents dans les civelles sauvages. Un seul métabolite a été identifié, le nordiazépam, un métabolite bien connu du diazépam, probablement en raison du faible taux métabolique des civelles à ce stade. Le dépistage des xénobiotiques a également confirmé la présence d'autres xénobiotiques dans les civelles sauvages, notamment les produits pharmaceutiques exémestane, primidone, iloprost et noréthandrolone.

Comme les civelles sont continuellement exposées à une contamination tout au long de leur parcours migratoire dans les estuaires, dans une certaine mesure, la chute de la population de cette espèce menacée pourrait être due à cette exposition, qui est particulièrement aiguë dans les estuaires soumis à une forte pression urbaine. La métabolomique a été utilisée dans le troisième et dernier chapitre de cette thèse pour évaluer les effets des contaminants sélectionnés précédemment, le diazépam et l'irbesartan, sur les civelles. Une expérience d'exposition au diazépam, à l'irbesartan et à leur mélange a été réalisée pendant 7 jours suivis de 7 jours de phase de dépuration. Après l'exposition, les civelles ont été tuées individuellement à l'aide d'un bain létal d'anesthésie, puis une méthode d'extraction d'échantillons non biaisée a été utilisée pour extraire séparément le métabolome polaire et le lipidome. Le métabolome polaire a été soumis à une analyse ciblée et non ciblée, tandis que pour le lipidome, seule l'analyse non ciblée a été réalisée. Une stratégie combinée utilisant l'analyse discriminante des moindres carrés partiels et l'analyse statistique (ANOVA, t-test et analyse des changements de plis) a été utilisée pour identifier les métabolites altérés dans les groupes exposés par rapport au groupe témoin. Les résultats de l'analyse du métabolome polaire ont révélé que les civelles exposées au mélange diazépam-irbesartan étaient les plus touchées, avec des niveaux altérés pour 11 métabolites, certains d'entre eux appartenant au métabolisme énergétique, qui a été confirmé comme étant sensible à ces contaminants. De plus, sept lipides ont également été dérégulés après l'exposition au mélange. De manière similaire, la même approche métabolomique a été utilisée dans la deuxième partie du troisième chapitre pour trouver les différences dans le profil métabolique des deux phénotypes comportementaux des civelles. Les civelles non migrantes ont montré des valeurs métaboliques globales inférieures à celles des civelles migrantes, et l'analyse du lipidome a également montré des altérations dans les niveaux de huit esters de cholestérol. Ces deux résultats offrent de nouvelles perspectives de recherche pour expliquer cette migration facultative des civelles et confirment la pertinence de la métabolomique pour tenter d'expliquer les différences entre les civelles migrantes et non migrantes.

ABBREVIATIONS

ACN	*Acetonitrile*
ADP	*Adenosine 5'-diphosphate*
AMP	*Adenosine 5'-monophosphate*
ANOVA	*Analysis of variance*
APCI	*Atmospheric pressure chemical ionization*
APPI	*Atmospheric pressure photoionization*
BCF	*Bioconcentration factor*
CD	*Compound discoverer*
CE	*Cholesterol esters*
CEC	*Contaminant of emerging concern*
DDA	*Data-dependant acquisition*
DIA	*Data-independent acquisition*
DMSO	*Dimethyl sulfoxide*
EC	*Emerging contaminant*
EDA	*Effect-directed analysis*
EDC	*Endocrine disrupting compound*
EPA	*Environmental protection agency*
EQS	*Environmental quality standards*
ERA	*Environmental risk assessment*
ESI	*Electrospray ionization*
FC	*Fold change*
FTICR-MS	*Fourier Transform Ion Cyclotron Resonance Mass Spectrometers*
FWHM	*Full widths at half maximum*
G6P	*D-Glucose-6-phosphate*
GABA	*Gamma-aminobutyric acid*
GC	*Gas chromatography*
GSH	*Glutathione reduced*
GSSG	*Glutathione oxidized*
HCD	*Higher-energy collision dissociation*
HESI	*Heated ESI*
HILIC	*Hydrophilic interaction chromatography*
HMDB	*Human metabolome database*
hpf	*Hours post-fertilization*
HPLS-DA	*Hierarchical partial least squares discriminant analysis*
HRMS	*High-resolution mass spectrometry*

HTS	*High-throughput screening*
ICIS	*Intelligent Component Integration System*
IPA	*2-propanol*
IT	*Index of toxicity*
LC	*Liquid chromatography*
LV	*Latent variable*
LV-SPE	*Large-volume solid phase extraction*
m/z	*Mass to charge*
MEC	*Measured experimental concentration*
MTBE	*Methyl tert-butyl ether*
NCE	*Normalized collision energy*
OPLS-DA	*Orthogonal partial least squares discriminant analysis*
PA	*Phosphatidic acids*
PC	*Phosphatidylcholine*
PCA	*Principal component analysis*
PC-O	*Oxidized phosphatidylcholine*
PE	*Phosphatidylethanolamines*
PFAS	*Perfluoroalkyl substances*
PI	*Phosphatidylinositol*
PLS-DA	*Partial least squares discriminant analysis*
PNEC	*Predicted no-effect concentration*
PS	*Phosphatidylserine*
ROC	*Receiver operating characteristic*
RQ	*Risk quotient*
RTI	*Retention Time Indices*
SET	*Sea urchin embryo test*
SNTS	*Suspect and non-targeted screening*
SPE	*Solid phase extraction*
SPLS-DA	*Sparse partial least squares discriminant analysis*
SWOT	*Strengths, Weaknesses, Opportunities, and Threats*
TG	*Triglyceride*
TIE	*Toxicity identification evaluation*
TOF	*Time-of-flight*
TP	*Transformation product*
UHPLC	*Ultra-high performance liquid chromatography*
VIE	*Visible Implant Elastomer*
VIP	*Variable importance in projection*

WFD *Water framework directive*
WHO *World health organization*
WWTP *Wastewater treatment plant*

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1. INTRODUCTION

“Wanderer above the Sea of Smog”

by Caspar David Friedrich

1.1 SUMMARY

Throughout this thesis, I will show and discuss environmentally relevant issues closely linked to the elucidation of toxic contaminants in complex samples. The core work aims to provide insight into this analytical and ecotoxicological challenge and it goes from the contribution to the automation of effect-based analytical approaches, to the understanding of the mechanism of action of prioritized contaminants by metabolomic approaches. Although the work was not intended to follow a straight path, from the occurrence to the toxic effects, we have emphasized this approach to accommodate an appropriate narrative. Therefore, the initial work details the development of an image analysis-based tool to support the application of a standard bioassay in effect-based contaminant identification strategies. In fact, this image-analysis tool became a freely available app key to supporting the effect-directed analysis of one of two main wastewater treatment plants releasing the effluents to the Adour (Aturri) estuary. The combination of this analysis and the environmental risk assessment of the effluent allowed us to face the study of the effects that the main contaminants released to one estuary might lead to the European glass eels (*Anguilla anguilla*). In fact, based on controlled exposures, we were able to study the fate of some selected contaminants in glass eels, assess their effects, and understand observed behaviour that might explain their migration patterns in the estuary.

This work was performed in the framework of a cotutelle PhD between the UPPA and UPV/EHU through the collaboration of IBeA and IPREM research groups. The rationale of the work was to join the experiences in eel behaviour and the analysis of contaminants of emerging concern to carry out this work.

Comentado [i.1]: Komentatu Mathilderi zelan jarri

Comentado [MM2]: I don't know in spain but in france we are used to add figures/illustrations in the intro section

Comentado [MM3]: ¿??

1.2 CONTAMINANTS OF EMERGING CONCERN (CECs)

It is nothing new to say that in the last century we have brought the Earth to an unsustainable point that is causing serious problems for the ecosystems as we know them. From the human and environmental health perspective, we now must face some threats that can be readily seen such as climate change or biodiversity loss as well as some others that may not be directly perceived but have equally or even greater harmful implications. One of these invisible threats, and the one concerning us in this work, is chemical pollution, which has been recently reported by the WHO ("Preventing disease through healthy environments," n.d.) and The Lancet commission (Landrigan et al., 2018) as a crisis of utmost urgency. The former report estimates that environmental risk factors such as air, water, and soil pollution, chemical exposure, climate change, and ultraviolet radiation, contribute to 23% of all deaths and are closely connected to noncommunicable diseases (i.e., cancer, cardiovascular disease, diabetes, respiratory disease, and mental health) (Herrick, 2020). In 2005, Christian Wild called for the need to develop new methods to measure an individual's environmental exposure and its consequences (Cp, 2005). This wild claim gave birth to a new term, the exposome. The exposome encompasses the impact of not only

chemical exposures, but also infections, radiation, and other factors such as the microbiome, metabolism, and lifestyle.

Although the exposome term was conceived to refer to the impact on humans, it is important to recognize the interactions between humans, animals, and our environment when assessing the evolution and emergence of health threats. This thesis has been developed and written during a pandemic that stresses the importance of that interaction, COVID-19 (Trilla, 2020), caused by a zoonotic virus spread between people and animals. Another important term emerged from the need to establish new approaches to address the complexity and interdisciplinarity nature of interactions between biology and medicine on the one hand, and public health, social sciences, economics, and political sciences on the other. This way the "One Health" ("One Health," n.d.) concept was coined and new calls for action have been claimed (Brack et al., 2022; Wang et al., 2021). It is worth mentioning that I am writing this chapter in the middle of one of the hottest summers since we have records, and that the chances to face a harsh winter are part of the daily breaking news. This scenario opens greenwashing issues closely linked to the "One Health" such as whether the use of fossil fuels (coal) is convenient, or if nuclear energy is a green source.

In addition to this, within the context of chemical pollution and the environmental risk perception, some pollutants still have a greater impact on people's minds while many others have emerged out of the blue. These trends have been driven by several factors including developments in health, agriculture, manufacturing, etc., as well as political and economic factors that have contributed to our well-being. However, thanks to the scientific and technological development in chemical analysis, ecotoxicology and risk assessment, we are now more aware than ever about the trade-offs of this wealth (Rockström et al., 2009). At the beginning of the last century, the spotlight was put on air pollution and heavy metal pollution that was inherited from the previous decades. Later, with the release of a certain book, society became
organic chemicals released into the environment could bec
has always surfed between two waves, sometimes adaptir
and sometimes exposing those needs. The former is clear from the examples mentioned above, and great evidence of the latter is the recently introduced contaminants of emerging concern (CECs). Since chemical pollution is not perceptible in most cases, we have previously referred to it as the invisible threat, being so, contaminants of emerging concern are not only invisible, but also non-expected, overlooked and missed (Daughton, 2004). Most of them are found in such low concentrations that cannot be detected even with the most advanced instrumentation. This heterogeneous group includes chemicals with very different properties with two common characteristics: they are unregulated, and the scientific community cannot guarantee that they are not hazardous to the environment (Diamond and Burton Jr., 2021). Though some other contaminant families (e.g., rare earth elements (Gwenzi et al., 2018), microplastics… (Lambert and Wagner, 2018)) could fit the definition, the CECs that will be mentioned in this work are organic micropollutants that can be

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primarily classified between personal care products, pharmaceuticals, and industrial products (Diamond and Burton Jr., 2021).

Due to the growing use of these chemicals and the wider occurrence of CECs shown in the literature, the effect of CECs on the environment and human health has become one of the most complex environmental problems of this decade (Landrigan et al., 2018). Different public bodies such as European Environmental Agency or the US Environmental Protection Agency have increased their concern and included some of these contaminants in their regulatory water directives (e.g., the European Water Framework Directive, WFD) for their monitorization. These entities have also created the so-called watch lists which collect contaminant candidates for future incorporation in the monitorization directives. Among these, we find a large representation of pharmaceutical products including antibiotics (e.g., amoxicillin, azithromycin, ciprofloxacin…), natural and synthetic hormones (e.g., estrone, 17-beta-estradiol…) non-steroidal anti-inflammatories (e.g., diclofenac…), as well as industrial products such as perfluoroalkyl substances (PFAS) (e.g., perfluorooctanoic acid and perfluorooctane sulfonic acid) and plasticizers (e.g., nonylphenols…), or pesticides and pesticide by-products (e.g., 3-hydroxycarbofuran…) (Joint Research Centre (European Commission) et al., 2020; US EPA, 2015). These directives are all focused on water monitoring, which should not be surprising since the aquatic environment is typically the fate for many CECs and stands out as one of the most sensitive compartments.

Some of the main players in the release of emerging contaminants in the aquatic environment are the wastewater treatment plants (WWTPs). Though it may seem contradictory now, WWTPs are part of the successful achievements of urban development. WWTPs were designed and built to remove most of the organic wastes and mineral nutrients from sewages long before society was aware about the CECs. Now we know that WWTPs can barely remove CECs efficiently from wastewater, making their effluents an unwanted source of aquatic contamination (Loos et al., 2013). These effluents are discharged directly into rivers or coastal ecosystems where, depending on the dilution factor of the effluent with respect to the water body, they can have a greater or lesser impact on the environment. Additionally, the efficiency of the treatments used in WWTPs is highly pollutant-dependant, which makes it even more difficult to find standardized procedures for their removal (Bijlsma et al., 2021; Fernández-López et al., 2016; Papageorgiou et al., 2016). Since the concentrations of these contaminants in the environment are usually below the nanograms per millilitre levels and the detection limits can hinder their identification in other matrices, WWTP effluents are the most studied samples for their analysis. However, with the development of more sophisticated and sensitive analytical instrumentation, the analysis of CECs has been extended to a wide variety of matrices including biological matrices (Álvarez-Ruiz and Picó, 2020; Ziarrusta et al., 2016), sediment and soil samples (Ma et al., 2022; Vethaak et al., 2017), and even air (López et al., 2016; Röhler et al., 2021). This way, environmental analytical chemists have accepted the challenge, and new scientific papers report the occurrence of CECs at ever more remote locations and samples. CECs can be found in water and biota samples in Antarctica (Corsolini, 2009;

Comentado [MM5]: Not really scientific…

Comentado [i.6R5]: We have intentionally included some not very scientific terms in the thesis. I'd say that it is just to make it easier to read but it is probably just my way to write. Nestor has already changed almost all of these "licenses", but has allowed me to keep some of them. I have also prepared a post scriptum where I can write in my own terms.

Szopińska et al., 2022), in different shark species (Chynel et al., 2021), in several Himalayan rivers (Quincey et al., 2022), and, of course, in most of our estuaries (Mijangos et al., 2018).

Indeed, the most concerning problem with CECs is their continuous release into the environment. Since the release rate is equal or higher than the degradation rate, even for those compounds that are not supposed to be persistent in the environment, their levels are in a steady state that makes the accumulation an important issue. The scenario is even worse for some compounds with high persistency (e.g., PFAS, polychlorinated biphenyls…) (Cousins et al., 2022). Cutting the emissions of these compounds is no longer a solution. Therefore, their presence and effects will be with us for a long time. For all this, the scientific community agrees on the importance of assessing the effects that these compounds can cause at sub-lethal concentrations.

1.3 PRIORITIZATION STRATEGIES: ASSESSMENT OF T

Comentado [MM7]: I would split this section with several sub-sections
The idea is to give an overview of the different existing methoologies (bioassays, eda, set,…) showing advantages and drawbacks of each
Maybe a figure to present the different approach and summarizing advantages and drawbacks

Assessing the impact of CECs at environmentally relevant co
achieving an effective regulation. This means that we hav
certain compounds with the disruptive effects that may hap
from the molecular to the ecosystem level). The developm
tailor-made analytical methods or ecotoxicological bioassay
candidates seem, therefore, unachievable.

1.3.1. Traditional bioassay approaches

Many bioassays, both *in vivo* and *in vitro* have been developed and fine-tuned to understand the mechanisms of toxic action of the contaminants (Escher et al., 2021). *In vivo* bioassays using aquatic model organisms such as zebrafish, *daphnia magna,* etc., have been often used in water quality assessment as they offer a better understanding of the effect of the contaminant at the whole organism level. These toxicity tests can measure endpoints such as mortality, growth, and reproduction, but also more specific ones such as behaviour, specific malformations, or other sub-lethal effects. These model organisms used for toxicity testing are usually easy to maintain and manipulate, have physical traits that help measurements (e.g., transparent body), rapid development, high fecundity, and other characteristics favourable to scientific research. However, we must not forget that using these organisms is not suitable to assess the environmental health of any aquatic ecosystem. Since they have been adopted by several national bodies and have standardized guidelines, these toxicity tests are sometimes used questionably to assess the environmental health of ecosystems that are very different from the habitat of the organisms used (e.g., zebrafish, which is a tropical species, is often used to assess the health of marine coastal ecosystems). This work supports the use of autochthonous species as toxicity bioindicators to achieve a better representation of the ecosystem that is studied (Chen et al., 2019; Schreiber et al., 2018). On the other hand, *in vitro* tests offer an ethical

alternative to animal testing, using cultured bacterial, yeast, and even animal (including human) cells to test the toxicity of individual contaminants or mixtures. Cultured cells can also be genetically modified to enhance the sensitivity to specific endpoints. This way, *in vitro* tools are a sensitive, cost and time efficient, and reproducible alternative but can lead to false generalizations about an organism. *In vitro* bioassays are also the best solution to assess the human exposome and many tests have been developed to cover different mechanisms of action associated with toxicity in humans (Escher et al., 2021).

1.3.2 EDA approach

Both *in vivo* and *in vitro* tests are useful tools to assess the toxicity of a given contaminant, effluent, or any other mixture but in the latter case, they do not provide information about the cause of the effects observed. The ecotoxicological and analytical challenges lie in the complexity of these mixtures and the relationships between the presence of certain compounds and the toxicological endpoints (Krewski et al., 2010). Thus, these bioassays are often coupled with chemical analysis techniques to explain these cause-effect relationships. The best example of the combination of toxicity testing and chemical analysis is the strategy known as effect-directed analysis (EDA) (Brack et al., 2016). This strategy assumes that the toxicity of the sample is composed of the contribution of its components and that the toxicity of most of these components is negligible against the most toxic compounds. So, for a tested toxic sample, this approach first uses a separation technique, typically chromatography, to fractionate the sample, reducing its complexity and removing the fractions that do not test toxic in the selected bioassay. This process can be performed iteratively to remove the contaminants that do not contribute to the total toxicity of the sample and prioritize the ones that do contribute over them. The toxic fractions that remain at the end of this process are then analysed to identify the candidates for the observed toxicity. One might think that after this fractionation the identification of the toxic compounds should be straightforward, but the reality is that this identification is only possible thanks to very sophisticated analytical strategies that allow clarifying the composition of the fractions without the need for standards. This identification is commonly done using high-resolution mass spectrometry using non-target screening approaches, as we will discuss later.

EDA can be applied to just prioritize between contaminants and provide a compound list of possible candidates responsible for the total toxicity but often a deeper explanation of the contributions is sought. When that is the case, the standards of the identified compounds are used to test the individual and the mixture toxicity and the results are compared to the toxicity of the raw sample obtaining the actual contribution of each of the candidates in terms of different bioanalytical units (toxic units, bioanalytical equivalents…) (Escher et al., 2021).

The main drawback of EDA and generally effect-based strategies is that they are time-consuming and labour-intensive, which makes their implementation in routine

monitoring unfeasible. In this regard, implementing high-throughput screening (HTS) methods is crucial to deal with this elucidation (Villeneuve et al., 2019) and many HTS platforms have taken a big step toward deploying complex and laborious methodologies in routine analysis through *in vitro* (Arini et al., 2017; Jonkers et al., 2020) and in vivo (Letamendia et al., 2012) bioassays. The current trend in the implementation of HTS-EDA for contaminant monitoring includes the enhancement of both the fractionation and the toxicity testing using 96-well microplates to collect the fractions and run the bioassays. This is complemented with high-performance data processing workflows and in the most ambitious cases with the application of bioassay batteries to cover several mechanisms of action (Jonkers et al., 2022).

1.3.3. Sea urchin embryo test (SET) approach

As mentioned above, the use of local organisms can give a clearer picture of the impact of contaminants in the study area. In this sense, the sea urchin (*Paracentrotus lividus*) embryo test (SET) has arisen as a key *in vivo* assay for coastal marine ecosystems. Apart from being affordable for use and maintenance, many studies have proven their sensitivity to emerging contaminants (Gambardella et al., 2016; Vethaak et al., 2017) and the suitability of this bioassay in EDA strategies (Mijangos et al., 2020). The SET has been gaining importance in the last few years and more than 30 papers have been published since 2020 including the toxicity testing of crude and bunker oils (DeMiguel-Jiménez et al., 2022), sewage sludge-ash (Prabhakar et al., 2022), and micro- and nano-plastics (Manzo and Schiavo, 2022).

Two endpoints are typically measured by microscopic observation in this bioassay, the growth of the larvae and their malformation level. The former is easily calculated by measuring the size of eggs at the beginning of the experiment and the size of the larvae at 48 hours post-fertilization (hpf). The malformation level is given according to the four levels proposed by Carballeira et. al., (Carballeira et al., 2012), which would be: normal development (level 0), incorrect location of skeletal rods (level 1), incomplete or absence of skeletal rods (level 2), and blocked development (level 3). Every larva in the sample is given a value between 0 and 3 corresponding to the malformation level and the index of toxicity (IT) is calculated by applying the % of larvae found at every level as shown in equation 1. IT returns a value between 0 (best possible condition) and 3 (worst condition) and the toxicity measures can be calculated from dose-response curves as usually done in any toxicity test.

$$IT = \frac{0 \times \%level\ 0 + 1 \times \%level\ 1 + 2 \times \%level\ 2 + 3\ \times \%level\ 3}{100} \quad (1)$$

For statistical significance, at least 35 larvae need to be measured and annotated for malformation in each sample. This way, when the number of samples is too large it becomes cumbersome, not only because the measurements require long times, but also because the larvae need to be fixed and the shape of the larvae can be affected by formalin if the study takes too long. The second drawback of this test is that the classification of the larvae is based on the accumulated experience of the observer and

thus, the results may be biased. Since implementing SET on a routine basis to tackle high-throughput analysis demands a large number of assays, image analysis, and machine learning techniques may find their way towards the automation of this bioassay (Nyffeler et al., 2020).

1.4 AUTOMATION OF THE SET

Reducing times and eliminating user bias are two of the most common problems that are usually addressed by computational methods. The automation of the SET is a two-part puzzle, the two parts being its two endpoints. Measuring the growth of the larvae can be achieved by image analysis alone, but certainly, to be able to apply image analysis, first, an image is required. Indeed, having the instrumentation capable of automatically reading images from microplates was the main factor that pushed us to start the implementation of this HTS method.

For a lay reader, an image (in this case a greyscale image) is nothing but a matrix of numbers of X x Y dimensions (the dimensions of the picture) where each number corresponds to a pixel and is given an intensity that ranges from 0 (black) to 255 (white) (Figure 1.1). Thus, working with images follows the rules of linear algebra. Without going too deeply into the subtleties of image analysis, the most important technique used in our work is object detection. In each image we can find several larvae and, since the automation aims to measure and classify the larvae individually, first we need to detect each of them, and then extract them to start the individual analysis. That is achieved by object detection algorithms. The most advanced machine learning techniques include the use of deep learning, convolutional neural networks, support vector machines, among others (Kakishita et al., 2022; Kubera et al., 2022; Park et al., 2022). Since the larvae can be differentiated from the lighter background, pixel intensity based thresholding can also be helpful to distinguish the pixels that correspond to larvae from the background.

Comentado [NE8]: Lehen irakurketan sortu zitzaidan arazoa da asmatzea non dagoen muga spoiler bat ez egiteko. Horregatik, saiatu naiz geroko kontuak pixka bat urruntzen

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Comentado [i.10R9]: Kendu ditut egindakoaren spoilerra egiten zuten esaldiak

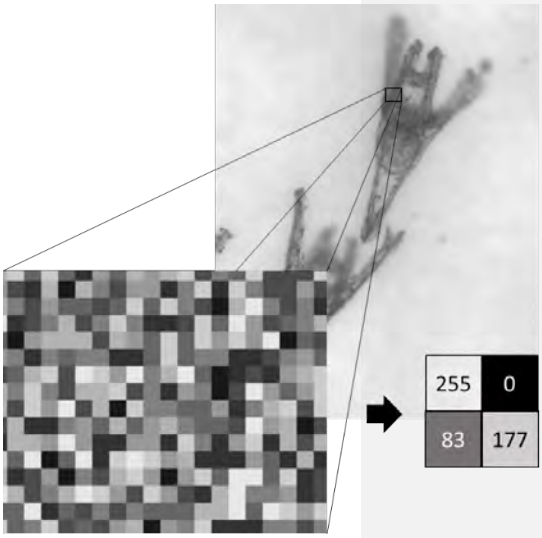

Figure 1.1*: The greyscale is represented as discrete values from 0 to 255. The range of the pixel values depends on the colour depth. Here we have 256 greyscales.*

Once the object detection has been successfully applied, Euclidean distance calculation is typically used when these objects need to be measured, which could be a perfect solution for the first endpoint. However, the second endpoint requires a higher level of parametrization and the building of classification models (Jeanray et al., 2015).

1.5 ENVIRONMENTAL RISK ASSESSMENT (ERA)

As mentioned previously, the number of contaminants that we are able to detect in surface and wastewaters has been increasing over the last few years thanks to the development of new wide-scope screening methods that can even include multi-target contaminant lists of >2000 contaminants (Alygizakis et al., 2019a; Gago-Ferrero et al., 2020). These advances in detection also require the improvement of methods for their characterization to allow us to prioritize those with certain properties of concern (e.g., toxicity, persistency, high frequency of detection…) (Finckh et al., 2022; von der Ohe et al., 2011). One of the most extended ways for toxicological risk characterization is the risk quotient (RQ) based environmental risk assessment (ERA). RQs are easy-to-understand bioanalytical units that measure the toxic risk of a contaminant and classify it into one of the three toxic levels. When the RQ is > 1 the contaminant is considered to have a high risk potential, RQs between 0.1-1 indicate a moderate risk potential, and RQs < 0.1 indicates that the environmental risk is negligible. This approach was adopted by the European Union technical Guidance Document (*Regulation (EC) No 1907/2006 of the European Parliament…* 2019) and makes risk communication understandable to all audiences. RQs are calculated for each compound as the ratio between the experimental concentration measured in the sample (MEC) and its corresponding

predicted no-effect concentration (PNEC, preferably calculated from experimental ecotoxicity data) or the environmental quality standards (EQS) under the WFD (Alygizakis et al., 2019a; Lopez-Herguedas et al., 2021). What this metric provides is basically how contaminated our sample is concerning a safe concentration. Specific RQs can be calculated for any species or trophic level using their PNEC/EQS, but typically all the available data are considered and the biological compartment showing the lowest safe concentration (most sensitive compartment) is used to calculate an RQ that is representative for the entire ecosystem health. A clear example of this is the case of diuron, an algaecide that usually appears in wastewater and poses very high toxicity for algae but low risk for the rest of the trophic levels. In such cases, the overall risk to the environment is as high as the risk to its weakest link, and the RQ would be calculated from the toxicological data for algae.

If we have experimental or empirical information regarding the PNEC values, ERA can be used as a prioritization tool. In this sense, we can use it to focus on those compounds that are most toxic when it comes to establishing regulations, choosing which compounds should be prioritized in WWTP treatments, or knowing which ones deserve to be studied more closely.

Comentado [NE11]: If we have experimental or empirical information regarding the PNEC values, ERA can be used as a prioritization tool

1.6 EUROPEAN GLASS EEL

Comentado [112]: Agerikoa da atal honetan dauden erreferentziak eta aurrekoetan erabili dituzunak

One of the key players of this thesis is the European glass eel (*Anguilla anguilla*). Glass eel is the stage from the larval stage (leptocephalus) to the pigmented juvenile stage (elver stage) of any species of eel. In our case, our target species is the European glass eel, which has one of the most extraordinary and still unclear migratory cycles in entire animal kingdom. Adult eels spawn in the Sargasso Sea and the leptocephali (i.e., the larvae stage) drift with the Gulf Stream to reach the European continental shelf, but the mechanism behind their orientation over this 5000 km and their swimming behaviour is unknown (Cresci, 2020). Once on the European coast they metamorphose and turn into the so-called glass eel (Tesch, 1980). In their second migratory phase, glass eels migrate up estuaries to join rivers, taking advantage of the current during the upstream flood tide and hiding in the river bed during ebb tide (Gascuel, 1986). Along the estuarine migration, glass eels enter a state of starvation and use the energy stores collected during the leptocephali stage to undergo several changes including morphological, physiological, and behavioural changes (Elie and Rochard, 1994), as well as pigmentation process (Ciccotti et al., 1993), gut development, osmotic adaptations (Ciccotti et al., 1993), and hormonal modifications (Jegstrup and Rosenkilde, 2003). However, these processes are often dependent on the internal conditions of each individual (e.g., energy status and body conditions (Liu et al., 2019)) and several studies have described partial migratory patterns with less than a 20% of the glass eels migrating successfully (Beaulaton and Castelnaud, 2005). The reasons that promote glass eels to migrate or settle are still unknown and, although many studies have aimed to understand the mechanism behind the differentiation of these two migratory

Comentado [MM13]: I would oriente this section to why glass eel could a good model organism (compared to organisms used for bioassays)

Comentado [i.14R13]: Hmmm I think that this is not the point of this section. Actually, I think that glass eels would be a very bad model organism. We are studying the impact on glass eels because we are really interested in that specific organism but I can't see how an endangered species could become a model organism.

phenotypes (i.e., active glass eels that can use the tide to migrate efficiently, and inactive ones that settle in estuaries (Liu et al., 2019)), a physiological explanation is still pending.

Since the 1980s, the glass eel recruitment has drastically decreased, and this species is now considered endangered. Among other important factors such as habitat modification or fishery (Starkie, 2003), several studies point out that contamination might be one of the causes behind this decrease (Palstra et al., 2006; Robinet and Feunteun, 2002). The accumulation of toxic substances including polychlorinated biphenyls (Freese et al., 2016; Maes et al., 2012), polycyclic aromatic hydrocarbons (Kammann et al., 2014), metals (Claveau et al., 2015; Figueiredo et al., 2018; Maes et al., 2012), etc. has been previously studied, especially in sub-adult (yellow eel) or adult (silver eel) stages, due to their diet (bottom-dwelling predators) and their high body fat contents where lipophilic contaminants are accumulated. However, the number of such studies in the glass eel stage can be counted on the fingers of one hand. Only the effects of three chemicals/chemical families have been assessed in glass eels: the adverse effects and accumulation of lanthanum were studied by Figueiredo et. al. (Figueiredo et al., 2018); methylmercury occurrence and its effect on the migratory behaviour and energy metabolism were also studied by Bolliet and colleagues (Bolliet et al., 2017; Claveau et al., 2015; Liu et al., 2019; Monperrus et al., 2020); and the bioaccumulation of one chemical family of emerging concern, polybrominated diphenyl ethers, was also analysed by Sühring et.al. (Sühring et al., 2014). It is especially interesting the work by Liu et. al. (Liu et al., 2020), where the impact of methylmercury exposure is related to the migratory behaviour of glass eels. Since the migration is also related to the sex determinism of eels (Geffroy and Bardonnet, 2016), and this could hinder their reproduction, it is important to examine these relationships if a deeper explanation of the population decrease is sought. During their migration through estuaries, glass eels are particularly exposed to the contaminants released by wastewater treatment plants including some endocrine disrupting compounds (EDCs) and neurotoxins, so, we therefore believe it is important to evaluate the impact of these effluents on glass eels, which, to our knowledge, has never been studied.

1.7 BIOCONCENTRATION AND BIOTRANSFORMATION

1.7.1. Bioconcentration

The accumulation of contaminants in fish can be defined by two more specific terms: bioaccumulation and bioconcentration. The difference between these two concepts relies on the source of the contaminants that are absorbed ("Aquatic Bioconcentration/Bioaccumulation," n.d.). In the case of bioconcentration, the term covers only the uptake of pollutants from water, whereas, in the case of bioaccumulation, all possible sources are considered (e.g.,
Since glass eels fast during their migration, they bioconcentr
water so, from now on, we will refer to this process as bioconcentration. The measure

Comentado [115]: Edonola ere, hori horrela ezagutu dut aspaldi

typically used for this is the bioconcentration factor (BCF). This dimensionless magnitude is calculated as the ratio of equilibrium (steady state) concentrations in two phases, in this case, the fish and the water (Equation 2).

$$BCF = \frac{C_{fish}}{C_{water}}$$

Most of the contaminants detected in WWTP effluents are found in concentrations that can range from the few ng/L levels to several hundreds of µg/L (Finckh et al., 2022), but when they reach aquatic organisms these values can be multiplied up to >5000 times depending on their physicochemical properties (i.e. partition coefficient), and especially when the exposure is chronic (Chen et al., 2021). Thus, bioconcentration and bioaccumulation are key factors to understanding the real concentrations that aquatic organisms are exposed to, and ignoring them may lead to misleading results.

A quick bibliographic search can give us an idea of the relevance that the study of the accumulation of pollutants from wastewater treatment plant effluents has been gaining in recent years. Among the most concerning studies, Fan et. al., (Fan et al., 2019) described the bioaccumulation factor higher than 5000 for some EDCs such as 4-nonylphenol, bisphenol A and tert-octylphenol; Duarte et. al. (Duarte et al., 2022) assessed the bioconcentration of neuroactive pharmaceuticals based on literature data and stressed the need for experimental data in BCF calculations since the bioconcentration of these compounds seemed to be poorly correlated to lipophilicity and the prediction tools were not appropriate. Another recent review compiled information on PFAS bioconcentration and bioaccumulation in different aquatic species, compiling information on the known high accumulation capacity of these very persistent compounds (Burkhard, 2021). Finally, McCallum et. al., studied two pharmaceuticals particularly interesting for this thesis, irbesartan, and temazepam, and their results showed that only temazepam was bioconcentrated on sea trout (McCallum et al., 2019).

1.7.2. Biotransformation

After the contaminant uptake, it can be metabolized following a known set of enzymatic reactions. Many studies show that CECs can also be extensively biotransformed resulting in metabolites with equal or higher toxicity and bioaccumulation potential as the parent compound (Chen et al., 2017; Zind et al., 2021). When the uptake of a contaminant occurs, this may be accumulated, eliminated without being metabolized, biotransformed for excretion, or even accumulated after biotransformation. Although the concept biotransformation is sometimes used to describe the changes produced by microorganisms, in this work metabolization and biotransformation are used without distinction to refer to the changes that contaminants undergo in the glass eel organism. The usual biotransformation processes in fish are phase I metabolization reactions catalysed primarily by enzymes of the cytochrome P450, and phase II metabolization reactions, where the contaminants are mainly transformed into glucuronide, sulphate, or glutathione

Comentado [116]: Another brick in the Wall
Hona helduta arakatu dut pixka bat bibliografia eta hara non biotransformazioa erabiltzen da mikrobioek eragindako aldaketak adierazteko eta again guk adierazi nahi duguna metabolizazioa da.

Comentado [i.17R16]: Mmm ba arazo bat daukagu bai, aldatuko ditut biotransformed guztiak orduan? Again batzuk erabili ahal dira sinonimo bezala. Berez ez gaude guztiz ziur benetan angulek metabolizatzen dutenik…

Comentado [118]: Metabolized?

conjugates. Although most of the metabolization processes occur for the excretion of pollutants by increasing their polarity, since the accumulation is not always related to the lipophilicity some of these transformation products (TPs) can still be highly accumulated.

Comentado [119]: Esaldia luze da eta bira asko daude. Agian sinplikatzu argitzeko

Even if targeted strategies have been used to quantify TPs in biota (Miller et al., 2017), the availability of standards for these metabolites is very limited, so often these metabolites are completely overlooked in the usual targeted analysis. A feasible alternative is to implement an enzymatic derivatization to transform the phase II metabolite and get back the parent (or phase I) compound (Yang et al., 2016). This type of analysis also requires prior information on the metabolization of the contaminants, which is not very common to have. Still, some free tools have been implemented to predict *in silico* the possible biotransformation of the compounds of interest, something that can be of great help if later in the analytical method. In our case, the article by Ziarrusta et. al. (Ziarrusta et al., 2017) served as a reference to carry out our study. In this work, an exposure experiment to amitriptyline was carried out to calculate its bioconcentration and identify its transformation products in gilt-head bream. The suspect screening workflow that was used to identify 33 amitriptyline phase I and phase II metabolites will be discussed in the study 2 and some new strategies that were not available at the time will be included.

1.8 SUSPECT AND NON-TARGETED SCREENING (SNTS)

One of the latest tipping points in environmental analytical chemistry is the spread use of high-resolution mass spectrometry coupled to chromatographic systems. Thanks to the instrumental development, upgraded data processing procedures and the community work of many research groups we are witnessing the standardization of the suspect and non-targeted screening methods (SNTS). The basic concepts of non-targeted analysis were foreshadowed to us in the 1950s with a very basic idea (Beynon, 1954). If the mass of any ion is measured with sufficient precision, its elemental composition can be easily deduced and from its mass spectrum, not only its empirical formula but also information about its structure can be obtained. However, this statement does not necessarily define the HRMS the most important matter here is how accurate the measurement should be. The answer is also simple: enough so that the number of candidates obtained for that exact mass is manageable.

As we explain in our recent review (González-Gaya et al., 2021), SNTS is a strategy for dealing with the identification of unknowns that ranges from the experimental design itself, including sampling and sample processing, to data acquisition, processing, and reporting of results. Though the discussion about suspect vs non-target screening can be extended *ad infinitum*, in this text the difference I will make between one strategy and the other is simple: just as any analysis that uses standards is referred to as targeted analysis, any strategy that does not use standards will be called non-targeted analysis. Within the non-targeted, we can differentiate the suspect analysis in those

cases in which the study is focused from the beginning on the detection of a few known compounds and, therefore, the whole workflow is biased towards them. Therefore, the two main advantages of non-targeted analysis, related precisely to the possibility of identifying compounds without needing their standard, are the possibility to analyse very long lists of compounds that would not be economically possible otherwise, and to be able to identify compounds for which there is not even a standard available (e.g., TPs).

1.8.1. Water sampling and treatment for SNTS

Since the method is key to understand the message, it is important to revise briefly the main topics. As for water sampling, grab sampling, composite sampling, and time-integrated active and passive samplings are the available strategies (Menger et al., 2020). Grab sampling is cheap and fast, but spot collection limits the representativeness of the sample (Bernard et al., 2019). This can be avoided by collecting composite samples over time (from hours to days) but the main drawbacks of this method are the possibility of losing punctual contaminant spikes and the possible degradation of the analytes until sample collection due to environmental reasons. Time-integrated active sampling is a good alternative to avoid the disadvantages of composite sampling. Usually, on-site extraction instrumentation is used to pass large volumes of water and concentrate the contaminants in SPE cartridges ensuring their stability (Schulze et al., 2017). Similarly, passive samplers can accumulate the contaminants in sorbents or films and are used for unattended monitoring (Bernard et al., 2019; Soulier et al., 2016). The instrument employed for water collection in studies 1 and 2 was a large volume sampler coupled to solid phase extraction (LV-SPE). This device uses an SPE cartridge to extract contaminants from water so, even if this cartridge is composed of three different types of solid phases to achieve the retention of as many contaminants as possible, in this step we already have a small bias that takes us away from the ideal non-targeted screening. Concerning the sample treatment, SPE is the most frequent method used in SNTS, while as for the sorbents used, the possibility of simultaneously extracting as many contaminants as possible is always sought (e.g., using commercial cartridges such as HLB or multi-layer cartridges consisting of reversed-phase and ion exchangers, both commercial and homemade) (Daniels et al., 2020).

Comentado [120]: McLuhan style The media is the message

Comentado [i.21R20]: Gustatu zait

1.8.2 Biological sample treatment

On the contrary, biological samples could be whole organisms or organism pools, specific tissues, organs, or even biofluids. Biological matrices are much more complex, and the large number of endogenous compounds present may interfere depending on the purpose of the study. For all the solid samples, the homogenization of the sample followed by solid-liquid extraction is a must. The solvents used in the solid-liquid extraction can also bias the identification of the analytes, thus, a combination of polar, semi-polar, and non-polar solvents can be used to cover their wide polarity range. The

extraction process can be enhanced using different techniques such as ultrasonication, and tissue homogenizers (Mijangos et al., 2019; Ziarrusta et al., 2017).

1.8.3 Analytical techniques in SNTS

In terms of mass detectors, the development of instrumentation with this high precision mentioned above was achieved at the beginning of the 21st century, thanks to the development of more sophisticated mass spectrometers with new ionization techniques that allowed interfacing with different separations techniques. Chromatographic separation techniques are of great help to reduce the complexity of the sample and offer very useful orthogonal information in the identification of compounds, although sometimes overconfidence in mass spectrometry can make us mistakenly leave them in the background. The demonstrated capacity to separate a great range of polarities has made liquid chromatography (LC) the most widely used separation technique, particularly the advanced adaptations such as high or ultra-high performance liquid chromatography (HPLC or UHPLC). This tendency to identify everything in the sample has also led to the use of gas chromatography (GC) as a complementary tool to identify the most non-polar analytes. The choice of the chromatographic column and the mobile phases are other factors that can introduce a bias in the analysis. Within liquid chromatography, it is also impossible to choose a column capable of covering the polarity range of the sum of all compounds and it is sometimes necessary to resort to combinations between columns for the analysis of semi-polar and non-polar compounds (e.g., C18 columns) with others for the analysis of polar compounds (e.g., HILIC or mixed mode columns). About the interface of the chromatograph and the mass spectrometer, the ionization source, electrospray ionization (ESI), in both polarity modes, is the most common choice in HRMS, being complemented with atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) sources for the ionization of more non-polar substances that cannot be ionized by ESI.

Finally, regarding the instrumentation for the mass analysis, three are the most common technologies: Time-of-Flight (TOF) mass analysers, including the hybrid versions of quadrupole-TOF (Q-TOF), the Orbitrap mass analysers, also including hybrid (Q-Orbitrap) and the new tribrid versions, and Fourier Transform Ion Cyclotron Resonance Mass Spectrometers (FTICR-MS), which offers the highest mass resolution but its use is less extended due to its cost and user limitations. Orbitrap analysers outperform TOFs in resolution and can also be externally calibrated offering better mass accuracy, but still have a slower scanning speed than TOFs (i.e., less scans per time are performed) and the resolution in Orbitrap is inversely proportional to mass (the nominal resolution is provided at m/z 200). In terms of data acquisition, two modes stand out in SNTS, data-dependant acquisition (DDA) and data-independent acquisition (DIA). Since it facilitates the structure elucidation by giving fragmentation spectra that are easier to interpret, the former is the most extended acquisition mode. Acquisition in DDA mode starts with full-scan (MS1) acquisition that switches into the fragmentation of the precursor ion (MS2) when an analyte appears. The MS2 spectra

of all the analytes in a sample can be obtained in the same analytical run but this approach requires longer cycle times which reduces the quality (the resolving power) of the full scan. This is even more noticeable in the Orbitrap analysers (at least until the new tribrid generation), since, as mentioned above, the scanning speed is slower than in the TOFs. To avoid this, the number of MS2 scans can be reduced by setting a minimum intensity threshold, which gives more time to scan the rest of the analytes that exceed that threshold. By contrast, in DIA all the precursor ions are separated into consecutive small mass-to-charge (m/z) windows and then fragmented at high energy with no preselection. This mode of acquisition allows more complete fragmentation spectra to be obtained, but the data lack the information linking a precursor to its fragments, so the use of algorithms for further deconvolution is essential.

1.9 SNTS DATA PROCESSING

Regardless of the acquisition mode used, raw data processing follows three common steps: data pre-processing, feature prioritization, and compound identification and annotation. Data pre-processing is typically applied to correct possible problems that may have arisen during data acquisition (e.g., RT alignment and mass correction), and to reduce the complexity of the data (e.g., peak picking, componentization, signal grouping, signal-to-noise ratio thresholding…) (González-Gaya et al., 2021; Menger et al., 2020). In particular, the peak picking step is the main bottleneck of SNTS data treatment (Hollender et al., 2017). In both commercial and open-source software, programmed algorithms oversee peak picking, and although some parameters can be controlled, most of their functions are a black box for the average user. Peak picking process is responsible for defining which features will be considered from then on, and those that are not, are missed definitively. After the peak picking, we obtain the so-called features. Features are those peaks that have passed the selection process and are therefore relevant and should be worked-out. However, as the number of features that are returned after peak picking is enormous, different prioritization strategies are usually applied, some common to all studies (sample/blank ratio thresholding, peak intensity thresholding, mass accuracy thresholding…) and others dependent on the specific research questions (e.g., constraints on elemental composition, characteristic adduct ions, variable selection based on chemometric approaches…).

One may think that after all the pre-processing and prioritization, all the remaining features are identified without any problem but that is still far from the reality of SNTS, even though the scientific community has made enormous collaborative efforts in the development of databases and tools for the elucidation of these features (Alygizakis et al., 2019b; “The NORMAN Suspect List Exchange (NORMAN-SLE),” 2022). The most common strategy to achieve the identification is the comparison between the information of the features obtained experimentally with spectral libraries. Some of these databases can provide information on fragmentation spectra (e.g., mzCloud, massBank…), but others only serve to find candidates for each feature based on the

exact mass of the parent (e.g., NORMAN SLE, Pubchem, HMDB…). In the latter case, *in silico* fragmentation tools such as Metfrag (Ruttkies et al., 2016) or Mass Frontier are very useful to predict the fragmentation of our candidates and compare it with the experimental data.

Some databases have been built by the community to address specific cases, for example in the Norman Suspect List Exchange section (https://www.norman-network.com/nds/SLE/) we not only find unified databases for the analysis of CECs very appropriate for this work, but there are also others for families or more specific groups including EDCs, bisphenols, antibiotics.... In the same way, suspect screening methods have emerged as the best option to identify TPs not only in biota, but also in wastewaters (Llorca et al., 2016; Wang et al., 2020), sediments(Li et al., 2014), and other water bodies (Eysseric et al., 2022). In this case, the data treatment does not deviate much from what has been explained before, but a masslist including information on TPs is necessary. A good option is to use the previously mentioned webtool, BioTransformer, to obtain a TP list of the compounds we are interested in, which can be used as a masslist in the non-directed analysis.

Last but not least, the annotation of compounds in SNTS is done in a consensual manner as proposed by Schymanski et. al. (Schymanski et al., 2014). In this paper, the authors proposed the classification of compounds according to the level of confidence with which they can be identified. This way, if the structure is confirmed by reference standards, the compound is classified as level 1; if a probable structure is elucidated but confirmation by reference standards is not possible then it is classified as level 2. When there is evidence of possible structures but not enough to choose an exact one (e.g., positional isomers) it is level 3. These three levels are the most commonly reported in scientific articles since levels 4 (only the molecular formula is known) and 5 (only exact mass is known) are not very informative.

1.10 METABOLOMIC APPROACH

Among all the ways to evaluate the effect of pollutants, there is one that I have not yet mentioned in this introduction that belongs to a field of science that is rapidly growing and acquiring great importance, omics. Since we are interested in the exposure effects on organisms, the study of the metabolomic profiles and patterns can provide complementary information that is not achievable by previous methods, such as bioassays or bioaccumulation. Broadly saying metabolomics can be seen as the last stage of the omics pipeline (genomics, transcriptomics, proteomics, and metabolomics). In fact, it refers to the large-scale analysis of the endogenous metabolites (chemical compounds with a mass range between 70 and 1500 Da) in target cells, biofluids, tissues, or organisms. The main interest of this particular -omic lies in the versatility of the alterations that may be observed as a consequence of stressing events. The underlying approach is that the study and the analysis of slight modifications in the metabolic profile would provide key information regarding chemical events that happen when, for instance, any organism is exposed to a

contaminant. We can say that the metabolic profile of a healthy/unaltered organism can be used as a fingerprint of the status of the living being. The revelation of changes in the metabolic profile is so useful to get information about the physiological state, the progression of a disease, and, overall, the health status of the subject.

Since the metabolic profile is highly sensitive to many changes (more sensitive than genomics or proteomics), metabolomics has gained importance in many scientific fields such as medicine, nutrition, clinical pharmacology, and the one we are particularly interested in, environmental toxicology (Lankadurai et al., 2013). The ability of metabolomics to detect biomarkers that indicate the impact of xenobiotic compounds on an organism, as well as to help elucidate the mechanisms of action of these compounds, has contributed to the development of a new discipline called environmental metabolomics. This discipline has helped investigate the effects of contaminants directly on organisms obtained from their natural habitat under environmental exposure, obtained from their habitat and exposed under laboratory conditions, or raised and exposed under laboratory conditions. To carry out a proper application of the metabolomics approach, we must ensure that all factors that are not relevant to the scientific question, and that may have an impact on the metabolome, are kept constant between organisms. This means that the experiments should be carefully designed taking into account the sources of variation (i.e., genotype, phenotype, age, sex, feeding…) and minimizing the effect of all the non-relevant ones, something that can be complicated when wild animals are used. To give a few examples, Meador et. al., (Meador et al., 2020) studied wild Chinook salmons and confirmed that fish in effluent-receiving estuaries exhibited altered metabolomes. Similarly, Ekman et. al., (Ekman et al., 2018) deployed male fathead minnows in cages near WWTP effluents and discovered that the concentration of one contaminant, estrone, was correlated with some biological responses demonstrating the importance of monitoring this compound. Ziarrusta et. al., (Ziarrusta et al., 2018) were the pioneers in our research group in applying non-targeted environmental metabolomics. Juvenile gilt-head breams were exposed to the UV filter oxybenzone under laboratory conditions, and they confirmed the adverse effects of this contaminant based on the liver and serum metabolome. Moreover, within metabolomics there are signs of independence emerging from the branch of metabolomics that studies specifically the lipid profile, this branch is called lipidomics. Since lipids play important roles in cellular functions, such as cellular signalling, structural functions, or energy storage, many studies have successfully attempted to link changes in lipid profile to many diseases including diabetes, cancer, obesity, and neurodegeneration. Likewise, lipidomics has also found a great opportunity in toxicology, and some studies such as the one by Xu et. al., (Xu et al., 2010) have demonstrated the relation between lipid dysregulation and exposure to some emerging contaminants. Moreover, environmental metabolomics is not only applied to evaluate the impact of pollutants, it has also been used to study the effect of other important abiotic factors including temperature (Roh et al., 2020; Zhang et al., 2021), pH (Sun et al., 2018) and salinity (Sun et al., 2021) among others. Even in a less toxicological context, metabolomics also allows us to

discover physiological differences between individuals with different phenotypes (Jansma et al., 2022; Sawada et al., 2019; Sutour et al., 2017).

Targeted analysis and SNTS can complement each other when some of the interesting metabolites to be analysed are known, and analytical standards that can solve specific purposes of the study are available. This can be the case when the biomarkers to be followed are known and the quest is based on their profile. Among others, the targeted strategies often include metabolites belonging to general metabolic pathways such as macronutrient metabolism (mainly glucose metabolism), fatty acid metabolism, protein metabolism, being the liver responsible for all these processes, as well as others much more concrete such as the neurotransmitter synthesis, hormone regulation, antioxidant system, etc.

As in the analysis of CECs, metabolomic studies involving SNTS are designed to identify unknowns with enormous chemical variability. Most of the factors to be taken into account during the experimental design have already been explained above, but we must add some that are characteristic of metabolomics. First, when sampling, two factors must be considered: the homogeneity of the organisms to be studied, including size, weight, age, sex, etc., and the suitability of the sample that will be collected. Being the main responsible for animal metabolism, the liver is the sample of choice for metabolomic studies in animals. However, as its extraction is completely invasive and usually involves the death of the animal experimental subject, it can be replaced by biofluids such as blood or urine. Other parts more sensitive to specific metabolites may also be used depending on the purpose, for instance, the brain for neurotransmitter analysis (Gu et al., 2015). Metabolomics can also be applied to cell cultures, and one of the most recent applications is its use to determine the metabolic profile of individual cells (Liu and Yang, 2021). Regardless of the sample, its collection is one of the most critical points of the whole procedure. The objective of metabolomics is to study the metabolic profile of the organism as it was at the time the sample was taken, which implies that any metabolic activity after collection is an error in the measurement. It is therefore important to stop this activity as soon as possible. The most common is the snap-freezing of the sample after collection in liquid nitrogen or at temperatures of -80°C or below. Even after freezing the sample, it is essential to maintain the cold chain until analysis. The chemical nature of the metabolome makes it impossible to analyse using a single method since the metabolites range from very polar dicarboxylic compounds involved in the Krebs cycle to cholesterols or other extremely hydrophobic lipids. One of the most common strategies is the separation of the polar metabolome from the lipidome (Chen et al., 2013). For this purpose, samples can be treated completely separately in each of the two methods, but, as biological samples are usually scarce, some extraction methods use insoluble solvents to separate the lipid phase from the polar phase by liquid-liquid extractions. Of course, when the sample is solid, this type of separation must be done after homogenizing and extracting the metabolites from the sample. In any case, extractions are usually done by keeping the temperature below 4°C and organic solvents are usually used first in order to be able to stop the enzymatic activity. Regarding the analysis, there are two techniques used

for the non-targeted identification of the metabolic profile, nuclear magnetic resonance (NMR) and gas and liquid chromatography coupled to HRMS, but the latter is certainly emerging since the SNTS strategies have been optimized (Segers et al., 2019). Gas chromatography has found a niche in the metabolomic analysis of gaseous samples such as breath, but it can also be complementary to the undoubtedly leading technique LC-HMRS.

Although there is not much to add about data acquisition and analyte identification to what has already been explained in the SNTS section, there is a whole world to discover for this type of data in its post-identification or post-quantitation treatment. Data processing in SNTS workflows is usually quite simple since the main objective is the characterization of the sample or, where applicable, the quantification of contaminants. However, in metabolomic studies, especially when developed for toxicological purposes, identification or quantification is only the first step. In these studies, the important thing is not the set of metabolites that are identified, but to find the differences in these sets between samples from different experimental groups. These comparisons can be carried out using the concentrations of the metabolites in the targeted analysis or their area in the SNTS. The three main steps in the treatment of metabolomic data are commonly pre-processing, biomarker identification, and interpretation, which can be carried out in different software including chemometric and statistical tools, either open-source software such as some packs like metabolomicsR (https://github.com/XikunHan/metabolomicsR), for R or the webtool Metaboanalyst (https://www.metaboanalyst.ca/), or proprietary tools like the (PLS-Toolbox https://eigenvector.com/software/pls-toolbox/) in MATLAB, LipidSearch (Taguchi and Ishikawa, 2010), etc. All these programs allow you to carry out the first two steps but some of them like Metaboanalyst are especially interesting as they also help you to interpret the results.

The next step, biomarker identification, is usually approached as a question of variable selection by chemometric means, but it is always advisable to start studying the data by exploratory analyses such as principal component analysis (PCA). As this type of data usually has a very high intrinsic variability due to the nature of the biological samples, if the differences between experimental groups are detectable with a PCA, the end of the study is likely to be easily achieved. In cases where a PCA does not provide information on the differentiation of the groups, there is no need to worry, since there are supervised techniques that help us to force the differentiation of the groups and thus find the variables responsible for this differentiation (Yi et al., 2016). Possibly the most widely used algorithm for this purpose today (and my favourite as you will see in study 1) is the partial least squares discriminant analysis (PLS-DA), at least until deep learning techniques become more accessible to the public. The popularity of this algorithm is probably due to the ease of interpretation, something very important in metabolomics since we can easily study loadings or variable importance in projection (VIP) to select important metabolites. Different adaptations of this algorithm (e.g., SPLS-DA or OPLS-DA), heatmaps, random forest, and many other algorithms can be used as well, as long as you know how to properly interpret the results. However, the

application of these unsupervised methods alone is not sufficient to tell which metabolites are responsible for the differences between groups. To be sure that the differences are significant, it is necessary to apply statistical tests (e.g., ANOVA, t-test...) before confirming which metabolites are biomarkers.

Finally, once the biomarkers have been identified, it is necessary to interpret the significance of these compounds being altered with respect to the control group. This requires interdisciplinarity since analytical chemists usually lack the necessary skills to make this interpretation. Interpretation is usually done by studying the metabolic pathways to which the identified biomarkers belong and looking for the relationship between these pathways and the scientific question under investigation. Figure 1.2 gives a very general overview of the usual workflow of metabolomic studies.

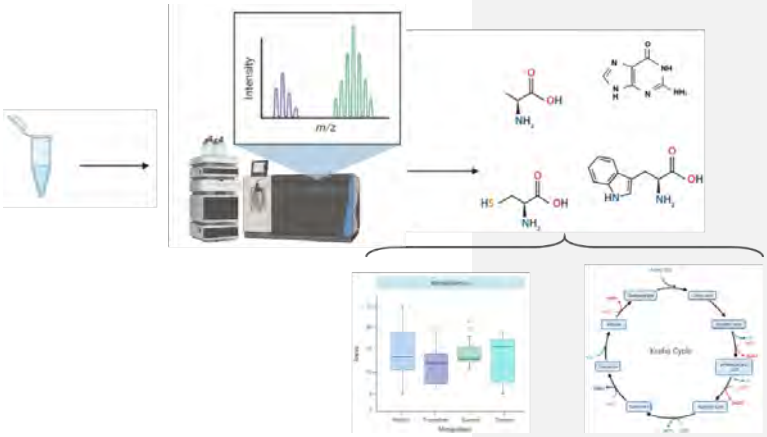

***Figure 1.2**: General metabolomic workflow*

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2. OBJECTIVES

"Water-Lilies – The water treatment plant" by Claude Monet

In the context of this cotutelle PhD, our key aim was **to understand the influence of environmental relevant emerging contaminants released by the local WWTPs and found in the Adour estuary on the migration patterns of glass eels**. In order to address this ambitious goal efficiently, we have designed a tiered set of operative objectives:

- The first step was to establish a diagnosis of the presence of CECs in the effluents of one of the WWTPs in Bayonne and the toxic effects in the estuary. In this case, we have carried out a twofold study. The first one was the automation of the sea urchin embryo test (SET) to support a more user-friendly application of the effect-directed analysis (EDA) of the effluent. The second one was the application of the EDA and a complementary ERA to study the fate of the main contaminants bioaccumulated by glass eels.
- The second step was to study in detail the effect of the exposure of glass eels to the main contaminants found previously. In this case, we also carried out a twofold metabolomic study. In the first one, we studied the global variation of the metabolomics profile through the exposure to the main contaminants both individually and in mixtures. In the second one, we studied the metabolic characteristics of the behavioural pattern variation (migration) because of a long exposure to diazepam.

Each of those three studies are arranged as independent chapters and the specific aims of each of them can be summarized as follows:

The two primary objectives of this thesis were to bridge the gap between effect-directed analysis and routine analysis, and to study the exposome of glass eels in the Adour estuary. The first objective was addressed in the entitled "*SETApp: A machine learning and image analysis based application to automate the sea urchin embryo test"* that was published in May 2022. The second objective encompasses different aspects of the exposome such as the identification of contaminants present in the habitat of glass eels, their fate and their impact on the eels. A second research article headlined as "*Prioritization based on risk assessment to study the bioconcentration and biotransformation of pharmaceuticals in glass eels (Anguilla anguilla) from the Adour estuary (Basque Country, France)*", where the occurrence and fate of contaminants within glass eels is studied, was recently published in August 2022. The effect of selected contaminants was also assessed by metabolomics in the first part of the 3rd Study and the results were collected in a paper that will be submitted for publication later this month (October 2022). Lastly, metabolomics was also used to gain further insight into the complex mechanism of migratory behaviour glass eels. This work is part of a larger collaborative effort to decipher the reasons why some glass eels are able to migrate and while others settle in the estuary.

The specific objectives of each study are listed below:

Study 1

- Assess the suitability of image analysis and chemometrics to automate the sea urchin embryo test.
- Develop an efficient, fast, reproducible, and user-friendly tool to automate the SET.
- Test the tool in a real and demanding scenario, the effect-directed analysis of Bayonne's (Basque Country, France) Pont de l'aveugle WWTP effluent.

Study 2

- Conduct a risk-assessment study to find the most relevant CECs in the WWTP effluent that is released to the glass eel habitat.
- Evaluate the bioconcentration potential of the selected contaminants in glass eels.
- Identify the biotransformation products of the selected contaminants in glass eels.
- Study the occurrence of non-targeted contaminants in wild glass eels.

Study 3

- Assess the impact of the selected contaminants using metabolomics to detect the altered metabolites in both the polar metabolome and the lipidome.
- Study the polar metabolome and the lipidome of migrant and non-migrant glass eels to find their differences.
- Explore the phenotype-related sensitivity differences to diazepam.

3. STUDY 1:

SETApp: A Machine Learning and Image Analysis Based Application to Automate the Sea Urchin Embryo Test

Sea Urchins – Van Gogh

3.1. INTRODUCTION

Efforts to better understand the chemical and ecotoxicological characterization of the wastes released into the environment have addressed a set of organic micropollutants as contaminants of emerging concern (CECs) (Pontius, 2021; Tang et al., 2020). Some studies pointed out that wastewater treatment plants (WWTPs) can barely remove them from wastewater, making WWTP effluents a complex mixture of compounds with a high range of ecotoxicological effects (Loos et al., 2013; McCance et al., 2018; Mijangos et al., 2018). The ecotoxicological and analytical challenges lie in the complexity of these mixtures and the relationships between the presence of certain compounds and the toxicological endpoints [6]. Although many bioassays, both *in vitro* and *in vivo*, are commonly used for risk assessment, they do not provide further information about the cause of the effects observed. Thus, *in vivo* bioassays are often coupled to chemical analysis techniques to explain these cause-effect relationships.

Effect-directed analysis (EDA) and toxicity identification evaluation (TIE) are the best available strategies for toxicant identification approaches (Brack et al., 2016; Burgess et al., 2013). Whichever the approach is, these strategies eventually require a long study to elucidate the cause-effect relation. In this regard, implementing high-throughput screening (HTS) methods is crucial to deal with this elucidation (Villeneuve et al., 2019) since many HTS platforms have taken a big step towards deploying complex and laborious methodologies in routine analysis through *in vitro* (Arini et al., 2017; Wetmore et al., 2015) and *in vivo* (Letamendia et al., 2012) bioassays.

The sea urchin (*Paracentrotus lividus*) embryo test (SET) has arisen as a key *in vivo* assay for coastal marine ecosystems. Apart from being affordable for use and maintenance, many studies have proven their sensitivity to emerging contaminants (Gambardella et al., 2016; Vethaak et al., 2017) and the suitability of this bioassay in EDA strategies (Mijangos et al., 2020). The two endpoints of this bioassay, the size increase, and the malformation level, are typically assessed by optical microscope observation. In the former case, the size increase from the egg to the 48 h larvae is compared. In addition to this, the reference criteria for malformation classification differentiate four malformation levels including, normal development (level 0), incorrect location of skeletal rods (level 1), incomplete or absence of skeletal rods (level 2), and blocked development (level 3) (Carballeira et al., 2012). Since implementing SET on a routine basis to tackle high-throughput analysis demands a large number of assays, image analysis, and machine learning techniques may find their way towards the automation of this bioassay (Nyffeler et al., 2020; Ramakumar et al., 2015).

The use of high-resolution digital microscope images combined with chemometric tools provides fast procedures to address high-throughput analysis, as recently shown in food control (Tormena et al., 2021) or microplastic analysis [20] and more affordable than those based on deep learning microscopy (von Chamier et al., 2021). Within these

tools, partial least squares discriminant analysis (PLS-DA) models are widely spread as they combine dimensionality reduction and discriminant analysis for predictive and descriptive modelling (Brereton, 2000). The method builds a linear model and classification boundaries that can be used as image classifiers (McEvoy and Amigo, 2013). Thus, the model requires a training set containing enough information to optimize the algorithm parameters to establish these boundaries. Validation of the built classifier is the most important step in any machine learning application; therefore, a test set must be used to construct the model (and calculate the boundaries); and a validation set must be used to test the performance of the previously created model.

This study aimed to develop an image analysis and machine learning-based pipeline to automate the SET. The proposed methodology would replace the traditional microscopic inspection of the larvae, providing comparable and observer-independent results reducing the workload and times in a user-friendly format. For a given image set of larvae exposed to any sample, the developed tool would give quantitative results for the two SET endpoints and build dose-response curves according to the concentration that the user introduces. In addition, to show the outputs obtained from this novel HTS method and prove its benefits, the developed app was implemented in the effect-directed analysis of Bayonne's WWTP effluent.

3.2. EXPERIMENTAL SECTION

3.2.1. Sea urchin embryo test

Adult sea urchins were collected from the intertidal area of Armintza (43.43347° N, 2.89889° W, Basque Country) and maintained in aquaria at the Plentzia Marine Station (PiE). The temperature of the water was kept at 15 ± 1 °C, and the room was programmed with a natural photoperiod. Gametes and embryos were obtained following the procedure described by Mijangos et al. (Mijangos et al., 2020)

Fertilized sea urchin eggs were exposed for 48h to four compounds independently and in five different concentrations (1 $\mu g \cdot L^{-1}$ - 5 $\mu g \cdot L^{-1}$ - 50 $\mu g \cdot L^{-1}$ - 500 $\mu g \cdot L^{-1}$ and 5 $mg \cdot L^{-1}$, 2 mL, n = 2) in 20 mL glass vials with 100 $egg \cdot mL^{-1}$ density. The selected compounds were albendazole, amitriptyline, copper (II) chloride and caffeine (Sigma Aldrich; St. Louis, MO, USA). In previous works, albendazole and amitriptyline were pointed out as important contributors to the toxicity of a WWTP effluent in EDA using the SET. Copper (II) chloride is a reagent typically used as a positive control in the SET methodology, and the morphological changes that this compound causes to sea urchin larvae have been deeply studied (Saco-Álvarez et al., 2010). Finally, caffeine was chosen for its mode of action (central-nervous-system stimulator), which differs from the aforementioned compounds. At 48hpf, larvae were fixed with formalin and transferred to 24-well-microplates. Each microplate row corresponded to six replicates of 200 µL.

3.2.2. Image acquisition and processing

Training and test image sets for the classification model construction were created using bright field pictures of sea-urchin larvae at 48 hours post-fertilization (hpf). Images of non-exposed control embryos were also taken. Cytation™ 5 (4x magnification objective, BIOTEK) image reader was used to automatically obtain an image mapping of 25 pictures per well all over the plates. The imaging conditions suggested for an optimal larva separation using Cytation5 are LED intensity = 2; IT = 115; CG = 4 and Focus Height = 2750µm. The imaging conditions were optimized using GEN5 software (BIOTEK) to ensure an appropriate focus and contrast of the larvae. Raw images were saved as *Tagged Image File Format* (.tif).

The images were processed to obtain a collection of up to 272 orientated and normalized larva pictures (Figure 3.1) using homemade scripts in MATLAB® (Mathworks®, R2019b) supported by the Image Processing Toolbox™. The larvae image collection was classified by experts into three groups according to their degree of alteration as proposed by Carballeira et., al. (Carballeira et., al., 2012), but combining the two intermediate states (levels 1 and 2). The image set was randomly split into two groups: a training set (~90%) consisting of 242 larva images to train the model and a test set of 30 images (~10%) to estimate the performance of the measurements. The training set was revised to embrace all kinds of malformations (i.e., all the phenotypes described by Carballeira et., al. (Carballeira et., al., 2012): crossed tip, separated tip fused arms, incomplete or absent skeletal rods, absence of skeletal rods, and folded tip, fractured ectoderm, undeveloped embryos). This way, the model was not biased towards any particular phenotype, and every larva was considered independent regardless of the experiment they came.

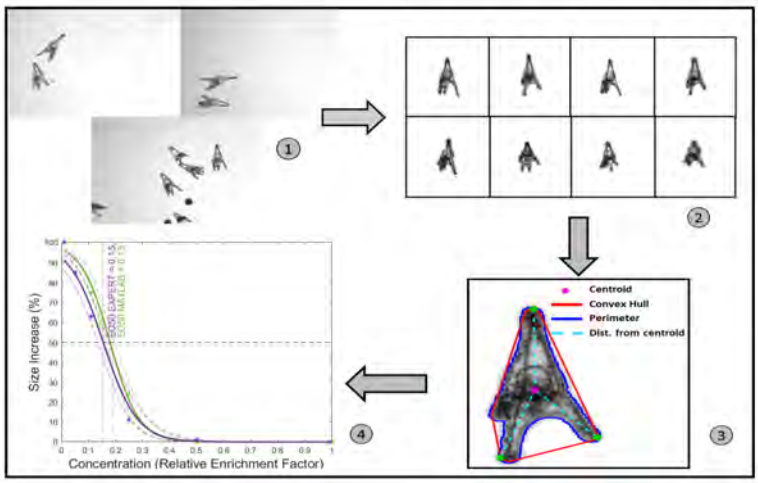

***Figure 3.1**: Flow chart of the image processing. (1) Raw images. (2) Processed images. (3) Image parametrization. (4) Dose-response curves built from image parameters.*

The image of each larva was parametrized, calculating N=14 image parameters concerning the shape of the larva (Table 3.1). Following this strategy, each larva was encoded to be the m^{th} row of a matrix **X** (*M* x *N*), where the values of each parameter were placed in the *N* columns. The PLS-DA models were built using the Classification Toolbox built by Ballabio and Consonni (Ballabio and Consonni, 2013) working under a Matlab environment using randomized subset cross-validation to assure the independence of the validation subsets. The external validation was carried out by predicting the malformation level of the larvae belonging to the test set and comparing them to the level determined by the experts. The sensitivity (estimation of the model ability to avoid false negatives), specificity (estimation of the model ability to avoid false positives), and the prediction error were calculated as outlined by Ballabio et al. (Ballabio et al., 2018)

Table 3.1: *Parameters used in the classification model to predict the malformation level.*

PARAMETER	**DEFINITION**
Alpha	Angle between the centroid-left leg and centroid-right leg vectors
Area	Area of the extracted larva image
Area Ratio	Ratio between the extracted larva image area and the convex hull associated with that image
Beta	Angle between the top-left leg and top-right leg vectors
Circularity	Circularity of the extracted larva image
Convex Hull Area	Area of the convex hull associated with the extracted larva image
Left Leg Distance	Euclidean distance between the centroid and the left leg
Leg Size Ratio	Ratio between Left Leg Distance and Right Leg Distance
Mayor Axis Length	Euclidean distance of the extracted mayor axis of the larva
Minor Axis Length	Euclidean distance of the extracted minor axis of the larva
Perimeter	Perimeter of the extracted larva image
Right Leg Distance	Euclidean distance between the centroid and the right leg
Size	Euclidean distance from the top to the furthest point of the leg
TopSum	Number of pixels within the first ten image rows (gives information about the top shape of the larva)

An additional experiment to validate the size increase measurement was carried out by exposing the larvae to copper (II) chloride. The approach described by Carballeira et

al. was implemented by two experts measuring the larvae under an inverted microscope coupled to an electronic camera and using NIS-Elements image analysis software v4.30 (Nikon Instruments BV, Europe). Three larvae groups exposed to different concentrations of $CuCl_2$ were measured and compared to the size measurements obtained in the parametrization.

The MATLAB App Designer was used to compile the image processing, analysis, and modelling scripts in a standalone app. The classification models built in the Classification Toolbox were also compiled within the app and used to predict a new outcome from the image sets that the user loads. The logit function was implemented for dose-response curves to fit the quantified endpoints to the concentration data that the user can introduce.

3.2.3. Implementation of the SETApp on effect-directed analysis

To test the performance of the SETApp in a real and demanding scenario, we implemented it in the effect-directed analysis of Bayonne's (France) Pont de l'aveugle WWTP effluent to assess the impact of this effluent in the Adour estuary. The benefits of using this new approach over the traditional strategy were studied, and both methodologies were compared.

An automatic large volume solid-phase extraction system (LV-SPE, MAXX Mess-u. Probenahmetechnik GmbH, Rangendingen, Germany) was used to sample 17L of Bayonne's (France) Pont de l'aveugle WWTP effluent. Reverse-phase, cationic exchanger, and anionic exchanger-based in-house cartridges (6g Strata HR-X, 2g Strata ZT-WAX, and 2g Strata ZT-WCX) were used for analyte extraction. The extracts were pooled and evaporated in a rotary evaporator (LABOROTA 4000, Heidolph Laborota 4000, Schwabach, Germany, and Büchi B-480 water bath, Flawil, Switzerland) to 15 mL obtaining a relative enrichment factor (REF) of 1133.

The raw sample was then subjected to a fractioning step using semi-preparative reverse-phase liquid chromatography (C_{18} column, 250 x 10 mm, 5 µm particle size, Phenomenex Gemini®, CA, USA) coupled to an automatic fraction collector (Agilent 1260 Infinity II, Santa Clara, CA, USA) under the control of ChemStation C.01.08 software. The fractioning conditions described by Mijangos et al. (Mijangos et al., 2020) were followed to obtain 17 fractions, and SET was applied to fractions 3 to 17 (no compounds were expected in samples 1 and 2 due to the dead volume of the system). Suspect screening using UHPLC-HRMS was restricted to toxic fractions, and raw and recombined samples. The conditions of the chemical analysis and the suspect screening workflow (Figure SI.3.1) are described in the supporting information (SI.3.6.1 and SI.3.6.2).

For fractions 3 to 17, larvae (100 eggs/mL, 2 mL, n = 2) were exposed for 48h to each sample at REF 30 (H_2O, 0.1% DMSO) in 10 mL vials. After 48h, larvae were fixed with formalin, transferred to 24-well microplates, and the images were read and processed using the SETApp. Four control groups were also prepared in H_2O, 0.1% DMSO. Two were fixed at t = 0h and used as the egg control group; two were fixed at t = 48h and

used as the developed control group. In total, 17 microplates were submitted for image acquisition. Dose-response curves were built for fractions showing toxicity at REF 30, and EC_{50} values were calculated.

3.3. RESULTS & DISCUSSION

3.3.1. SET automation

Image binarization based on pixel intensity was applied to extract the individual pictures of the larvae from the raw image containing an undefined number of larvae. The binary image was submitted to a dilation, removing small artifacts and elements in the borders of the picture. This processed binary image was used to localize individual larvae and extract, rotate and normalize each larva from the original image. The individual larvae pictures are then saved in 500x400, *.tiff* format. The full workflow is described within the supporting information (SI.3.6.3).

A set of 242 larvae pictures covering a wide range of different degrees of physical alteration was obtained to build the training set and 30 extra images as the test set. The expert panel classified 37 larvae as level 0, 107 as level 1 (corresponding to levels 1 and 2 of Carballeira et. al., (Carballeira et al., 2012)), and 98 as level 2. The image parameters chosen to explain the differences between alteration levels are summarized in Table 1 and used as input for the two PLS-DA models tested. Whereas the first model gave optimal results with 4 latent variables (59%, 11%, 9%, and 5% of the total explained variance), 3 latent variables were selected in each of the sequential models of the HPLS-DA (51%, 8% and 9% of the total explained variance for the first model and 32%,10% and 10% for the second). The scores and loadings scatter plots of latent variable 1 against latent variable 2 are displayed in Figure 3.2. Different groups can be observed in the scores scatter plot. These results were compared to those obtained by a Hierarchical PLS-DA (HPLS-DA) built by two consecutive PLS-DA and, thus, having 2 stages. The first stage separates levels 0 and 1 from level 2, and the second one separates levels 0 from 1.

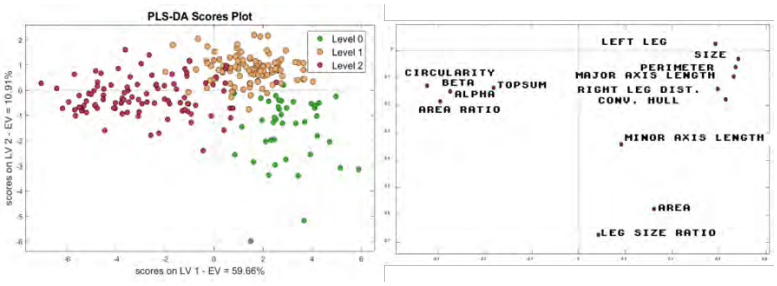

Figure 3.2: *Scores and Loadings plots of the PLS-DA model built with three classes. Each color corresponds to a malformation level.*

3.3.2. Validation of the classification model

Both approaches were built and validated with the same training and test sets, and their performance was compared. Overall performance figures of the two approaches are shown in Table 3.2, while for the selected model, the classification performance for each of the three levels and the confusion matrix is deeper explained in Table SI.3.1. In general, more accurate performance in sensitivity (84%), specificity (95%) and prediction error can be observed in the Hierarchical PLS-DA. The total classification uncertainty (15.6%) was in agreement with the one expected from the panel of experts. We have demonstrated that a concise and comprehensive parametrization of the samples, followed by a linear classification method gives accurate results.

Table 3.2: *Comparison between the studied classification performance of the model*

	HPLS-DA		**PLS-DA**	
	TRAINING	TEST	TRAINING	TEST
Sensitivity	0.916	0.844	0.908	0.806
Specificity	0.954	0.952	0.920	0.902
Prediction error	0.084	0.156	0.092	0.194

3.3.3. Validation of the size-increase measurement

In order to validate our approach, a new experiment was run. The length of three exposed ($CuCl_2$ 0.01, 0.1, 0.5 $mg{\cdot}L^{-1}$) embryo groups and an egg control group were measured in parallel by two experts under the microscope and the automatized pipeline. The comparative results are displayed in SI.3.6.5. In order to compare the size increase between groups, the mean length of the eggs was subtracted from each experimental measure (e.g., 1st expert Eggs subtracted to $CuCl_2$ 0.01 $mg{\cdot}L^{-1}$). We observed that the difference between groups increases as the size of the larvae decreases. Still, comparable results were obtained (for $CuCl_2$ 0.01, 0.1 & 0.5 $mg{\cdot}L^{-1}$; ρ-level > 0.05) between the three methods at all the concentration levels.

Furthermore, dose-response curves of a contaminated water sample were built simultaneously using manual measurements from expert and automatized measurements (Figure 3.3), and comparable results were obtained within a 95% confidence level.

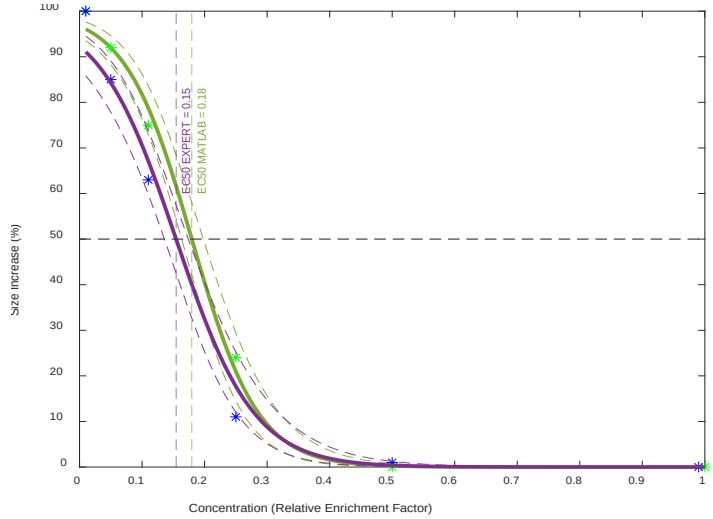

***Figure 3.3**: Dose-response curves of the same sample, built by Sea Urchin App (green) and an expert (purple).*

3.3.4. SETApp (v1.0)

Considering the obtained results, HPLS-DA was chosen to implement a MATLAB-based Application (SETApp) in the automatized sea urchin embryo test pipeline. This way, a standalone app (SETApp) has been released and is freely accessible at https://github.com/UPV-EHU-IBeA/SETApp. Overall, the SETApp allows the user to measure and classify every sea-urchin larvae in a given digital microscope image and export the results and dose-response curves. The simplified graphical interface enables the user to select the path to folders, process, measure, and classify the images in a very intuitive manner.

The SETApp includes two tabs, the Measurement, and the Classification tabs. The Measurement tab contains three folder browser buttons, egg group image selection (needed for growth calculation), control group selection, and sample selection (see SI.3.6.6). Thus, the only input that SETApp requires is the path to the image folders. Worth mentioning that the possibility to have subfolders inside the sample folder was also considered so every subfolder would be analysed as parallel sample groups. A tutorial on the app's functioning with interface pictures can be found in Supporting Information.

The process button starts the image processing of the images within the selected folders. No output is shown in the app after processing, but new images with the extracted larvae will automatically appear in each folder. The user can check these images in search of any artifact that could have been mistaken for actual larvae and

remove it. Measure button reads the extracted larvae and measures them. The results are displayed in a bar plot and a table including the mean length ($\bar{l}$) of the larvae in each folder, the standard deviation, and the size increase (SI) percentage, calculated with the following equation:

$$SI(\%) = \frac{\bar{l}_{sample} - \bar{l}_{egg}}{\bar{l}_{control} - \bar{l}_{egg}} \times 100 \qquad (3.1)$$

If necessary, the Measure Tab also includes a dose-response curve building option. The exposure concentrations can be introduced in the boxes included. Since the most common curve fitting algorithms in dose-response relationships are logistic regression analysis (logit) and probit analysis, logit has been implemented in the app, and dose-response curve charts can be visualized by pressing the *Build Curve* button.

On the other hand, the Classification Tab uses the already processed images to parametrize them and predict the malformation level of each larva using the chosen HPLS-DA model. In this case, the index of toxicity (IT) equation has been slightly modified from Carballeira et., al., (Carballeira et al., 2012) to:

$$IT = \frac{0 \times \%level\ 0 + 1.5 \times \%level\ 1 + 3 \times \%level\ 2}{100} \qquad (3.2)$$

The unification of levels 1 and 2 into new level 1 forces us to adapt the middle weight to 1.5, outlining the agreement between both methodologies. The combination of levels 1 and 2 could result in a loss of information for interpretation purposes. Thus, the processed images are saved in the folders and can be checked for further studies. IT results are displayed in a bar plot and a table, and they can also be used to build a dose-response curve.

Shortly, when the dataset is large enough, we will address the implementation of a new version based on deep convolutional architecture to improve the classification performance. Improvements in the classification model would bring the distinction between levels 1 and 2, and, at best, the classification between phenotypes, which would offer valuable information about the mode of action of the contaminants.

3.3.5. Implementation of the SETApp on effect-directed analysis of Bayonne's WWTP effluent

After the fractionation of the sample, images of the microplates with exposed larvae, developed controls, and egg controls were obtained. The results acquired from the SETApp after loading, processing, and measuring the images showed that fractions F4, F5, F7, F10, and F12 exhibited embryo growth inhibition at REF 20 (SI < 80%) (Figure 3.4.A). When category 2 bioassays (i.e., non-specific bioassays indicative of adaptive stress responses or apical endpoints) such as the SET are applied, the distribution of the bioactivity over multiple fractions is typically spread (Escher et al., 2021), as was observed in these results. For further prioritization between toxic fractions, REF 30 was also tested (Figure 3.4. B). The bioassay carried out at REF 30 pointed out F10 and F4 as the main concerning fractions.

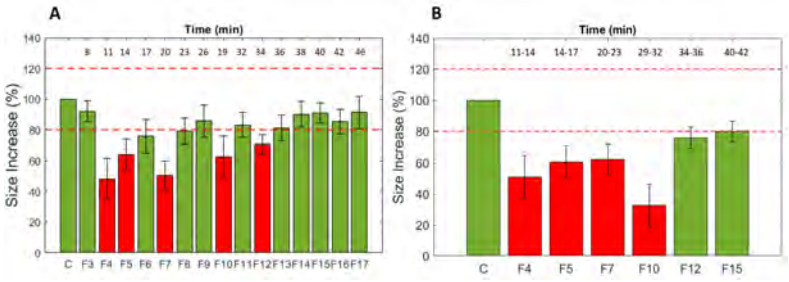

Figure 3.4: *Response in SET of all the fractions at REF 20 (A) and toxic fractions at REF 30 (B). Red bars refer to toxic fractions. F15 was included at REF 30 as the negative control of the samples.*

Dose-response curves of F4 and F10 were built in the SETApp (Figure 3.5). F10 showed significantly higher toxicity ($EC_{50,F10}$ = 9.30; $EC_{50,F4}$ = 38) than F4. Therefore, toxicant identification efforts were focused on F10, and suspect screening was restricted to this fraction.

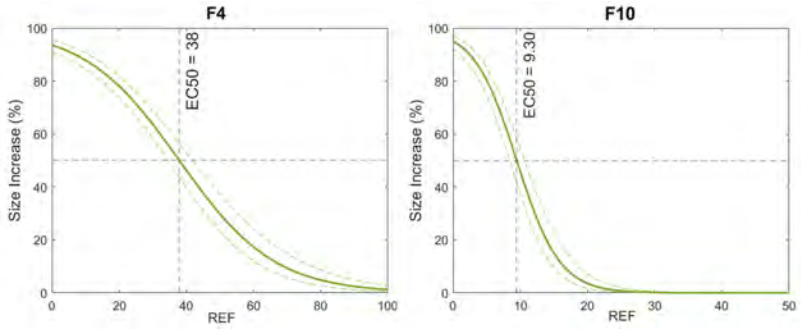

Figure 3.5: *Dose-response curve of the toxic fractions (F4 and F10), including EC_{50} values*

The implementation of the SETApp reduced the time to assess the toxicity from days to hours, and we can conclude that the developed application can be used as an efficient, fast, cost-effective, and reproducible tool to emulate the SET that fits remarkably well in EDA.

F10, raw eta recombined samples were analysed to identify the most likely toxic candidates following the workflow described in Section 1 of the Supporting Information. The preliminary output from this analysis rendered more than 2500 features. The list of candidates was drastically reduced to < 70 after applying a set of postrun filters. Among them, 4 were identified (Level 1) and 2 tentatively identified as probable structures (Level 2a) according to the classification by Schymanski et al., (Schymanski et al., 2014) (Table 3.3).

Table 3.3: *Overview of the seven identified features in F10.*

Compound	Formula	ESI mode	m/z	t_R (min)	mzCloud Match	Level
Carbamazepine	$C_{15}H_{12}N_2O$	[M+H]	236.09496	7.80	99	1
Cetirizine	$C_{21}H_{27}Cl_3N_2O_3$	[M+H]	388.15537	7.40	89.1	1
Terbutryn	$C_{10}H_{19}N_5S$	[M+H]	241.13611	8.70	97.7	1
2-Hydroxybenzothiazole	C_7H_5NOS	[M+H]	151.00918	5.70	93.7	1
4-Methylbenzotriazole	$C_7H_7N_3$	[M+H]	133.06387	4.49	98.3	2a
Terbumeton	$C_{10}H_{19}N_5O$	[M+H]	225.1587	5.73	71.4	2a

3.4. CONCLUSIONS

In conclusion, we have developed a novel predictive expert system, the SETApp, that can be used to automatically quantify the two endpoints of the sea urchin embryo test from a given image set. We have demonstrated that chemometrics, and especially multivariate linear classification models, can be successfully implemented in bioassay automation to avoid the cumbersome measurement of the embryo sizes and malformation levels. Furthermore, the SETApp provides the numerical estimation of the endpoints, including the dose-effect curve fitting and the estimation of the ECx values, if required. In addition, we have shown the efficiency of this HTS in a very demanding scenario, the EDA of Bayonne's (France) Pont de l'aveugle WWTP effluent. This EDA study concluded that the SETApp is an efficient, fast, cost-effective, and reproducible tool that can approach EDA to routine analysis.

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3.6 APPENDIX STUDY 1

SI.3.6.1: Chemical analysis: UHPLC-Q exactive conditions

Sample fractions, raw sample and blank sample were analyzed in a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap mass spectrometer (UHPLC-q-Orbitrap) equipped with a heated ESI source (HESI, Thermo-Fisher Scientific, Waltham, MA, USA).

Extracts were injected on a C18 column (XB-C18 column, 150 x 2.1 mm, 2.6 µm particle size, Phenomenex Kinetex®, CA, USA) with a pre-filter (2.1 mm ID, 0.2 mm) from Thermo-Fisher Scientific. For positive ionization mode, a mobile phase consisting of water and acetonitrile (ACN), both containing 0.1% HCOOH was used. In the negative mode, 5 mmol/L ammonium acetate was used in both solvents. The separation was carried out at 0.3 mL/min flow rate and 50 °C temperature. The gradient started at 13% ACN with a linear increase to 30% at 4 min, another linear change to 50% ACN at 8 min (hold 12 min), and further to 90% ACN at 30 min. After 5 min at 95% ACN, the gradient was returned to initial conditions for 5 min. Three 5 µL injections of all the sample extracts were performed (both in the positive and in the negative ionization modes) while the autosampler was maintained at 5 °C.

The q-Orbitrap was operated in full scan – data dependant MS2 (Full MS-ddMS^2) discovery acquisition mode for both positive and negative ionization, choosing the most intense ions from the full scan for fragmentation, with an intensity threshold of $8.0 \cdot 10^3$ and a dynamic exclusion of 8 s. One full scan at a resolution of 70,000 full widths at half maximum (FWHM) at *m/z* 200 over a scan range of *m/z* 70-1050 was followed by three ddMS^2 scans at a resolution of 17,500 FWHM at *m/z* 200, with an isolation window of 3.0 *m/z*. The stepped normalized collision energy (NCE) in the higher-energy collision dissociation (HCD) call was set at 30 eV. HESI source parameters were set at 3.2 and 3.5kV spray voltage in positive and negative respectively, 320 °C capillary temperature, 40 arbitrary units (au) sheath gas (nitrogen), 15 au auxiliary gas, 280 °C auxiliary gas heater and S-lens RF level 55.0. Pierce LTQ ESI calibration solutions (Thermo-Fisher Scientific, Waltham, MA, USA) were used for external instrument calibration every three days. The instrument was controlled by Xcalibur 4.0 software (Thermo-Fisher Scientific, Waltham, MA, USA).

SI.3.6.2: Suspect screening workflow

Suspect screening analysis was carried out using the Compound Discoverer 3.2 program (CD; Thermo-Fisher Scientific, Waltham, MA, USA). The workflow used for the data analysis with the CD is shown in figure SI.3.1. As the effluent of a wastewater treatment plant was analysed in this work, a database with many emerging pollutants was necessary. In this case, we have used the Norman list of contaminants of emerging concern (https://www.norman-network.com/nds/) including at that time more than 80.000 compounds.

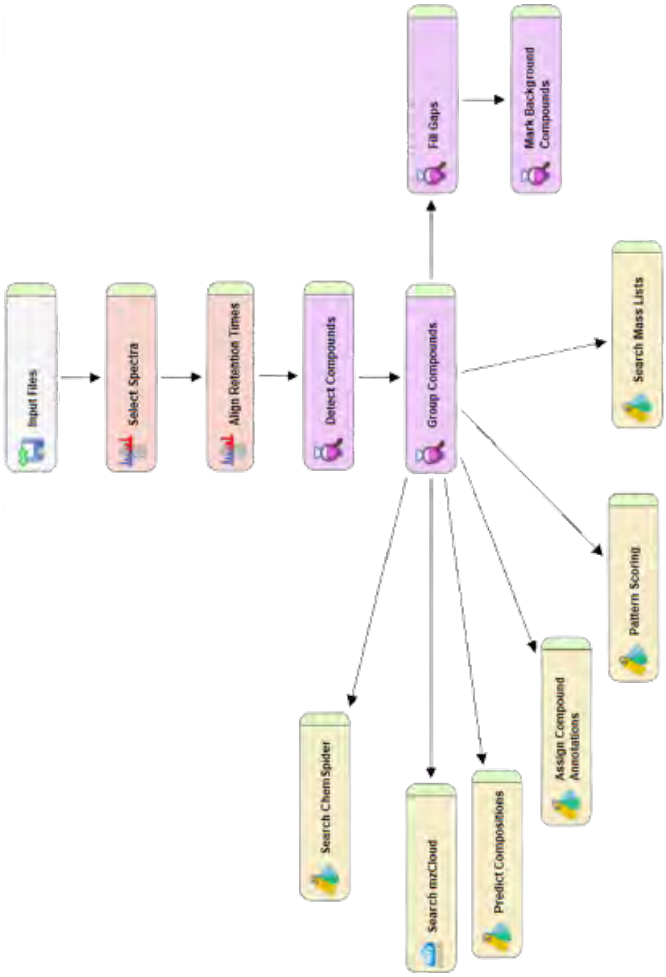

***Figure SI.3.1**: Workflow employed in Compound Discoverer 3.2*

To identify suspects, only features with a Lorentzian chromatographic peak shape were considered. Some post run considerations were taken into account, the molecular formula proposed by Compound Discoverer was only considered when the error compared to the exact mass was less than 5 ppm, when the measured and expected isotope patterns fit at least 30% and the peak has a minimum area of 10^6. When the MS2 was available, it was compared with the corresponding spectra in mzCloud library (https://www.mzcloud.org/) and a threshold value of 70% was considered for positive identification. And finally, minimum peak ratios between procedural blanks and samples above 20 were included. Tentative candidates were also searched in Chemspider (http://www.chemspider.com/).

Retention time was considered before confirmation. When the pure standard was available, a deviation of ± 0.1 min was admitted for positive identification and when it was not, an estimation of the theoretical retention time was performed using the retention time index platform (http://rti.chem.uoa.gr/).

SI.3.6.3: Workflow of the image processing

***Figure SI.1.2**: Schematic workflow of the data processing pipeline implemented in the SETApp*

SI.3.6.4: Performance figures of the HPLS-DA classification model

Table SI.3.1*: Confusion matrix and performance figures of the HPLS-DA training (top) and test (bottom) set.*

	True Class Training						
	Level 1	**Level 2**	**Level 3**		**Level 1**	**Level 2**	**Level 3**
Predicted 1	33	4	2	**Sensitivity**	0.77	0.93	0.98
Predicted 2	10	97		**Specificity**	0.97	0.91	0.98
Predicted 3	3		94	**Prediction error**	0.11		

	True Class Test						
	Level 1	**Level 2**	**Level 3**		**Level 1**	**Level 2**	**Level 3**
Predicted 1	5	2	0	**Sensitivity**	0.71	0.82	1
Predicted 2	0	9		**Specificity**	0.90	1	1
Predicted 3	0	10		**Prediction error**	0.16		

SI.3.6.5: Larva size measurement comparison

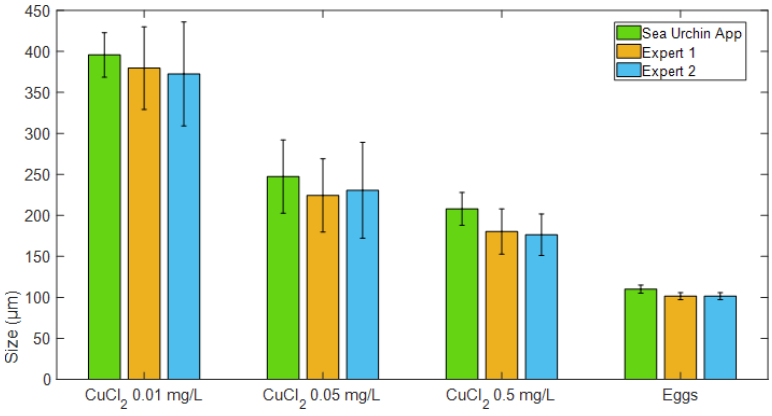

***Figure 3.3**: Comparative results of the measurements of the SETApp and the two experts.*

SI.3.6.6: SETApp Tutorial

4. STUDY 2:

Prioritization based on risk assessment to study the bioconcentration and biotransformation of pharmaceuticals in glass eels (*Anguilla anguilla*) from the Adour estuary (Basque Country, France)

The Starr-eel night – Van Gogh

4.1. INTRODUCTION

In the literature, we share the term emerging contaminants (ECs) or contaminants of emerging concern (CECs) for those compounds that are not included in any priority list of contaminants and whose effects on the environment are not yet known (Diamond and Burton Jr., 2021). This heterogeneous group includes chemicals with very different properties with two common characteristics: they are unregulated, and the scientific community cannot guarantee that they are not hazardous to the environment (Diamond and Burton Jr., 2021). Different sub-classifications can be defined according to the use (e.g., pharmaceuticals, personal-care products, industrial products…), common physicochemical properties (e.g., persistent organic pollutants), or specific chemical families (e.g., per- and polyfluoroalkyl substances, polybrominated diphenyl ethers…). Due to the growing use of many chemicals and the wider occurrence of CECs shown in the literature, the effect of CECs on the environment and human health has become one of the most complex environmental problems of this decade (Landrigan et al., 2018). The aquatic environment is typically the destination for CECs and stands out as one of the most sensitive compartments. Some studies point out that wastewater treatment plants (WWTPs) can barely remove CECs efficiently from wastewater, making their effluents an unwanted source of aquatic contamination (Loos et al., 2013; Mijangos et al., 2018).

Undoubtedly, the presence of CECs in impacted ecosystems has a direct consequence on aquatic organisms and can alter their living functions (Duarte et al., 2020; McCallum et al., 2019; Merola et al., 2022; Vossen et al., 2020). These contaminants are often metabolized and excreted but they can also be accumulated and spread through the food chain. The occurrence of CECs has been reported in aquatic organisms that comprise from invertebrate species (Marigómez et al., 2013) to several fish species including apex predators (Álvarez-Muñoz et al., 2015; Chiesa et al., 2019; Chynel et al., 2021; Madenjian et al., 2020). Recognizing which of these compounds are truly a concern is a complex issue due to the lack of information about them.

The main assessments to approach this issue are the study of alarming chemical properties (e.g., bioconcentration or bioaccumulation) and the evaluation of potential effects. The environmental risk assessment of CECs in a sample is often addressed by measuring the risk quotients (RQ). These values are calculated for each compound as the ratio between the experimental concentration measured in the sample and the expected no-effect concentration. This strategy can be used to prioritize between CECs when measures need to be taken or further research is needed to confirm if those contaminants are indeed a concern (Lopez-Herguedas et al., 2021).

Bioconcentration refers to the intake from water, and retention of a given contaminant and is one of the core properties to perceive the environmental risk. Contaminants that are bioconcentrated in aquatic organisms can build up to higher trophic levels (i.e., biomagnification) and even reach humans in the worst scenario. This retention can be measured as the bioconcentration factor (BCF) which is defined as the concentration of the contaminant in an organism, divided by its equilibrium concentration in water. Many studies indicate that CECs can also be extensively biotransformed resulting in

metabolites with equal or higher toxicity and bioaccumulation potential as the parent compound (Chen et al., 2021; Zind et al., 2021). Thus, the estimation of the bioconcentration focusing only on the parent contaminants could underestimate the true extent of the exposure.

In this work, we have studied the occurrence of CECs in a distinctive aquatic species, the European eel (*Anguilla anguilla*) by means of both target and non-targeted methods. The larvae of this species (*leptocephali*) cross from the Sargasso Sea to the European coast following ocean currents. At this stage, they accumulate the energy stores needed to metamorphose into glass eels and then migrate up estuaries to reach the river. Glass eel recruitment has drastically decreased since the early 1980s and the species is now below its safe biological limit. Among other confounding factors, several studies point out contamination as one of the causes behind this decrease (Palstra et al., 2006; Robinet and Feunteun, 2002). The accumulation of toxic substances including polychlorinated biphenyls (Freese et al., 2016; Maes et al., 2012), polycyclic aromatic hydrocarbons (Kammann et al., 2014), metals (Claveau et al., 2015; Figueiredo et al., 2018; Maes et al., 2012), etc., has been previously studied, especially in sub-adult (*yellow eel*) or adult (*silver eel*) stages, due to their diet (bottom-dwelling predators) and their high body fat contents. Glass eels are also likely exposed to chemicals in estuaries, which are considered a sink for various contaminants. In addition, it is now well accepted that all glass eels do not migrate up the estuary, some of them just settle in the estuary. These different patterns of migration could have a strong impact on the fate of the population because of the sex determinism in eels (Geffroy and Bardonnet, 2016; Tesch and Greenwood, 1977). It is, therefore, crucial to understand constraints on glass eels to either settle in or migrate up the marine/river continuum. A recent study suggested that glass eels exposed to methylmercury may present a lower propensity to migrate (Liu et al., 2019) and it becomes necessary to characterize the potentially hazardous compounds that glass eels are exposed to and understand their behaviour within their organism. In this study, therefore, we aim to gain further insight into the uptake of CECs by glass eels and elucidate their metabolization products. A risk assessment study was first conducted on the WWTP effluent that is released directly into the glass-eel habitat to address the most relevant CECs. The selected contaminants were used to perform a controlled exposure experiment with captured wild glass eels and evaluate their bioconcentration potential. Quantitative analyses of the targeted molecules and non-targeted analyses of their metabolites were carried out using a UHPLC-Q Exactive Orbitrap.

4.2. EXPERIMENTAL SECTION

4.2.1. Standards and reagents

Information regarding the analytical standards used in the targeted analysis is provided in section 4.6.4 (Table SI.4.1). This list includes a wide range of CECs known to be frequently detected in WWTP's effluents and some of them are prone to be included in future monitoring programs. Working solutions containing all the target compounds

and surrogates at 2 µg/g and 10 µg/g, respectively, were prepared in MeOH:H_2O (50:50, v/v; UHPLC-MS, Scharlab, Barcelona, Spain).

The solvents used in the SPE procedure were MeOH (HPLC, 99.9%, Sigma-Aldrich, St. Louis, MO, USA), ethyl acetate (HPLC, 99.9%, Sigma-Aldrich), ammonia (25%, Sigma-Aldrich) and formic acid (>98%, Panreac, Barcelona, Spain). For the UHPLC-q-Orbitrap analysis, formic acid, water, acetonitrile (UHPLC-MS grade, Fischer Scientific, Geel, Belgium), and ammonium acetate (≥ 99 %, Scharlab) were used in the mobile phases.

4.2.2. Multi-target analysis of Bayonne's WWTP effluent

An automatic large volume solid-phase extraction system (LV-SPE, MAXX Mess-u. Probenahmetechnik GmbH, Rangendingen, Germany) was used to sample 17L of Bayonne's (France) Pont de l'aveugle WWTP (111,667 population capacity, primary and secondary treatments) effluent in 12 h on the (November 30th, 2020). An in-house cartridge was prepared by filling a PFTE cartridge with 6g Strata HR-X and 2g of both Strata ZT-WAX and ZT-WCX, and then conditioned with 200 mL EtOAc:MeOH (50:50, v/v) followed by 200 mL Milli-Q. After the loading of the 17L of the sample, the cartridge was dried under a nitrogen stream and eluted using 300 mL EtOAc:MeOH (50:50, v/v) with 2% ammonium hydroxide and 300 mL of EtOAc:MeOH (50:50, v/v) with 1.7% formic acid. The extracts were pooled and evaporated in a rotary evaporator (LABOROTA 4000, Heidolph Laborota 4000, Schwabach, Germany) to 15 mL obtaining a relative enrichment factor (REF) of 1133. An aliquot of 55 µL was evaporated to dryness, reconstituted in 250 µL MeOH:H_2O (50:50, v/v; REF 250), and subjected to a multi-target analysis including 284 CECs.

4.2.3. Environmental Risk Assessment

An Environmental Risk Assessment (ERA) was carried out following the RQ approach described by Lopez-Herguedas et. al.(Lopez-Herguedas et al., 2021) Briefly, the measured environmental concentration (MEC) was divided by the minimum predicted no-effect concentration (PNEC) among three trophic levels to calculate the RQ of the ecosystem. RQ values > 1 indicate a high potential environmental risk, values between 0.1 and 1 indicate moderate risks, and for RQs < 0.1 the environmental risk was negligible. RQs were used to prioritize between CEC for further bioconcentration study. For each quantified compound, the available toxicity data (i.e. NOEC or EC50 values) was collected from ECOTOX (https://cfpub.epa.gov/ecotox/), NORMAN Network (https://www.norman-network.com/nds/) and Pesticide Properties databases (http://sitem.herts.ac.uk/aeru/ppdb/) or literature (Paíga et al., 2016; Papageorgiou et al., 2016) as annotated in Table SI.4.2 (Section 4.6.4.). When no experimental toxicity data were available, ECOSAR™ v. 2.0 software was used to predict the NOEC.

4.2.4. Fish Collection and Exposure

Procedures used in this study have been validated by the ethics committee N°073 'Aquitaine Poissons-Oiseaux' (ref: APAFIS#28511-2020120213191896 v3). The experiment was carried out in strict accordance with the EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e., Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2013-118, February 1st, 2013).

Wild glass eels were captured near the mouth of the Adour estuary using a dip net at night during flood tide. Once in the laboratory, glass eels were kept in an aerated tank with water from the sampling site. For the next 48h, the seawater was progressively diluted with fresh water. Fish were kept under 12 °C and a photoperiod of 12 L/12 D with a very low light intensity during the photophase (0.2–0.3 µW/cm^2).

After acclimatisation, glass eels were exposed to a continuous flow of a mix of diazepam, irbesartan, and propranolol at 3 ng·mL^{-1}, 3 ng·mL$^{-1,}$ and 0.1 ng·mL^{-1} nominal concentration respectively (i.e., concentrations found in WWTP effluent). Two tanks with four eels each (n = 8) were used in the exposure experiment and 5 samplings times were fixed: One before the exposure (control group t_0), three after 30h (t_{30h}), 5 days (t_{5d}), and 7 days (t_{7d}) of continuous exposure and the last one after 7 days of exposure followed by 7 days of depuration (t_{14d}). Sampled fish were killed using a lethal bath of anaesthesia (benzocaine, 0.05 mg·L^{-1}), individually measured for the wet weight (± 1.0 mg) and length (± 0.5 mm), and then flash-frozen in liquid nitrogen and stored at -80˚C before analysis. Additionally, 100 mL of water from tanks was also sampled at each sampling time and stored at -20°C for future determination of actual concentrations in water.

4.2.5. Sample Treatment

The glass eels were homogenized and extracted in pools of 2 eels for each exposure condition (n = 4x2). The homogenization of the pools was carried out in 7 mL MeOH:H_2O (95:5, v/v) using FUSLE (focused ultrasound solid-liquid extraction) first, as described by Mijangos et. al. (Mijangos et al., 2019), and second, the Precellys 24 Tissue Homogenizer (3x60s-6400rpm) under controlled cooled temperature (4 °C) (Cryolys, Bertin Technologies, Montigny-le-Bretonneux, France). The samples were then centrifuged for 15 min at 21,000 rpm (Centrifuge Allegra X-30R, F2402H, Beckman Coulter, High Wycombe, UK) to get the supernatant which was evaporated to 1mL under a gentle stream of nitrogen (Horizon Technology XcelVap, Lake Forest, CA, USA). The extraction of the samples was carried out following the method validated by González-Gaya et al. (González-Gaya et al., 2021) Briefly, samples were diluted to 6 mL in Milli Q water and loaded into homemade 0.5 g SPE cartridges (Strata HR-X/ZT-WCX/ZT-WAX, 3/1/1) previously conditioned with 6 mL MeOH and 6 mL Milli Q water. After loading the sample, the cartridge was rinsed with 5 mL of Milli-Q water and dried overnight under a vacuum. The cartridge was subsequently eluted first with 12 mL of EtOAc:MeOH (50:50, v/v) with 2% ammonium hydroxide and then 12 mL of

EtOAc:MeOH (50:50, v/v) with 1.7% formic acid. Eluate was concentrated to dryness under nitrogen stream, and reconstituted in 250µL MeOH:H_2O (50:50, v/v) spiked with 70 µg/L of azoxystrobin-d4 as internal standard. Water samples collected from each tank were also extracted following the same SPE procedure. Two additional pools of two glass eels, spiked with 50 µg/L of diazepam and irbesartan and 15 µg/L of propranolol, were also prepared to assess the suitability of the method and calculate the recoveries.

4.2.6 Instrumental Analysis

WWTP effluent, exposure tank water, and glass eel extracts were injected using the same chromatographic and mass spectrometry conditions in a Thermo Scientific Dionex UltiMate 3000 UHPLC running an XB-C18 column (150 x 2.1 mm, 2.6 µm, Phenomenex Kinetex®, CA, USA) with a pre-filter (2.1 mm, 0.2 µm, Phenomenex Kinetex®, CA, USA), coupled to a Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap mass spectrometer (UHPLC-q-Orbitrap) equipped with a heated ESI source (HESI, Thermo-Fisher Scientific, Waltham, MA, USA), operating in full scan−data dependent MS2 (Full MS-ddMS2) acquisition mode. Operating conditions described by Lopez-Herguedas et. al. (Lopez-Herguedas et al., 2021) were followed for optimal chromatographic separation and data acquisition (SI.4.6.1).

4.2.7. Data Handling

Four independent data managing strategies were implemented: i) multi-target screening of CECs for WWTP effluent, ii) target analysis of the selected contaminants in glass eels and exposure water, iii) suspect screening of potential metabolites of these selected contaminants in glass eels, and iv) suspect screening of CECs in glass eels.

For the quantitative analysis in target screening approaches, TraceFinder 5.1 software (Thermo-Fisher Scientific, Waltham, MA, USA) was used to process the experimental data. From the multi-target analysis, concentrations found in effluent water were used as MEC values for RQ calculation. BCFs of the exposure contaminants were calculated as the ratio between tissue and water concentrations.

The suspect screening of CECs was carried out in t_0 control samples using Compound Discoverer 3.3 (Thermo-Fisher Scientific, Waltham, MA, USA). Peak picking and peak alignment were conducted with mass tolerances of 5 ppm and maximum retention time deviations of 30s. Annotated features compared to the Norman list of CECs (https://www.norman-network.com/nds/) and MS2 fragmentation were contrasted with the mzCloud database (https://www.mzcloud.org/). Detailed workflows and feature filtering information are described in SI.4.6.2. When the standards of the candidates were available, experimental retention time was confirmed with an allowed error of ±0.1 min. If not, retention times predicted using the Retention Time Indices (RTI) platform (http://rti.chem.uoa.gr/) were compared to the experimental data. Finally, candidates were classified according to Schymanski's (Schymanski et al., 2014) identification confidence level and only levels 1 and 2 were considered.

The suspect screening of potential metabolites in glass eel (iii) was addressed by two parallel strategies. The differences between both strategies lay in the way to predict the potential metabolites from the parent contaminant. Although both used *in silico* predictions, the first strategy used BioTransformer 3.0 to predict the phase I and phase II metabolites and build the suspect list. The second strategy used the transformation prediction node in the Compound Discoverer workflow (Djoumbou-Feunang et al., 2019). Again, metabolites were searched in the mass lists with a tolerance of 5 ppm, peaks were manually checked and MS2 spectra were studied for confirmation in Compound Discoverer and MetFrag (Ruttkies et al., 2016) when PubChem ID was available for that candidate.

4.3. RESULTS AND DISCUSSION

4.3.1 Multi-target Analysis of Bayonne's WWTP Effluent

The method recoveries and quantification limits of all the target compounds are shown in Table SI.4.1. (Section 4.6.4.). Absolute recoveries of the method were obtained from the validation of the method in a previous work by Gonzalez-Gaya et. al., (González-Gaya et al., 2021) and the instrumental quantification limits were set as the lowest concentration level where the RSD < 30% and the trueness between calculated and theoretical concentration > 70% after the injection of three replicates of the calibration points.

The multi-target analysis carried out in the effluent of the WWTP detected the presence of 56 CECs. Their mean concentrations and standard deviations are included in Table 4.1. Pharmaceuticals represent 69% of the detected compounds, being antihypertensives the ones found at higher concentrations. A lower occurrence of fungicides, industrial chemicals, and herbicides was also detected (8%, 8%, and 6% respectively). These results are in line with our previous studies conducted on WWTPs from the Basque Country and Spain (Lopez-Herguedas et al., 2021; Mijangos et al., 2018; Miossec et al., 2019). Broadly saying, the distribution of CECs follows similar patterns, since angiotensin-II receptor blockers (such as the sartans) and some antibiotics appear at the same levels and, on the contrary, metformin or gabapentin, follow very different ones. The high concentrations of the compounds found in the list agree with several studies that stress the poor removal efficiency of secondary treatments upon these compounds. Golovko et. al., (Golovko et al., 2021) also reported the low removal efficiency of WWTP for most of the compounds in the top part of our list (e.g., sotalol, irbesartan, telmisartan, valsartan, tramadol, azithromycin, atenolol…). This implies that the presence of these compounds is not an isolated case for this particular scenario, but probably many other migration sites for glass eels are also contaminated with these pharmaceuticals.

Table 4.1*: Results of the quantitative analysis and risk assessment of CECs in the WWTP effluent. RQs of the compounds detected in the WWTP effluent were calculated from the minimum PNEC value among three trophic levels (min. PNEC). *RQ value for Telmisartan considering fish PNEC was 0.4. ** RQ value for Diazepam considering fish*

PNEC was 0.52. Only diazepam and telmisartan had hazardous RQs for more than one trophic level. The RQs of those contaminants posing a high risk potential are coloured in red, whereas those with moderate risk potential are coloured in orange.

Compound	Family	Concentration (ng/L)			RQ	min. PNEC
Sotalol	β-blocker antihypertensive	3100	±	54	0.01	Invertebrate
Diazepam	Anxiolytic	3000	±	74	0.72	Invertebrate**
Irbesartan	Antihypertensive	2700	±	38	1.5	Fish
Telmisartan	Antihypertensive	2000	±	52	1	Green Algae*
Valsartan	Antihypertensive	1300	±	3.1	0.01	Fish
Tramadol	Analgesic	930	±	20	0.01	Fish
Azithromycin	Antibiotic	650	±	6.2	34	Green Algae
Atenolol	β-blocker antihypertensive	450	±	1.3	0.01	Invertebrate
Hydroxychloroquine	Malaria treatment	450	±	5.4	0.03	Invertebrate
Carbamazepine	Antidepressant	430	±	14	0.17	Invertebrate
Bisoprolol	β-blocker antihypertensive	410	±	3.8	0.01	Invertebrate
Cetirizine	Antihistaminic	380	±	6.1	0.03	Invertebrate
2-Hydroxybenzothiazole	Industrial chemical	270	±	2.4	0	Invertebrate
Losartan	Antihypertensive	260	±	5.6	0.09	Invertebrate
Gabapentin	Antiepileptic	250	±	0.5	0	Invertebrate
Metformin	Antidiabetic	200	±	0.5	0.05	Fish
Mycophenolic acid	Antibiotic	200	±	2.4	0	Fish
Ketoprofen	Anti-inflammatory	200	±	2.3	0.1	Green Algae
Metoprolol	β-blocker antihypertensive	170	±	2.1	0.02	Green Algae
Fluconazole	Antifungal	140	±	1.1	0.01	Fish
Amantadine	Antiviral	140	±	2.3	0.01	Invertebrate
Trimethoprim	Antibiotic	130	±	0.92	0.01	Fish
Lidocaine	Anaesthetic	130	±	0.77	0	Invertebrate
Caffeine	Stimulant	120	±	2.1	0.02	Fish
Propranolol	Anaesthetic	110	±	2.2	0.18	Fish
Sulphapyridine	Sulphonamide	110	±	3.7	0	Invertebrate
Bezafibrate	Lipid-regulator	100	±	1.4	0.01	Invertebrate
Omeprazole	Proton pump inhibitor	93	±	1.3	0.02	Invertebrate
Caprolactam	Industrial chemical	87	±	5.3	0	Invertebrate

Verapamil	Antiarrhythmic and antihypertensive	68	±	2.3	0.01	Invertebrate
Lorazepam	Anxiolytic	51	±	0.91	0	Fish
Cyclophosphamide	Anticarcinogenic	51	±	2.1	0	Fish
Diuron	Herbicide	46	±	0.4	930	Green Algae
Equilin	Hormone	45	±	3.2	0	Invertebrate
Amitriptyline	Antidepressant	42	±	0.74	0.04	Invertebrate
Imidacloprid	Insecticide	42	±	0.21	0	Fish
Terbutryn	Herbicide	35	±	0.33	0.22	Green Algae
EDDP	Fungicide	31	±	0.79	0.03	Invertebrate
Mirtazapine	Antidepressant	25	±	0.61	0	Fish
Propiconazole	Fungicide	21	±	0.21	0	Fish
4-Methylbenzophenone	Industrial chemical	21	±	0.23	0	Green Algae
Thiabendazole	Anthelminthic	18	±	0.3	0.02	Fish
Benzophenone-2	Ultraviolet absorber in cosmetics	13	±	1.1	0.07	Fish
Tebuconazole	Fungicide	13	±	0.23	0.01	Fish
Eprosartan	Antihypertensive	12	±	0.5	0.01	Invertebrate
Indomethacin	Anti-inflammatory	12	±	3.1	0	Invertebrate
4-Hydroxybenzophenone	Industrial chemical	11	±	0.2	0	Invertebrate
Propyphenazone	Anti-inflammatory	11	±	0.2	0.01	Fish
Triphenyl phosphate	Industrial chemical	9.2	±	0.62	0.02	Green Algae
Clomipramine	Antidepressant	7	±	0.44	0.02	Fish
Diphenhydramine	Antihistaminic	6	±	0.13	0	Invertebrate
Carbaryl	Insecticide	6	±	0.2	0.01	Green Algae
Glibenclamide	Antidiabetic	4.9	±	0.54	0	Green Algae
Desloratadine	Loratadine metabolite	4.3	±	0.33	0	Invertebrate
Ketoconazole	Antifungal	3.8	±	0.14	0	Invertebrate
Acetamiprid	Herbicide	3.5	±	0.29	0	Green Algae

4.3.2 Environmental Risk Assessment

RQs calculated from the minimum PNEC values among the three taxonomic groups are summarized in Table 4.1. The RQs calculated from the most impacted taxonomic group allow us to get an idea of the whole ecosystem impact but does not address the individual comparison of the taxonomic groups. For that, the disaggregated RQs for each of the three taxonomic groups are shown in Table SI.4.2 (Section 4.6.4). Nine detected compounds showed environmental risk potential (high or moderate risk potential), being algae the most affected taxonomic group with three contaminants, telmisartan, azithromycin, and diuron, above the high potential risk limit (RQ > 1). Special attention must be given to the photosynthesis inhibitor diuron used precisely as an algicide, and herbicide in agriculture, which shows an unquestionable risk with a RQ almost 1000 times over the threshold. On the other hand, two contaminants threaten the invertebrate group with moderate RQs, diazepam, and carbamazepine. Finally, for the taxonomic group that specially concerned to this study, fish, the contaminants with the highest RQs were irbesartan, diazepam, telmisartan, and propranolol. Irbesartan (antihypertensive) was the only compound with a high-risk potential and the first candidate chosen to conduct the exposure experiment in glass eels. Telmisartan was the second contaminant with the highest RQ but, since contaminants with different mechanisms of action were sought, and telmisartan and irbesartan both belong to the same pharmaceutical family, diazepam (anxiolytic) and propranolol (anaesthetic) were chosen instead. The three selected CECs have been previously reported in the literature as harmful to different fish species. For instance, endocrine disruption was reported by Overturf et. al., (Overturf et al., 2016) in channel catfish after the exposure to diazepam, and the reproductive behaviour of fathead minnow was affected by this compound according to Lorenzi et. al., (Lorenzi et al., 2016). Studies with zebrafish (Zuo et al., 2022) also report the impact of irbesartan on the hatching success and the heart rate. In the case of propranolol, this compound can affect to the energy metabolism of meagre (Duarte et al., 2020), and even the swimming pattern of some fish (Matus et al., 2018), two interesting findings that can be related to the ability of glass eels to migrate (Bureau Du Colombier et al., 2007).

4.3.3 Exposure Experiments and Accumulation in Glass Eels

Mortality or alteration of the well-being status of glass eels was not observed during the exposure experiments. Groups sampled at different times did not differ significantly in weight or length (ρ levels>>0.05), as shown in the values collected in Table 4.2. The samples used for the optimization of the method showed recoveries of 91%, 99% and 71% for diazepam, irbesartan and propranolol respectively (Section 4.6.4., table SI.4.3). The recoveries of the method were applied to calculate the concentration of real samples. Low RSD (< %5) were also obtained for each of the three compounds.

***Table 4.2**: Biometric measurements (mean ± standard deviation) of glass eels sampled at the different exposure times. "n" refers to the sample size.*

	Length (mm) p = 0.36	**Weight (mg) p = 0.92**	**n**
T0	240 ± 43	70 ± 4	8
T2d	240 ± 62	70 ± 5	10
T5d	200 ± 59	60 ± 5	10
T7d	240 ± 54	70 ± 5	10
T14d	200 ± 43	65 ± 4	10

Diazepam, irbesartan, and propranolol concentrations in water during the uptake phase and in glass eels at different sampling times are presented in Figure 4.1. Water concentrations at t_0 and t_{14d} were always below detection limits for the three compounds. However, propranolol was measured in glass eels at all exposure times, regardless the water levels were below the detection limits at t_0 and t_{14d}. On the contrary, diazepam and irbesartan levels in glass eels showed a constant accumulation up to ~60 $ng \cdot g^{-1}$ and ~20 $ng \cdot g^{-1}$ respectively at t_{7d} followed by a decrease during the depuration time. This particular trend of propranolol was also described by Miller et. al., (Miller et al., 2017). In the same work, the authors also estimated that the half-life period for diazepam and metabolites was ~12h, which would lead to the complete depuration of these contaminants as confirmed in our study. Therefore, we can conclude that the contamination of glass eels by these pollutants is not an irreversible problem, but their constant release would not allow their depuration.

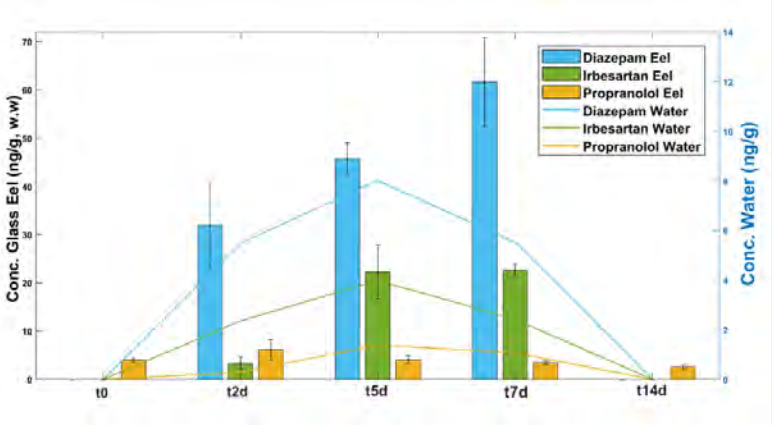

***Figure 4.1**: Concentrations of the three target contaminants in water (right axis) and glass eel (left axis) at five sampling times.*

The use of wild animals in bioaccumulation experiments has some implications that can hamper the results (i.e., unwanted contamination from environmental exposure) but, at the same time, they provide a real picture of the environmental issue that we must face. As mentioned, propranolol was found at low and constant concentrations over the experiment, even before exposure and after the depuration phase. At t_{2d} the concentration increased a 50%, an increment that was rapidly regulated by depuration or metabolization. Thus, propranolol was proven to be already bioconcentrated in advance from the wild and this hinders the calculation of its BCF within the experiment. This is not the first time that the presence of this pharmaceutical has been detected in fish, in fact, two studies reported high frequencies of detection in several wild fish species (Rojo et al., 2019; Xie et al., 2017).

Consequently, the evolution of BCFs overtime was only calculated for diazepam and irbesartan (Figure 4.2). Both compounds showed similar trends that reached a maximum BCF of 10 at t_{7d}. In the case of irbesartan, we can say that the bioaccumulation has reached steady state but, for diazepam, this fact is not entirely clear. In a recent review, Duarte et al. (Duarte et al., 2022) studied the bioconcentration of numerous neuroactive pharmaceuticals in fish and reported a mean BCF of 10 for diazepam. On the contrary, McCallum and colleagues (McCallum et al., 2019) did not find any evidence of bioconcentration for irbesartan in sea trout. Thus, diazepam bioconcentration results agree with those found in the literature but, to the best of our knowledge, this is the first time that a bioconcentration factor for irbesartan has been disclosed in fish. There is no evidence to suggest that the BCFs obtained in this experiment is related to log K_{ow} values of the selected compounds (log $K_{ow,\ Irbe}$ = 4.23, $K_{ow,\ Prop}$ = 3.48 & $K_{ow,\ Diaz}$ = 2.91), so the mechanism behind the accumulation of propranolol remains unknown.

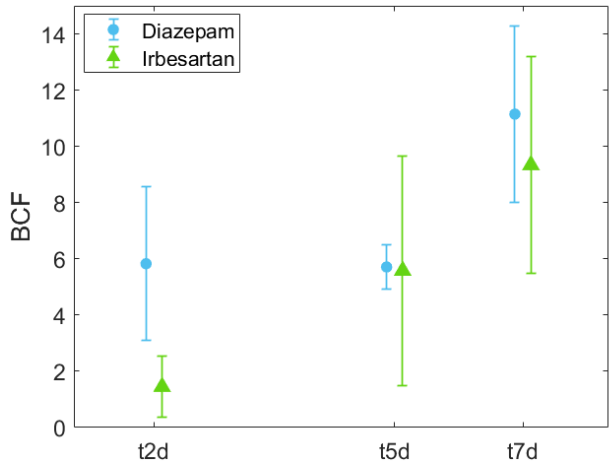

***Figure 4.2**: BCF values of diazepam and irbesartan over the exposure time.*

4.3.4 Metabolite identification

The suspect screening of biotransformation products (TPs) revealed the presence of one diazepam metabolite in glass eel (Figure 4.3). Structural assignments based on MS2 fragmentation data are available in SI.4.6.3. This metabolite corresponds to nordiazepam (*N*-methylation of the amine group) a well-known phase I metabolite that has been reported in many studies about diazepam pharmacokinetics (Greenblatt et al., 2021; Hooper et al., 1992; Zhou et al., 2020). These studies also describe two more active metabolites oxazepam and temazepam, but they were not detected in the analysis. Nordiazepam was identified within Schymanski's confidence level 2a (Schymanski et al., 2014) as no reference standard was available for confirmation. Thus, the quantification of this compound was not reached, but the transformation ratio was calculated based on the parent and the metabolite peak areas (Figure 4.4). The metabolization ratio of diazepam/nordiazepam at t_{2d} indicates that for the first 2 days almost no biotransformation of diazepam occurs in glass eels, therefore, the impact of this pollutant in acute exposures would be mostly due to the effect of the parent compound. This is not the case in long exposures where this ratio can be seen to decrease and nordiazepam would start to become relevant. As said before nordiazepam is an active metabolite, and it has higher bioavailability (Vernau and LeCouteur, 2008) and a longer elimination half-life (Wang et al., 2022) , which highlights once again the importance of monitoring the transformation products of CECs.

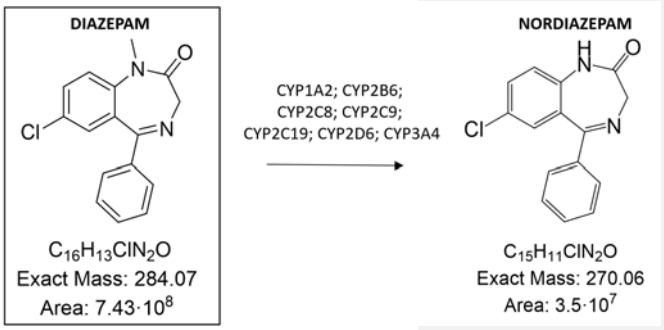

Figure 4.3: *Identified metabolites of the target compounds. Given areas indicate the maximum value found in the samples.*

Additionally, the suspect screening of TPs was also applied to the water samples of the tanks at different exposure times, but none of the mentioned metabolites was found above identification limits. However, we were able to identify one irbesartan TP which agrees with a minor surface water TP found by Boix et. al. (Boix et al., 2016) (SI.4.6.3.), but its occurrence could not be related to biotransformation since its presence in glass eels was not detected

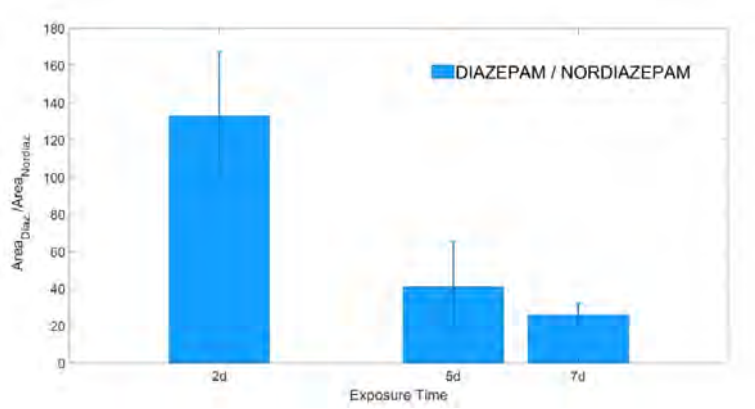

***Figure 4.4**: Metabolization ratios of diazepam and nordiazepam at different exposure times.*

4.3.5 Suspect screening of CECs in glass eel

Since we found propranolol in control samples, the analysis of other xenobiotic compounds that could be accumulated in wild glass eels gained interest. The suspect screening of CECs revealed a wide number of compounds, classified within *Schymanski*'s 1 and 2 classification levels (i.e., compounds confirmed with reference standards or structural library matching) (Table 4.3). Besides propranolol, the occurrence of two more pharmaceuticals was confirmed by reference

standards, primidone, and exemestane. It's worth mentioning that these two pharmaceuticals have been previously described as endocrine-disrupting chemicals (EDC) (Ismail et al., 2021; Jones-Lepp et al., 2015). In the case of exemestane, the exposure to this compound has been associated with female-to-male sex change in other fish species (Breton et al., 2019). Some studies point to a disparity between the distribution of females and males at different heights in estuaries (Harrison et al., 2014), an unexplained phenomenon for which we cannot rule out that exposure to endocrine disruptors has some kind of effect. To the best of our knowledge, most of the compounds identified in this suspect screening (e.g., Iloprost, Imazapic…) have never been reported in biological matrices. The chemical penetration enhancer 1-Dodecyl-2-pyrrolidinone was also identified in the samples (Godavarthy et al., 2009). Another benzodiazepine metabolite shows up within the identified compounds, 7-aminonimetazepam. Although they are structurally related, the metabolization of diazepam into this compound has not been described in the literature and it has only been related to nimetazepam and nitrazepam metabolization, which have no marketing authorization in France (Airagnes et al., 2019).

Table 4.3: *CECs identified in the suspect screening of t_0 control samples. Level 1 confirmation was only possible for the compounds included in our target list. (MW = Molecular Weight; rt = retention time).*

Compound	Formula	MW	rt	Confidence level	Use
Exemestane	$C_{20}H_{24}O_2$	296.17763	5.463	**1**	Pharmaceutical/Antineoplastic
Primidone	$C_{12}H_{14}N_2O_2$	218.10553	3.567	**1**	Pharmaceutical/Anticonvulsant
Propranolol	$C_{16}H_{21}NO_2$	259.15723	5.299	**1**	Pharmaceutical/Beta blocking agent
1-Dodecyl-2-pyrrolidinone	$C_{16}H_{31}NO$	253.24056	10.309	**2a**	Personal care products/ Cosmetics
2-(4-Morpholinyl) benzothiazole	$C_{11}H_{12}N_2OS$	220.06703	7.27	**2a**	Flame retardant
3-Hydroxybupivicaine	$C_{18}H_{28}N_2O_2$	304.21508	8.137	**2a**	Pharmaceutical/Anaesthetic
7-Aminonimetazepam	$C_{16}H_{15}N_3O$	265.12151	3.024	**2a**	Pharmaceutical/Benzodiazepine metabolite
Iloprost	$C_{22}H_{32}O_4$	360.23006	6.965	**2a**	Pharmaceutical/Antithrombic
Imazapic	$C_{14}H_{17}N_3O_3$	275.12699	2.074	**2a**	Herbicide
N, N'-Diphenylguanidine	$C_{13}H_{13}N_3$	211.11095	2.893	**2a**	Rubber component
Diisopropylethylamine	$C_8H_{19}N$	129.15175	2.206	**2a**	Plasticiser/Synthetic polymer
N-Ethylaniline	$C_8H_{11}N$	121.08915	5.663	**2a**	Industrial product
Norethandrolone	$C_{20}H_{30}O_2$	302.22458	13.188	**2a**	Anabolic steroid/Progestogen
Triethylene glycol monobutyl ether	$C_{10}H_{22}O_4$	206.15181	5.421	**2a**	Industrial product/Solvent

4.4. CONCLUSIONS

The exposure of glass eels to CECs was based on a previous prioritization of the most toxic and more abundant contaminants released from one of the main WWTPs of Bayonne. Based on the quantification of 56 CECs continuously discharged into the Adour estuary, we were able to calculate the risk quotients for three trophic levels. Our study concludes that the Adour estuary is, at least, threatened by 4 CECs that pose a high environmental risk potential, including the algicide diuron which exceeds the limits by almost 1000 times being a serious concern for the health of the algae together with telmisartan and azithromycin. The antihypertensive irbesartan also exceeds this limit, in this case being a threat to fish. The environmental risk assessment results were then used to select the contaminants with the highest impact on fish to conduct an exposure experiment on our target species, the glass-eel. The exposure to diazepam and irbesartan showed that these compounds were bioconcentrated up to 10 times in glass eels. Since the depuration period was sufficient to remove these two compounds from the glass eel organism, we can state that this contamination problem is not irreversible, but to solve it, the continuous release of these contaminants must be stopped. Surprisingly, propranolol was found at t_0, which suggests that glass eels accumulate low levels of this drug that are not fully eliminated neither after the quarantine period prior to the experiment nor the depuration phase of 7 days, which suggests a much more worrying contamination problem than in the previous case. In addition, we searched for metabolization products that could occur in the glass eels by suspect screening, but only one diazepam metabolite was identified, nordiazepam, an active metabolite with longer half-life and higher bioavailability, which stresses the importance of the monitoring of biotransformation products when the effects of CECs are assessed in biota. Finally, a suspect screening of CECs was also carried out to identify further cases of environmental contamination in t_0 glass eels. Two more CECs were confirmed as Schymanski's confidence level 1, exemestane and primidone, both being endocrine disruptors that could affect the sex differentiation of glass eels. Eleven more CECs were also identified as level 2a, some of them pharmaceuticals that had never been reported in fish. In summary, this work exposes the contamination problem faced by glass eels during their migratory stage. The habitat of this species is highly threatened by many contaminants that pose high risk potential, some of them, such as diazepam and irbesartan, being able to accumulate several times in their organism. Once again, it has been demonstrated that at this stage the glass eels are specially affected by contamination since they are directly exposed to contaminants released to estuaries from human activity, and thus, the protection of this endangered species also relies on the evaluation of the contaminants that are now part of its habitat. With this work we have addressed the identification of the main threats to glass eels and the information will be soon used to assess the effects of these contaminants and to study the connection between the glass eel exposome, their migratory behaviour and the population decrease.

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4.6 APPENDIX STUDY 2

SI.4.6.1: Chemical analysis: UHPLC-Q exactive conditions

WWTP effluent, exposure tank water, and glass eel extracts were analyzed in a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap mass spectrometer (UHPLC-q-Orbitrap) equipped with a heated ESI source (HESI, Thermo-Fisher Scientific, Waltham, MA, USA).

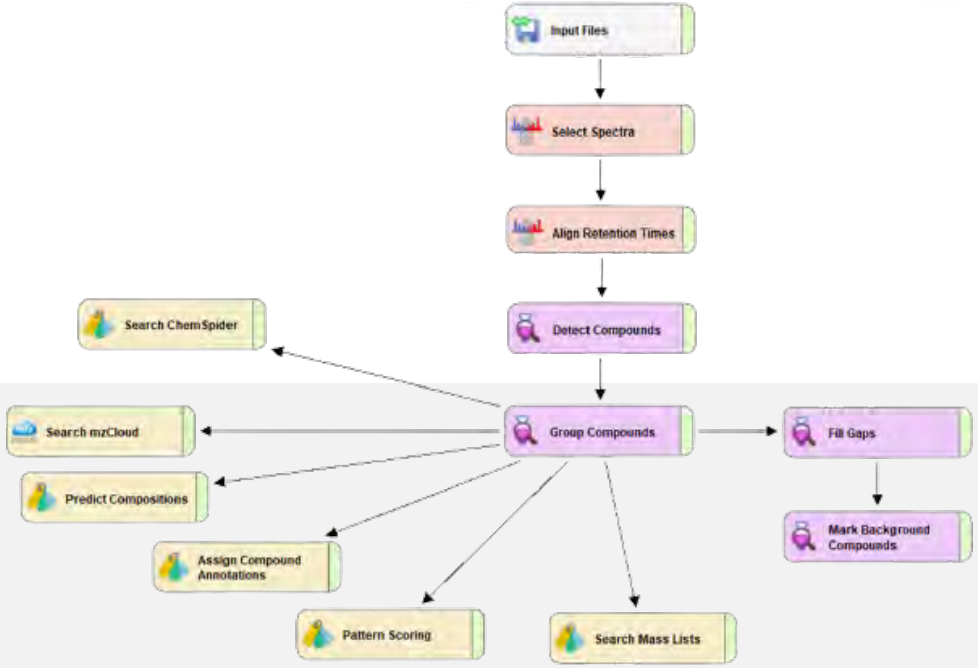
Extracts were injected on a C18 column (XB-C18 column, 150 x 2.1 mm, 2.6 µm particle size, Phenomenex Kinetex®, CA, USA) with a pre-filter (2.1 mm ID, 0.2 mm) from Thermo-Fisher Scientific. For positive ionization mode, a mobile phase consisting of water and acetonitrile (ACN), both containing 0.1% HCOOH was used. In the negative mode, 5 mmol/L ammonium acetate was used in both solvents. The separation was carried out at 0.3 mL/min flow rate and 50 °C temperature. The gradient started at 13% ACN with a linear increase to 30% at 4 min, another linear change to 50% ACN at 8 min (hold 12 min), and further to 90% ACN at 30 min. After 5 min at 95% ACN, the gradient was returned to initial conditions for 5 min. Three 5 µL injections of all the sample extracts were performed (both in the positive and in the negative ionization modes) while the autosampler was maintained at 5 °C.

The q-Orbitrap was operated in full scan – data dependant MS2 (Full MS-ddMS^2) discovery acquisition mode for both positive and negative ionization, choosing the most intense ions from the full scan for fragmentation, with an intensity threshold of $8.0 \cdot 10^3$ and a dynamic exclusion of 8 s. One full scan at a resolution of 70,000 full widths at half maximum (FWHM) at *m/z* 200 over a scan range of *m/z* 70-1050 was followed by three ddMS^2 scans at a resolution of 17,500 FWHM at *m/z* 200, with an isolation window of 3.0 *m/z*. The stepped normalized collision energy (NCE) in the higher-energy collision dissociation (HCD) call was set at 30 eV. HESI source parameters were set at 3.2 and 3.5kV spray voltage in positive and negative respectively, 320 °C capillary temperature, 40 arbitrary units (au) sheath gas (nitrogen), 15 au auxiliary gas, 280 °C auxiliary gas heater and S-lens RF level 55.0. Pierce LTQ ESI calibration solutions (Thermo-Fisher Scientific, Waltham, MA, USA) were used for external instrument calibration every three days. The instrument was controlled by Xcalibur 4.0 software (Thermo-Fisher Scientific, Waltham, MA, USA).

SI.4.6.2: Suspect screening workflows

Suspect screening of CECs in glass eels was carried out using the Compound Discoverer 3.3 program (CD; Thermo-Fisher Scientific, Waltham, MA, USA). The workflow used for the data analysis with the CD is shown in figure S2. ChemSpider and MzCloud libraries as well as the Norman list of contaminants of emerging concern (https://www.norman-network.com/nds/), including at that time more than 80.000 compounds, were used for contaminant identification. Tank water samples were used as procedural blanks to filter contamination coming from the input water (Caprolactam, Citroflex 2, Citroflex 4-A, Diethylphtalate, Diethylenetriamine… were some of the contaminants accumulated in glass eel from input water contamination). For the identification of the metabolites of the exposed contaminants, the first strategy used the same workflow shown in Figure S2 adding the suspect list built in BioTransformer 3.0. The second strategy used a parallel suspect screening workflow (Figure SI.4.1)

***Figure SI.4.1**: Workflow employed in Compound Discoverer 3.3 for suspect screening of CECs.*



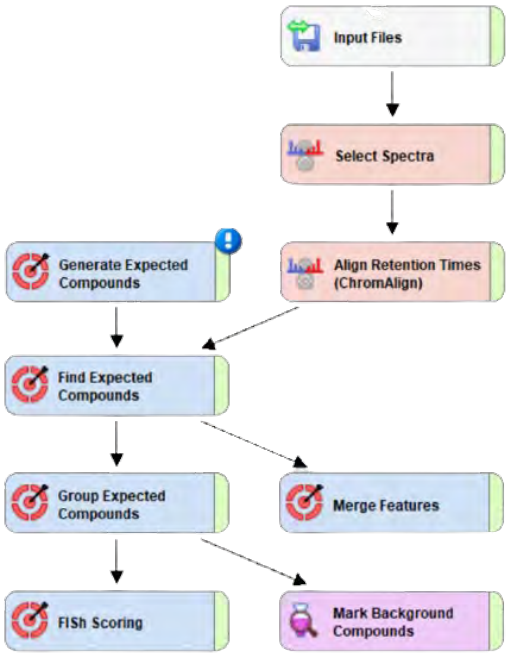


***Figure SI.4.2**: Workflow employed in Compound Discoverer 3.3 for prediction and identification of the transformation product of the selected contaminants.*

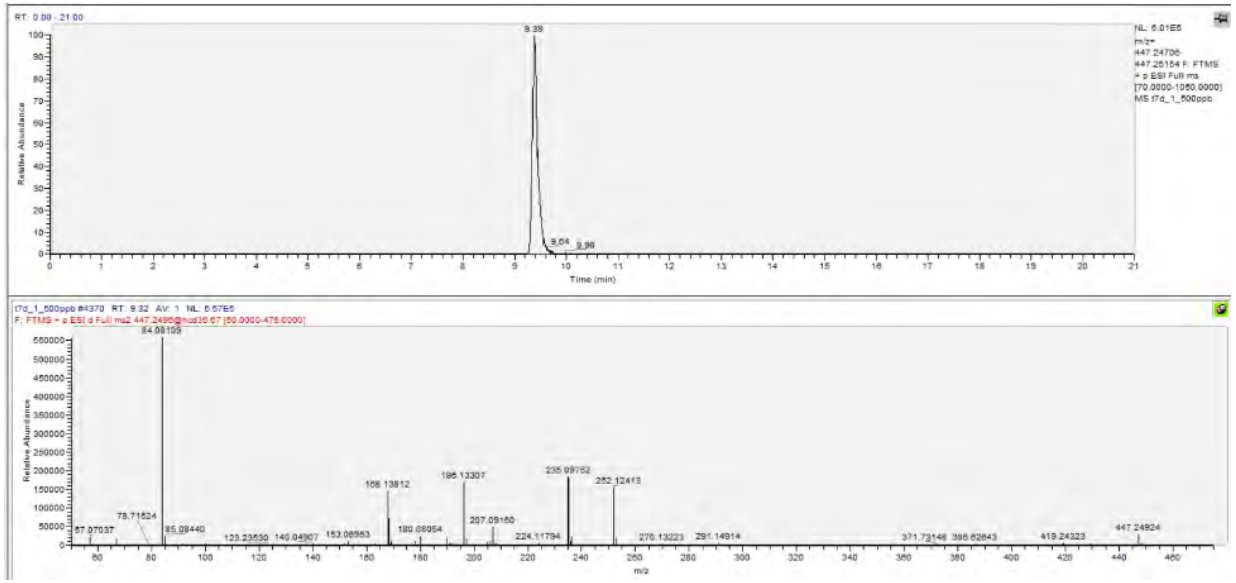
To identify suspects, only features with a Lorentzian chromatographic peak shape were considered. Some post-run considerations were taken into account, the molecular formula proposed by Compound Discoverer was only considered when the error compared to the exact mass was less than 5 ppm, when the measured and expected isotope patterns fit at least 30% and the peak has a minimum area of 10^6. When the MS2 was available, it was compared with the corresponding spectra in mzCloud library (https://www.mzcloud.org/) and a threshold value of 70% was considered for positive identification. And finally, minimum peak ratios between procedural blanks and samples above 20 were included.

Retention time was considered before confirmation. When the pure standard was available, a deviation of ± 0.1 min was admitted for positive identification and when it was not, an estimation of the theoretical retention time was performed using the retention time index platform (http://rti.chem.uoa.gr/).

SI.4.6.3: Proposed chemical structure and MS assignment for TPs.

Figure S3: *Experimental and mzCloud MS2 fragmentation matching for nordiazepam.*

Figure SI.4.3: *Experimental ion extracted chromatogram and MS2 fragmentation for Irbesartan's metabolite.*



Experimental data of the Irbesartan's metabolite found in water matches with the one found by Boix et. al., 2016[1]:

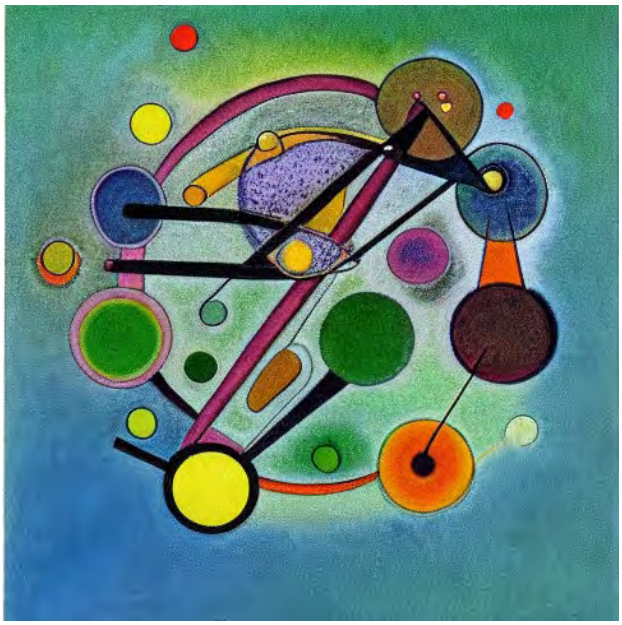
ISW1b	ESI+	7.3	C25H31N6O2	447.2495	−1.3	13.5	Hydroxylatio
			C5H10N	84.0814	0.1	1.5	+Hydrogenati
			C10H18NO	168.1390	0.2	2.5	
			C14H11N4	235.0995	1.1	11.5	
			C14H11N2	207.0922	0.0	10.5	
			C11H18NO2	196.1343	0.5	3.5	
			C13H10N	180.0814	−0.1	9.5	
			C14H10N	192.0810	−0.3	6.5	
			C14H8N	190.0659	0.2	11.5	

1 Boix, C. *et al.* Biotransformation of pharmaceuticals in surface water and during waste water treatment: Identification and occurrence of transformation products. *Journal of Hazardous Materials* **302**, 175–187 (2016).

SI.4.6.4: Tables SI.4.1/4.2/4.3

5. STUDY 3

Glass eel metabolomics: Studying the impact of diazepam and irbesartan and the differences in the migratory phenotypes



The Krebs Cycle – Wassily Kandinsky

5.1 INTRODUCTION

The migratory cycle of the European eel (*Anguilla anguilla*) is still one of the great unknown cycles within the animal kingdom. Adult eels spawn in the Sargasso Sea and the leptocephali (i.e., the larvae stage) drift with the Gulf Stream to reach the European continental shelf, where they metamorphose and turn into the so-called glass eel. In their second migratory phase, glass eels migrate up estuaries to join rivers, taking advantage of the current during the upstream flood tide and hiding in the estuarine bed during ebb tide (Gascuel, 1986). Along the estuarine migration, glass eels undergo several changes including morphological, physiological, and behavioural changes (Elie and Rochard, 1994), as well as pigmentation process (Elie et al., 1982), gut development, osmotic adaptations (Ciccotti et al., 1993), and hormonal modifications (Jegstrup and Rosenkilde, 2003). In addition, previous studies suggested that glass eels do not use every flood tide to migrate (Beaulaton and Castelnaud, 2005; Gascuel, 1986) and that some individuals may not migrate to rivers and complete their life cycle in coastal or estuarine waters (Daverat et al., 2006; Tsukamoto and Aoyama, 1998).

These different migratory patterns could have a strong impact on the fate of the population because, the European eel is a species with environmentally determined sex and males are generally observed to dominate in high-density environments, often associated with estuarine or lower river reaches, whereas females tend to become increasingly dominant with increasing distance from the sea (Davey and Jellyman, 2005; Harrison et al., 2014). Understanding glass eel migration is therefore a crucial issue for the species. Although many studies have aimed to understand the mechanism behind the differentiation of the two migratory phenotypes (i.e., active glass eels that are able to use the tide to migrate efficiently, and inactive ones that settle in estuaries (Liu et al., 2019a)), an accepted physiological explanation is still pending (Beaulaton and Castelnaud, 2005).

The energetic condition of the glass eels has been suggested in some studies to explain, at least partly, the facultative migration (Bureau Du Colombier et al., 2007; Edeline et al., 2007; Liu et al., 2019a). However, as described by Claveau et. al. (Claveau et al., 2015), contamination may also have an impact on migratory behaviour. Furthermore, the glass eels are specially affected by contamination since they are directly exposed to contaminants released to estuaries from human activity (e.g., WWTP effluents), thus, the protection of this endangered species also relies on the evaluation of the contaminants that are now part of its habitat.

In this vein, previous works by our research group have tried to address the fate of emerging contaminants (ECs) in the Adour estuary (Bayonne, Basque Country, France), which it is one of the main habitats for glass eels. In 2017, the three main WWTPs that release their effluents into the estuary were studied and the quantification of contaminants included in the Water Framework Directive (WFD) was performed (Cavalheiro et al., 2017). More recently, the environmental risk assessment study conducted in the previous study (Alvarez-Mora et al., 2022), led to the identification of

diazepam, irbesartan and propranolol as the most remarkable ECs released by the WWTP of Bayonne. Glass eels were then exposed to these ECs to study their bioconcentration and biotransformation potential. This work is a follow-up to that study and aims to assess the effects of the selected ECs by means of HPLC-HRMS metabolomics.

Metabolomics is the term we used to define the study of the small endogenous (<1.5kDa) metabolites in an organism or part of an organism. Alterations in the metabolite profile can provide a clear insight into the health status of an organism and is, thus, often used in toxicology to find the biochemical pathways disturbed by a stressing event (e.g., disease, nutritional imbalance, contaminant exposure…). Metabolomics applied to environmental assessment has been successfully applied to explore the cause-effect mechanisms of ECs in aquatic organisms (Bhagat et al., 2022; Colás-Ruiz et al., 2022; Labine et al., 2022; Xu et al., 2022) and it is one of the key elements in adverse outcome pathways (Brockmeier et al., 2017; Dumas et al., 2022).

The underlying approach is that the study and the analysis of slight modifications in the metabolic profile would provide key information regarding chemical events that happen when, for instance, we exposed the glass eels to a contaminant. To ensure the right application of this approach, we should assure that most of the remaining factors that might modify the metabolomic profile are kept constant (Villas-Boas et al., 2007). This means that the experiments should be carefully designed taking account of the sources of variation (i.e., genotype, phenotype, age, sex, feeding…) and minimizing the effect of all the non-relevant ones. As previously mentioned, sex and feeding are not influential since the sex of glass eels is still not defined and they fast until the juvenile stage. However, when working with wild glass eels, controlling the genotype is not possible as compared to laboratory animals with common genetic lines, and the migratory phenotype may also play an important role in the variability of the metabolic profile.

Comentado [VB22]: I am not sure to understand why you say that as you Will not study the two phenotypes. You could rather keep that for the discussion no?

The HRMS metabolomics analysis is typically carried out f
analysis strategy to benefit from the unbiased and holistic ap
In this way we lack the previous knowledge about the comp
and the aim is to discover the metabolites that show di
experimental conditions.

Comentado [i.23R22]: The idea is to present also the behaviour results in this part of the thesis. Once we prepare it to submit the paper I will remove all the info regarding the behaviour experiment

The intrinsic biological variation together with the large a
make it even more difficult to find potential or significan
multivariate data analysis is used to simplify this task (Worley and Powers, 2013). The lack of consensus about data pre-processing and multivariate analysis tools (especially because they are usually application-oriented) can lead to an incorrect/biased use that can therefore result in biased interpretations. Given the nature of the metabolomics studies, where the discrimination of different experimental conditions can lie in a small proportion of the total variance, unsupervised multivariate analysis tools such as PCA can fall short. By contrast, classification methods such as partial least squares

Comentado [NE24]: https://doi.org/10.1016/j.mam.2021.101006

discriminant analysis (PLS-DA) can be useful to find differences between the targeted groups and discover the metabolites related to the class separation, especially when combined with variance analysis (i.e., ANOVA) tools.

As a follow-up to the previous study on the fate of CECs in glass eels, the aim of this work was to investigate the effects of two of them in glass eels, diazepam and irbesartan. As mentioned above, these contaminants were selected from the previous environmental risk assessment study and their toxicity was tested in glass eels under controlled conditions at environmentally relevant concentrations. Glass eels were exposed to both contaminants separately, as well as their mixture, and their polar metabolome and lipidome were studied for signs of alterations related to the exposure. In addition to the environmental metabolomics study, a second set of glass eels was used to study the migratory behaviour of glass eels. For that purpose, glass eels were previously sorted according to their migratory behaviour and their metabolic profiles were analysed in order to find discriminating profiles between the two groups. In addition to this, the differences in the sensitivity of the two phenotypes to diazepam exposure was also studied. Target and non-targeted analysis of the polar metabolome and non-targeted analysis of the lipidome were carried out using a UHPLC-Q Exactive Orbitrap to address this double approach.

5.2. EXPERIMENTAL SECTION

5.2.1. Standards and reagents

Information regarding the metabolite standards used in the targeted analysis is provided in section 5.6.1 (Table SI.5.1). The list includes a wide range of metabolites representative of the main biochemical pathways usually assessed in environmental metabolomics. Diazepam and irbesartan standards were acquired from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol (MeOH), water, acetonitrile (ACN) and 2-propanol (IPA) were obtained from PanReac AppliChem (Barcelona, Spain). HPLC grade tert-butyl methyl ether (MTBE) was obtained from Sigma-Aldrich (St. Louis, MO, USA). MS grade formic acid (98% purity) and ammonium formate salt (≥99% purity) were purchased from Fisher Scientific (Madrid, Spain) and Fluka (Steinheim, Germany) respectively

5.2.2. Fish Collection

Procedures used in this study were validated by the ethics committee N°073 'Aquitaine Poissons-Oiseaux' (ref: APAFIS #32100-2021062317263102 v2). The experiment was carried out in strict accordance with the EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e., Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2013-118, February 1st, 2013).

The sampling site correspond to the estuarine mouth of a small river (French Basque coast France: 43.857822, -1.390253, map available in Bolliet et al., (Bolliet et al., 2017)).

The fishes were collected in December using a dip-net at night and during flood tide. Once collected, fish were transferred to the laboratory and maintained at 12 ± 0.5°C overnight in a tank containing aerated water from the fishing site. During the next week, 1/3 of the water was daily replaced by freshwater previously aerated during 24 hours. The room was maintained under a photoperiod of 10 L/ 14 D with a very low light intensity during the photophase (0.2-0.3 $\mu W/cm^2$). After this transition to freshwater, two batches of glass eels were separated, 60 were used to assess the effects of diazepam and irbesartan and 80 to study the differences between behavioral phenotypes. These latter glass eels were anesthetized by batches of 10 individuals (Benzocaine, 0.01 mg L^{-1}), individually measured for initial wet weight (± 1.0 mg) and length (± 0.5 mm) and tagged with Visible Implant Elastomer (VIE Tag). Each individual VIE tag was a unique combination of two or three hypodermic spots of four different colors on the back (Liu et al., 2019a). Once tagged and handled, all individuals were released in two buckets (40 individuals per bucket) to recover overnight prior to the exposure to diazepam.

5.2.3 Exposure conditions to assess the effects of diazepam and irbesartan on glass eels.

A graphical overview of the setup used for contaminant exposure is shown in section 5.6.2. Briefly, glass eels were exposed to diazepam and irbesartan at a nominal concentration of 3 $ng \cdot ml^{-1}$ pumping the 4L contaminant solutions using two peristaltic pumps. A third peristaltic pump was used to pump both solutions into a third tank to assess the impact of the contaminant mixture. To prepare the stock solutions the analytical standards of each contaminant were dissolved in water carried by a minimum amount of MeOH (< 0.1%). At the same time, two control tanks were prepared pumping water and water containing the above expected concentration of MeOH in the exposure tanks. Twelve glass eels were introduced in each of the five tanks all equipped with shelters for glass eels to hide. The temperature and photoperiod were maintained at 12°C and 12L/12D and checked daily. Six glass eels were sampled after 7 days of exposure and six after 7 extra days of depuration. This way the experimental groups were defined as follows: two control groups (C7 & C14), two diazepam-exposed groups (D7 & D14), two irbesartan-exposed groups (I7 & I14), two groups exposed to the mix of both contaminants (DI7 & DI14), and two groups exposed to the expected MeOH concentration (MC7 & MC14). Glass eels were anaesthetized, killed using a lethal bath of anesthesia (Benzocaine, 0.05 mg L^{-1}), individually measured for the wet weight (± 1.0 mg) and length (± 0.5 mm), and then flash-frozen in liquid nitrogen and stored at -80˚C before analysis.

5.2.4. Exposure conditions and behavioural test for phenotype differentiation.

Two groups of 40 glass eels were placed in two tanks supplied with the same water in open circuit with a peristaltic pump (10.0 mL/min). Another peristaltic pump (1ml/min) supplied one of the tanks with water and the other one with diazepam in order to obtain a concentration of 5ppb in the tank. Both tanks were equipped with shelters located at the opposite of the arrival of diazepam and there were submitted to the

same conditions of light intensity, photoperiod, and temperature than during freshwater acclimatization. Flows of water and diazepam, as well as water temperature were daily checked during the 7 days of exposure.

After one week of exposure, the 40 control and 40 contaminated glass eels were transferred into an annular tank installed in a temperature-controlled room as described by Liu et., al(Liu et al., 2019a). Two pumps fixed on the opposite ends of the tank allowed the change in the water current direction every 6.2 h to mimic the tides (alternately clockwise and counterclockwise water flow). The swimming behaviour of glass eels was tracked individually during 7 days by a camera programed to record 15 s every 40 min. In the wild, migrant glass eels synchronize their swimming activity to the tide, swimming with the water current during the flow and hiding in the substratum during the ebb tide. In our installation, glass eels synchronizing their swimming activity to the change in water current direction were considered as having a high probability of migration (active). Individuals remaining permanently or mostly hiding in the substrate, regardless of the water flow direction, were considered as having a low propensity to migrate (inactive) (Bolliet and Labonne, 2008; Bureau Du Colombier et al., 2007; Liu et al., 2019a). The room was maintained under the same conditions of light intensity, photoperiod, and temperature than during exposure and a constant UV light (0.6 µW/cm^2) was added to see the VIE Tag.
After 7 days of observation, active and inactive glass eels were removed from the tank and placed in sheltered, current-free tanks for 5 days before being euthanized so that swimming activity would not influence the results of the metabolomic analysis. During these 4 days, the glass eels remained in the shelters and no swimming activity was observed.

5.2.5. Metabolite Extraction

Individual glass eels were sliced using a scalpel and the sample extraction (~100 mg) was carried out via an unbiased extraction approach based on a two-phase extraction system. 300 µL MeOH and 100 µL of Milli Q water (UHPLC-MS quality) were added to the samples before being homogenized (6400 rpm – 60s x 3 – 15 s) (Precellys®, Bertin Instruments, Montigny-le-Breonneux, France) at 4 °C using homogenization tubes with ceramic beads (1.0 mm diameter). 900 µL MTBE and 250 µL Milli Q water were then added and the homogenization process was repeated (Chen et al., 2013). The extracts were centrifugated for 5 min (4°C, 21000G) (Allegra X-30R Centrifuge, Beckman Coulter®), the solid fraction was removed and the liquid centrifuged for 15 min (Ribbenstedt et al., 2018). The liquid phase had two phases, the organic fraction on top and the aqueous fraction at the bottom which were collected separately. The organic phase was evaporated under N_2 flow (XcelVap®), the residue was dissolved in 1.5 mL IPA:MeOH:water (4:3:1) mixture (Paglia and Astarita, 2017) and diluted for injection (1:10, v/v). In the same way, the polar phase was collected and diluted directly (1:10 and 1:50) using a mixture of water:methanol (3:1). For qualitative control purposes, a pool containing small aliquots from all the extracts was also prepared.

5.2.6. Instrumentation

Sample analysis was carried out using a Dionex Ultimate 3000 UHPLC (Thermo Scientific) coupled to a Q-Exactive Focus hybrid quadrupole-Orbitrap MS (Thermo Scientific, Waltham, Massachusetts, United States) with a heated electrospray ionization source in positive and negative modes (HESI, Thermo, CA, USA). For the polar metabolome analysis, the separation was achieved using a Kinetex® C_{18} (2.6 µm, 100 Å, LC Column 150 x 3 mm; Precolumn SecurityGuard® ULTRA Cartridges UHPLC C_{18} 2.1mm) column was used and for the lipidome analysis a Luna® Omega C_{18} (1.6 µm, 100 Å, LC Column 150 x 2.1 mm). Mobile phases in polar analysis were A: HPLC Water (10mM Ammonium formate in the negative mode and 0.1% Formic acid in the positive mode) and B: HPLC Water:AcN 5:95 (10mM Ammonium formate in the negative mode and 0.1% Formic acid in the positive mode). The gradient was as follows: 0 min 5% B; 1 min 5% B; 15 min 95% B; 20 min 95% B, 24 min 5% B, with flow rate of 0.3 mL/min, column temperature of 35°C, and injection volume of 5 µL. On the other hand, separation conditions for the lipidome analysis were adapted from Chen et. al., (Chen et al., 2013). Mobile phases were A: 60% ACN in water, and B was IPA:ACN (9:1), both containing 10 mM ammonium formate and 0.1% formic acid in both positive and negative modes. The gradient was as follows: 0 min 40% B; 3 min 70% B; 10 min 100% B; 22 min 100% B; 27 min 50% B; 28 min 50% B with a flow rate of 0.2 mL/min, column temperature of 35°C and injection volume of 5 µL. Data were acquired using the full scan data dependant MS2 (Full-Scan-dd-MS2) mode, where the full scan range was fixed between m/z 50-750 for polar compounds and 70-1000 for non-polar ones with a resolution of 70.000 in the Full MS scan. Three ddMS2-scans were completed for every full scan with a resolution of 17.500. The ionization conditions in the HESI were the followings: The sheath and the auxiliary gas flow rates were established at 55 and 10 units respectively, the ion spray voltage was 3.10 kV for positive ionization and 3.20 kV for negative, and temperatures were kept at 300°C for the capillary and 290°C for the auxiliary gas heater.

5.2.7. Data Handling

For the targeted analysis of the polar metabolome data were processed using Trace Finder 5.1 (Thermo Scientific). The integration of the chromatographic peak areas was carried out by the automatic function using the Intelligent Component Integration System (ICIS) algorithm. The correct integration was evaluated metabolite by metabolite over the calibration solutions. The non-targeted screening of metabolites was performed using Compound Discoverer 3.3 (Thermo-Fisher Scientific, Waltham, MA, USA). Features were detected following the non-targeted metabolomics workflow (Section SI.5.6.3) using a mass tolerance of 5ppm and a minimum peak intensity of 10^5. All the metabolomic features found were matched with freely available MS mass lists such as HMDB 5.0 (Wishart et al., 2022), LipidMaps (Fahy et al., 2007), and the endogenous metabolite database from Compound Discoverer and mzCloud MS libraries. Additionally, from the resulting list of candidates, a manual inspection was carried out to removed either noisy peaks or non-meaningful peaks (bad chromatographic shape, poor fragmentation), and only features with peak areas 10

times larger in samples than blanks were further considered. Through the Fish Scoring application, the *in silico* fragmentation of the final list of candidates was run to include the match level among the quality metrics. The statistical handling of the filtered data was carried out by MetaboAnalyst (v5.0) (Pang et al., 2022). PLS-DA was followed by statistical tests (ANOVA, t-tests and fold change analysis) to explore the effect of the different conditions and discover the altered metabolites. In parallel, to assess the robustness of the PLS-DA models and show it in the most intuitive manner, the PLS-Toolbox (v8.9.1, Eigenvector Research) was used to create the receiver operating characteristic (ROC) curves.

5.3. RESULTS AND DISCUSSION

5.3.1. Assessing the effects of diazepam and irbesartan on glass eels.

5.3.1.1. Analysis of the polar metabolome

The identified metabolites were annotated according to the confidence levels proposed by Schymanski et. al., (Schymanski et al., 2014). 44 metabolites detected by the target screening approach were annotated as a level 1, whereas for the non-target screening approach 14 metabolites were annotated as level 2, and 6 as level 3 (Table SI.5.2). Quality control samples were used to exclude all the metabolites showing an RSD > 40%. The data resulting from both screening methods were merged, normalized by the total sum of the metabolites in each sample, and autoscaled to homogenize the scale differences between concentrations and peak areas (from target and non-target screening respectively).

The work was based on two assumptions that were tested prior to any further statistical analysis of the data: the irrelevance of the 7-day time lapse in the metabolic status of the glass eels from the first sampling to the second and the lack of significant effect of the MeOH used to dissolve the standards. In the case of the relevance of time in the metabolic status of the animals, a PLS-DA model was used to assume two different classes and identify the most important variables in the differentiation of the groups, and a t-test was then applied to identify the metabolites significantly different among the experimental conditions. The scores plot of the PLS-DA model and its variable importance in projection (VIP) plot are shown in Figure 5.1. The robustness of the model can be checked via the ROC curves in SI.5.3 (section 5.6.1). The PLS-DA model clearly differentiates the two conditions and this fact is confirmed through the fold change (FC) analysis, showing that the levels of the first three metabolites in the VIP plot (i.e., tryptophan, creatinine, and cytosine) are significantly lower in C14 (SI.5.4). Therefore, we have to admit that the initial assumption was not valid.

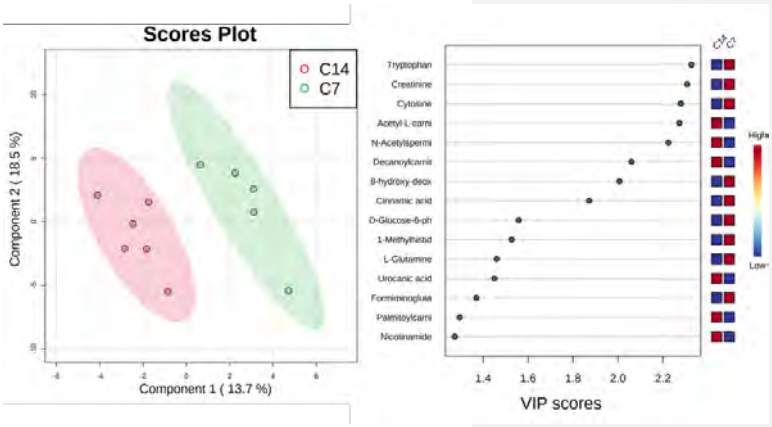


***Figure 5.1**: PLS-DA scores plot of the polar metabolites of the control groups at two sampling times (left) and its VIP scores (right).*

Comentado [VB25]: Sorry Iker, I am not familiar with these models but how can you explain that C7 and C14 overlap in fig 1 whhereas the PLS-DA model differentiates them in fig 2? It is a little bit confusing

Comentado [26]: *Respuesta a Valerie Bolliet (24/08/2022, 14:09): "..."*
The PLS-DA is a biased strategy where the model tries to find the maximun variance between the classes included in the model. In the previous case it tried to find the max. variance among 4 classes and here specifically between this C7 and C14. Thats why the discrimination power is much higher in this second model.

The suitability of methanol as a contaminant carrier was ch
control groups to the two methanol control groups (C7 vs
Volcano plot was used to find significant metabolites betw
but the results indicated that there were no significant diff
at either 7 (Figure 5.2) or 14 days (not shown). The re
concentration of methanol used in this work does not have
profile of the glass eels. This outcome agrees with the publica
et al., 2012), where the authors stress the suitability
contaminants in exposure experiments.

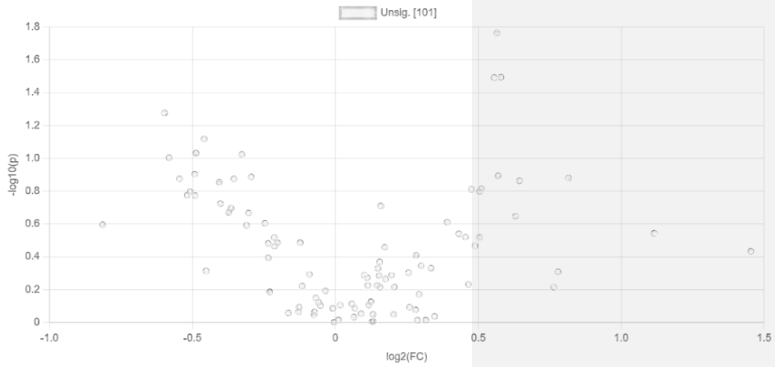


***Figure 5.2**: Volcano plot of C7 vs MC7. Y Axis shows the ρ values obtained from the t-test and x axis the fold change analysis.*

Although the relevance of the time was confirmed and the metabolic status at 7 days and 14 days in control samples are different, we run the comparison at the same exposure time between the exposed tank and the control one (i.e., D7 vs C7 and D14 vs C14) to i) study the effect of the contaminants compared to the control at 7 days and ii) in the case that the contaminant effect was confirmed, examine if the metabolic levels match the control group after the depuration phase.

To determine whether the exposure to the selected contaminants had any impact in the disturbance of the polar metabolic profile of glass eels, control samples were compared to the three exposure conditions (Figure 5.3). The cluster distance between control samples and the exposed samples can give us insight into the degree of their impact. The mixture of diazepam and irbesartan showed the highest distance from controls, and the ROC curves from cross-validation (SI.5.5) confirmed that this group was completely differentiated from the rest. An impact gradient can be retrieved from the PLS-DA scores plot with diazepam exposed samples barely differentiating from controls and irbesartan overlapping between controls and the mixture. Although the concentration addition of the two contaminants seems to be the simplest explanation for this, we cannot discard the synergic effect option.

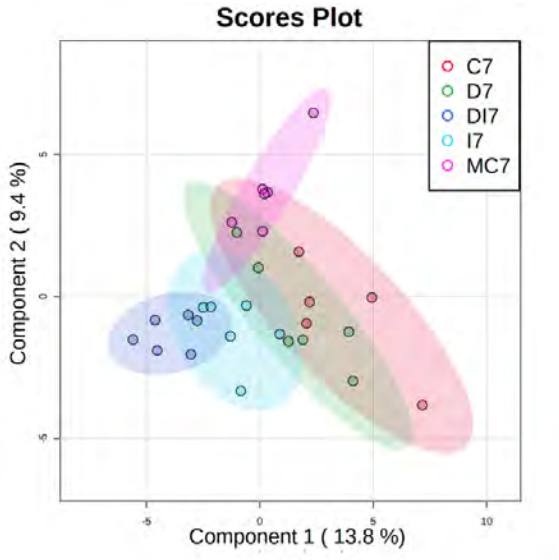


***Figure 5.1**: PLS-DA scores plot of the identified metabolites for all the experimental condition at day 7.*

The identification of metabolites significantly altered in DI7 was achieved by the direct comparison between this group and the control group. For higher statistical significance, C7 and MC7 control groups were merged. The t-test (SI.5.6) revealed that 11 metabolites were significantly altered (ρ value < 0.05). Among these compounds the

Comentado [VB27]: WHy didn't you merge them in fig 3?

Comentado [28]: *Respuesta a Valerie Bolliet (24/08/2022, 14:32): "..."*
After checking the results I definitely could, but since the MC had only been compared to controls I thought it was also a good idea to include them separately so we could have an actual idea of the distance difference from the most affected one to MC and C. This is the plot that tells us that MC7 and C7 are really overlapped compared to DI7 and justifies the merging of those groups afterwards.

fold change analysis (fold change threshold = 2) showed that adenosine 5'-monophosphate (AMP), D-Glucose-6-phosphate, L-Citrulline and Adenosine levels were remarkably reduced after the contaminant mixture exposure, whereas urocanic acid appeared enhanced within this group (Figure 5.4.A). The fold change analysis for irbesartan also revealed low D-Glucose-6-phosphate (G6P) and L-Citrulline levels and in the case of diazepam low L-Citrulline were also detected together with an increment in the Adenosine diphosphate levels.

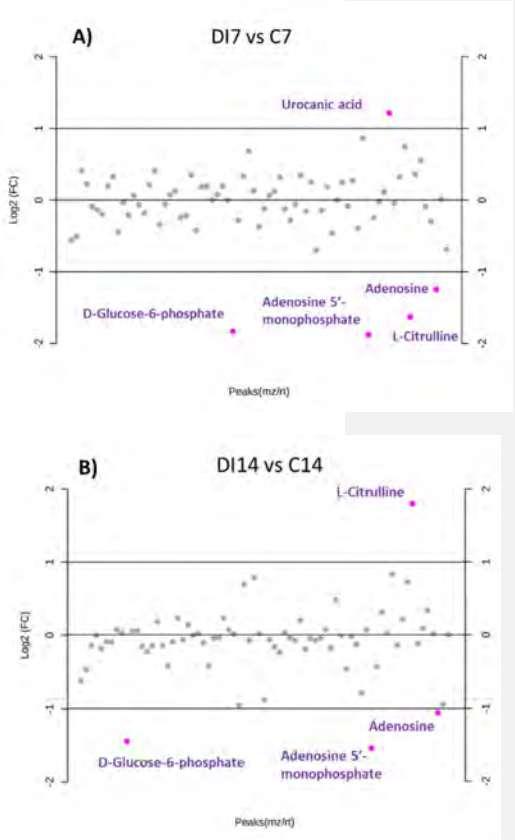


***Figure 5.2**: Results of the fold change analysis of A) DI7 vs C7 and B) DI14 vs C14. Coloured and out of the -1 to 1 interval are the metabolites showing fold change values higher or lower than the 2.0 or 0.5 thresholds respectively (i.e., 2-fold difference between conditions).*

The effectiveness of the depuration phase was checked comparing each exposure condition to the merged control group of C14 and MC14. After 7 days of depuration only urocanic acid returned to control levels, whereas the effect on L-Citrulline levels

was inverted (Figure 5.4.B). Therefore, we can conclude that the depuration phase was not sufficient for the fish to recover the basal levels.

5.3.1.2 Interpretation of the altered metabolites

In the previous section, the mismatch in the metabolic profile from the two control groups C7 and C14 was mainly attributed to the concentrations of three metabolites, the essential amino acid tryptophan, the by-product formed from energetic metabolism creatinine, and the nucleobase cytosine. The pathway analysis module of MetaboAnalyst was employed to further study the disturbed biochemical pathways, using the KEGG pathway library for fish (*Danio rerio*). The circles in the metabolome view graphic (Figure 5.5) represent the matched pathways, the pathway impact value corresponds to the number of metabolites identified within a given pathway, whereas the y-axis indicates whether the metabolites involved in those pathways are significantly changed or not. We can appreciate how the biochemical pathways related to tryptophan are the main responsible of the differentiation between the control groups. Even though tryptophan breakdown has been typically related to clinical conditions such as metabolic stress, inflammation, infection, or any other disease, its relation to nutritional changes, microbiota composition and the regulation of the immune system has been recently included (Gostner et al., 2020). The depletion of the tryptophan reserves (observed at C14) could be a mechanism to fight the metabolic stress caused by the handling and the adaptation to the experimental set-up. However, since tryptophan is an essential amino acid, dietary uptake is the main source this metabolite in fish, therefore, we cannot rule out that the drop in concentrations over time is simply related to the lack of uptake, i.e., fasting, since it cannot be synthesized in their organism. In addition, since the experiment involved the analysis of the whole organism, the role of the microbiome cannot be discarded.

Comentado [VB29]: Interesting for the next results in active and non active fish!, we found that after 7 days of behavioural test following 7 days of exposure to diazepam, which correspond to the depuration time here, the contaminated glass eels' swimming activity was still affected (even more than just after exposure).

Comentado [30]: *Respuesta a Valerie Bolliet (24/08/2022, 14:19): "..."*
That is really interesting indeed. We have to figure out how to link these results and the ones of the behaviour part with your results.

Comentado [VB31]: Maybe but Tryptophan is also not synthetized by the organism and the regulation of its metabolism is not so clear especially in fasting animals. metabolism Could it be related to fasting? Ap paper which could be interesting: doi.org/10.1111/raq.12223. I have just Access to the abstract. It is a review on the role of tryptophan in teleosts

Comentado [32]: *Respuesta a Valerie Bolliet (24/08/2022, 15:24): "..."*
I have read the article and yes, we definitely should consider that the decrease in the concentrations of any of the metabolites, and specially tryptophan, could be related to fasting.

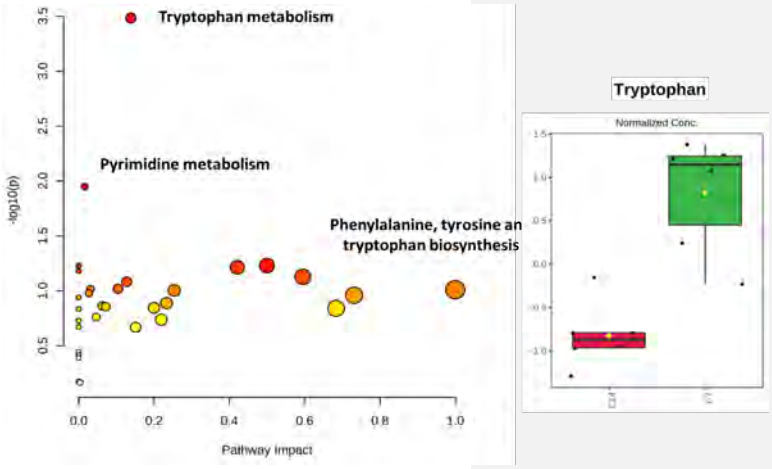


Figure 5.5: *Pathway analysis of the identified metabolites in C7 vs C14 (right). Colour intensity is linked to the relevance of that pathway in the condition differentiation and the size of the circle to % of identified metabolites within the pathway. In the left, box plot of the selected significant metabolite: tryptophan.*

The cocktail of diazepam and irbesartan was proven to have the highest impact in the polar metabolome among the exposure conditions. The mostly affected metabolites were AMP, a nucleotide involved in the energy regulation of cells; the purine nucleoside adenosine; the intracellular form of glucose, G6P; the α-amino acid citrulline; and the intermediate in the L-histidine catabolism, urocanic acid. The results obtained from the pathway analysis suggest that the main affected metabolic pathways are the glycolysis, the pentose phosphate pathway and the tryptophan metabolism (Figure 5.6). Besides the tryptophan metabolism, which seemed to have a similar behaviour in the DI7 and C14 groups, AMP and G6P are the metabolites involved in the main dysregulated biochemical pathways. AMP levels are sensitive indicators of energy stress. The energetic equilibrium of cells is regulated by the reaction 2ADP ↔ ATP + AMP which is driven towards ADP in unstressed cells (Hardie, 2014). However, since we were not able to identify enough metabolites related to the energy metabolism, we can only state that this biochemical pathway is sensitive to the contaminant mixture, but we cannot clarify to which direction it is displaced. Adenosine is produced in the cell through the hydrolysis of AMP, therefore, is not surprising that a higher availability of AMP would lead to higher concentrations of adenosine. This metabolite plays an important role in protecting against cell damage and combating organ dysfunction (Borea et al., 2016). G6P acts as the central hub of the carbohydrate metabolism (Rajas et al., 2019). This molecule works as substrate for the different metabolic pathways involved in energy production. Therefore, we can affirm that there is a correlation between the exposure to the contaminant mixture

and some metabolic changes, however the mechanism behind these changes is still unknown and will be tried to be solved in the future.

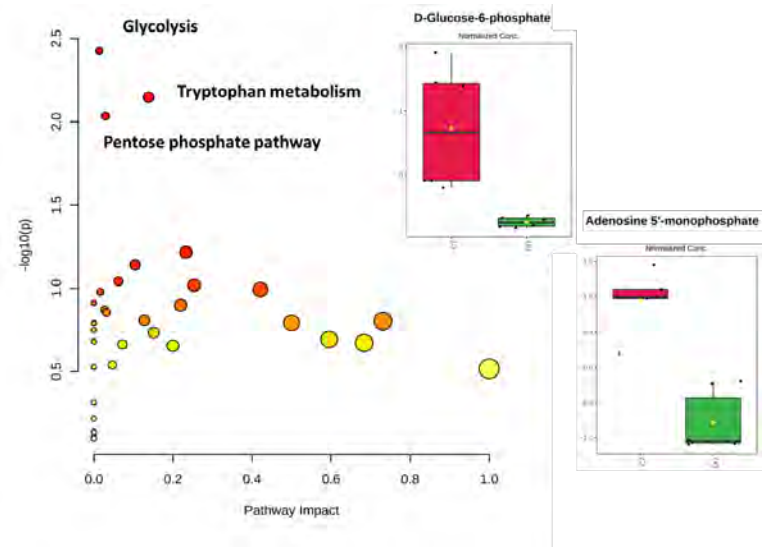


***Figure 5.3**: Pathway analysis of the identified metabolites in C7 vs DI7 (left). In the right, box plot of the selected significant metabolites: G6P and AMP.*

5.3.1.3 Lipidome analysis

Regarding the lipidome analysis, 221 lipids were identified by the non-target screening, most of them within a level 3 identification level (SI.5.7). Since a wide range of the identified lipids correspond to phospholipids with at least one unsaturated fatty acid, distinguishing between positional isomers becomes unfeasible, thus, the generic name was given to these lipids (i.e., the position of the double bond is not specified) and they were classified within the identification confidence level 3.

Data obtained from the analysis of the organic phase were statistically analysed following the same approach as for the polar metabolome. First, the distribution of the scores in the PLS-DA model was inspected to evaluate the differences between conditions (Figure 5.7). The first two latent variables were able to discriminate the C7 from DI7 (LV1) and C7 from D7 (LV2). Unlike in the polar metabolome, where the metabolite disturbance was reflected in a single latent variable, here two sources of variability seem to appear. Somehow, D7 and DI7 affect the lipidome in independent ways, disturbing the concentration levels of different lipids. To elucidate these two mechanisms, again, the exposure conditions were separately compared to the control samples. However, the paired T tests revealed that only DI7 exposure prompted a significant lipidome disruption. One ceramide, three phosphatidylcholines (PCs), one

phosphatidylserine (PS) and two phosphatidylethanolamines (PEs) were the lipids that showed significant ρ-values and fold changes higher than the 1.5 threshold (Table 5.1).

Table 5.2: *Fold change of significantly (t-test) altered lipids in DI7 compared to the control group.*

	DI7 vs C7		
Lipid	**Class**	**ρ-value**	**Fold Change**
Cer d18:1/22:0	Cer	7.3E-06	6.4
PC 17:2/17:2	PC	3.5E-05	0.2
PS 18:0/22:6	PS	6.9E-04	0.4
PE 18:1/0:0	PE	1.6E-03	1.7
PE 20:1/0:0	PE	3.1E-03	1.8
PC O-36:4	O-PC	3.3E-03	0.5
LPC 22:6	LPC	3.4E-03	2.9

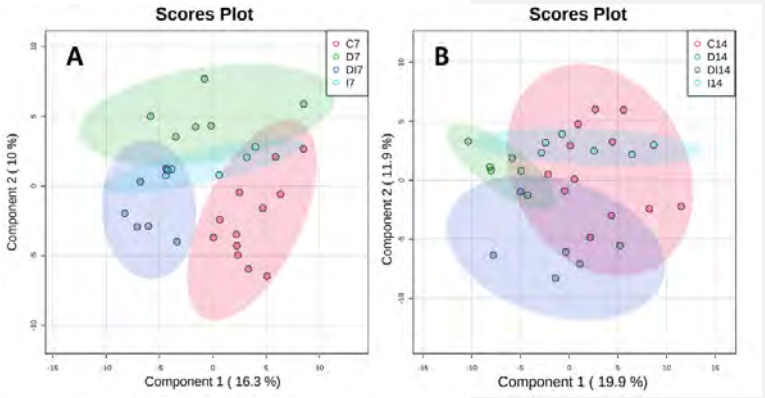


Figure 5.4*: PLS-DA scores plot of the identified lipids for all the conditions at day 7 (A) and day 14 (B).*

As for the effectiveness of the depuration phase, in this case the t-tests confirmed that the impact that the mix of diazepam and irbesartan has in the lipidome can no longer be observed, since none of the more than 200 metabolites is significantly different in this group. Nonetheless, even though diazepam showed no effect at 7 days, the statistical analysis hints that there might be a rebound effect seeing that the levels of 4 metabolites were altered after the depuration phase.

5.3.1.4 Interpretation of the altered lipidome

The results of the lipidomic analysis identified that the concentration levels of 7 lipids were significantly disturbed from C7 to DI7. The most striking compound was ceramide d18:1/22:0 (C22) which appeared enhanced up to six times in the exposed group. Ceramides were first thought to be mere structural elements or intermediates in the synthesis or metabolism of all the sphingolipids. However it is now known the role that this lipids play in the intra- and inter- cellular signalling and some studies also suggest that their role can be depend on their size and location (Turpin-Nolan and Brüning, 2020). More importantly, Spijkers et. al., (Spijkers et al., 2011), corroborated the importance of ceramides in the blood pressure control, so the exposure to an anti-hypertensive as irbesartan may be related to the abnormal ceramide levels (that were also observed in I7).

PCs and PEs are the most common phospholipids found in cell membranes. There are important precursors for a range of highly biologically active mediators of metabolism and physiology and phospholipids remodelling is certainly involved in homeoviscous adaptation of biological membranes, especially in temperature adaptation (Tocher et al., 2008). The low PC/PE ratios found in DI7 can influence energy metabolism and can be linked to disease progression (van der Veen et al., 2017) such us steatohepatitis, a disease characterized by inflammation of the liver and fat accumulation . At the same time, increased levels of LPC are related to pro-inflammatory status in fish and mammals (Aiyar et al., 2007; Tocher et al., 2008). PS dysregulation can also lead to immunological consequences in a variety of pathologies that include chronic inflammation. Finally, the presence of high levels of oxidized phospholipids is usually related to conditions of oxidative stress (Bochkov et al., 2010). Juvenile of Atlantic salmon exposed to long term handling stress showed an increase in concentration of PC-O that may result of the breakdown of phosphatidylcholine and be used as a biomarker of long term stress in fish (Karakach et al., 2009). Here, the ratio of PC-O could be another indicator of the metabolic stress that was observed in the control group.

5.3.2 Metabolomic analysis of the behavioural phenotypes.

5.3.2.1 Analysis of the polar metabolome

The identified metabolites were annotated according to the confidence levels proposed by Schymanski et. al., (Schymanski et al., 2014). The 40 metabolites quantified by the target screening approach were identified as level 1, whereas for the non-target screening approach 44 metabolites were annotated as level 2 and 6 as level 3 (SI.5.8). Quality control samples were used to exclude all the metabolites showing an RSD > 40%. The data resulting from both screening methods were merged, normalized by the actual weight of the samples and autoscaled to homogenize the scale differences between concentrations and peak areas (from target and non-target screening respectively).

The active controls were compared against the inactive ones using statistical univariate and multivariate analyses. At first glance, the differences between the two groups were

noticeable in the PLS-DA scores plot (Figure 5.8), since the 95% confidence level ellipses of the two groups were almost completely distinguished. Although, the VIP-scores plot of the first latent variable, which is responsible for the class separation, did not show any particularly high score, it showed that all the top 15 most important metabolites are enhanced in CA. In contrast, the mean weight of the glass eels was slightly lower in the CA so this enhancement was not related to the biometrics of the glass eels, which would have been corrected in the normalization anyway. The results of the t-test confirmed that these 15 metabolites were significantly altered (ρ-value < 0.05). All the metabolites in this list were identified as level 1, suggesting that the higher precision of the targeted analysis is strongly beneficial to discriminating between groups.

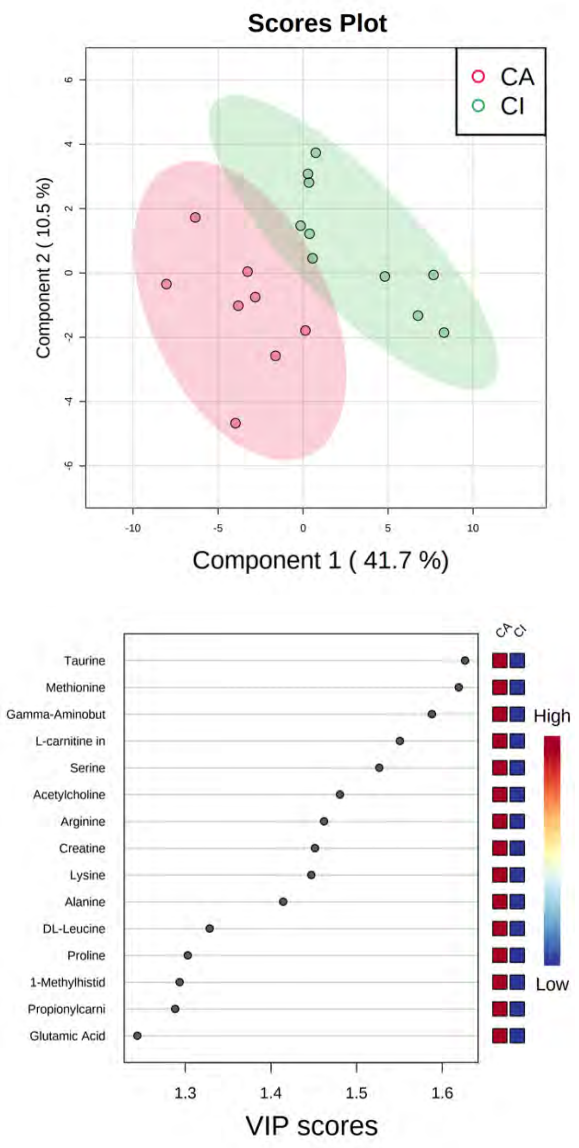


***Figure 5.8**: PLS-DA scores plot of the polar metabolites identified in active and inactive control groups (top) and its VIP scores (bottom).*

Following the behavioural test, glass eels were maintained without activity during five days prior to euthanasia and it is therefore unlikely that the observed results are the result of the difference in swimming activity between the two phenotypes. Rather than

that, we suggest that differences in the behaviour of glass eels could be related to a metabolic rate depression in inactive fish. This is a common mechanism used by organisms to endure harsh conditions such as temperature changes, anoxia, hypersalinity or low energy-limitations (Guppy and Withers, 1999). The fold change analysis (SI.5.9) shows that the metabolic rate of inactive glass eels was depressed between a 35-55% for the set of metabolites identified cor
Since the European glass eel stage is considered a non-f
phase, its energetic status depends on the energy store
leptocephalus stage. For some individuals those energy stor
continue with their migration and could lead to the ac
mechanism to reduce the energy consumption. However,
refused in two previous papers (Bolliet et al., 2017; Liu et al.,
pretentious to confirm it with this work without a deeper e
metabolism.

Regarding the pathway analysis, up to 12 biochemical pa
altered (SI.5.10). The scattering of the pathways represent
shows that a wide range of them were different among cla
aspartate and glutamate metabolism; the arginine biosy
proline metabolism; and the taurine and hypotaurine m
affected ones considering the impact (i.e., the number of inv
the significantly affected metabolic pathways. Since the
seemed to indiscriminately affect different types of metabolic pathways their individual exploration was avoided.

Comentado [VB33]: Are yo usure that your results give you an idea of basal metabolism which is an integrative process?
Basal metabolism is usually investigated using respirometry. In Liu et al., 2019, we did not see any difference in BMR between A and I in autumn or April
Here you look at protein metabolism

Comentado [i.34R33]: It was probably too ambitious to speak about basal metabolic rate considering only a small part of the metabolism. I have corrected this.

Comentado [NE35R33]: Nik esnago nuke aldaera baina basal hitza aipatu gabe

Comentado [VB36]: That was the hypothesis we tested in Bolliet et al 2017 and Liu et al 2019 and results did not confirm. I Will not go too far in the discussión at this stage

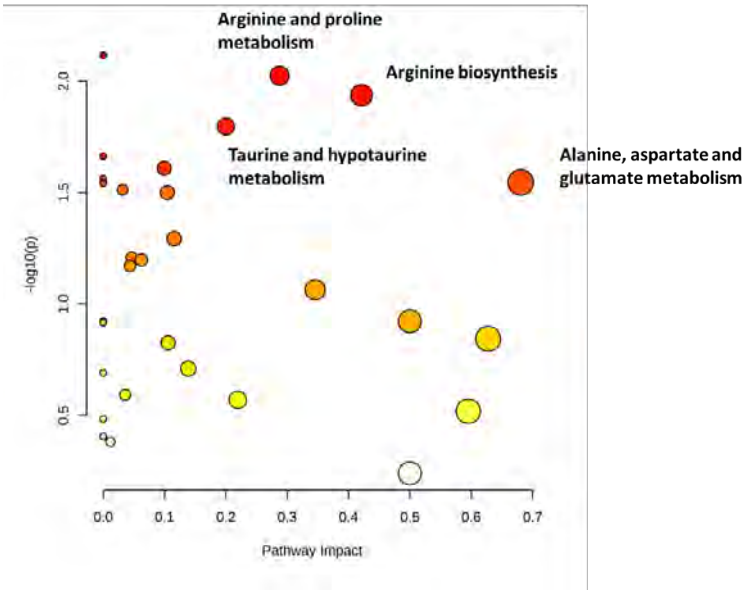


***Figure 5.9**: Pathways analysis of the main altered metabolic routes in CA vs CI.*

5.3.2.2 Lipidome analysis.

Unlike in the polar metabolome, where analytical standards were available, the lipidome analysis was carried out by non-target screening and hence, the identification of the lipids were classified within Schymanski´s confidence levels 2 and 3. 266 lipids were identified, most of them as level 3 (SI.5.11). 18 compounds that showed RSD > 40% in QC samples were excluded from the statistical analysis. The large number of lipids identified complicates the interpretation of the results, so instead, the interpretation was based on lipid families. This assumption was feasible because the lipids within the different families were well correlated in our samples (Figure 5.10). The correlation heatmap was built using Pearson's r to measure the variable distance. The colour trends observed can differentiate some of the lipid families found in the analysis, with the majority of the lipids belonging to phosphatidylethanolamines (PEs) followed by triglycerides (TGs), cholesterol esters (CEs) and diverse phospholipids such as phosphatidic acids (PAs), phosphatidylinositol (PIs) and their oxidized forms (O-PX).

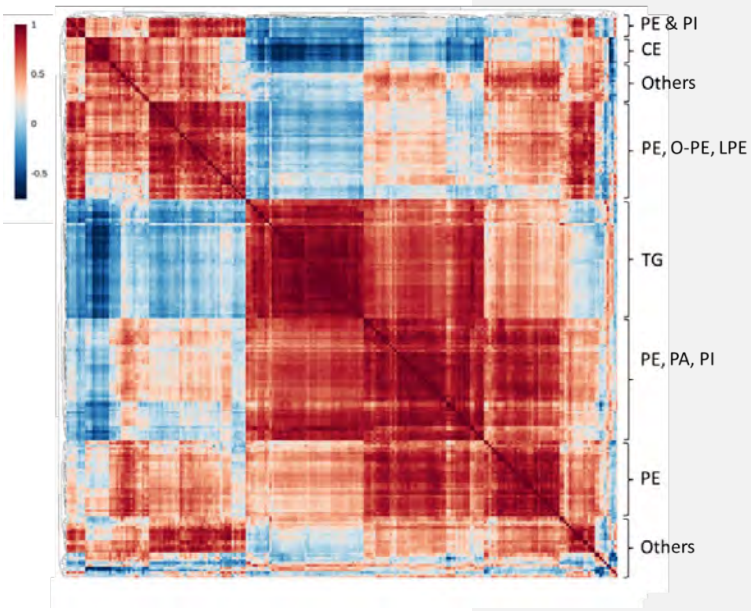


Figure 5.10: *Correlation heatmap of the identified lipids. Each pixel shows the correlation between the variables on the axes (symmetrical about the diagonal). Values close to 1 (red) correspond to positive correlation between variables whereas a value close to -1 (blue) correspond to negative correlation.*

To explore the differences between the control groups, autoscaled data were used to build the PLS-DA model. The classification model showed a poor performance separating the control groups (not shown). However, the univariate statistical analysis revealed that 9 compounds were significantly different between groups. Interestingly, 8 of these compounds all belong to the same lipid family, cholesterol esters. The fold change analysis indicates that the levels of all the identified cholesterol esters were, at least, 1.7 times higher in active glass eels than in the inactive ones (Table 5.2). The difference of these cholesterol esters lies in the length of the fatty acid chain that is bonded to the cholesterol, but they have all share a common function. The esterification of cholesterol is a mechanism used to store and transport cholesterol in lipoproteins and, this way, the toxicity of the excess of cellular cholesterol is avoided. In summary, the results of the statistical analysis indicate the depletion of the cholesterol storage in inactive controls. It is also noteworthy that cholesterol is the precursor of all steroid hormones, and thus, of all the sex hormones. As beforementioned, the connection between the sex differentiation of glass eels and the migration has been deeply investigated but remains unclear. Some studies have proposed that the fat content of glass eels can be involved in the facultative migration at the end of the season, when glass eels have less reserves, becoming a limiting factor (Bureau Du Colombier et al., 2007; Larsson et al., 1990), but this evidence is not met at

the beginning of the season (Liu et al., 2019b). However, this work offers new research perspectives to explain this facultative migration, by putting the focus on the free cholesterol levels (in the form of cholesteryl esters in this case) rather than in the total fat content. Among these perspectives, we must include the possibility that the changes in free cholesterol levels are due to the possible adaptation to the deed-water life where there is a food deficit and fish need to modify ratio of neutral lipids in the body, as demonstrated by Voronin et. al., (Voronin et al., 2021). In that study, they also describe the function of cholesterol esters in maintain in the adequate buoyancy and they relate it to the vertical migration in the water column, a mechanism that is also present in the European eel, especially in the leptocephali stage, and which is arguably even more unknown than its estuarine migration (Cresci, 2020).

Table 5.2: *Fold change analysis of the lipids significantly different between CA and CI*

Lipid	Family	Fold Change
16:1 Cholesterol Ester	CE	2.5
20:1 Cholesterol Ester	CE	2.2
20:3 Cholesterol Ester	CE	2.0
TG 16:0/16:0/16:0	TG	1.9
22:5 Cholesterol Ester	CE	1.9
18:3 Cholesterol Ester	CE	1.8
20:4 Cholesterol Ester	CE	1.8
18:1 Cholesterol Ester	CE	1.8
20:5 Cholesterol Ester	CE	1.7

5.3.2.3 Phenotype-based sensitivity to diazepam

The outcome of the phenotype discrimination gives reasons to think that these two groups can be differently affected by the exposure to diazepam. New PLS-DA models including control vs exposed eels were built. First, active controls were compared against active exposed eels and the PLS-DA scores plot showed a good discrimination between classes by the first and second LVs. According to our previous study (Study 2) (Alvarez-Mora et al., 2022) diazepam is completely eliminated from the glass eel organism by 7 days of depuration. Thus, it is worth mentioning that since the glass eels were introduced into the phenotype distinction tank after exposure to the contaminant and kept there for 7 days, it would be likely that the effect of the contaminant would no longer be visible. The relevant metabolites for this discrimination are displayed in the VIP plot in Figure 5.11.A.

Comentado [VB37]: I don't know what your references are. What has been suggested is that energy reserves would be involved in facultative migration, rather at the end of the season when glass eels have few reserves and energy can become a limiting factor. But at the beginning of the season, when reserves are high, we cannot explain why some individuals stop migration

Comentado [VB38]: In the table 1 there are more TG in active glass eels than in inactive ones? It would be surprising because we previously demonstrated that TG is a Good proxy of body weight and active presented a lower weight than inactive ones because of swimming activity (Liu et al 2019)

Comentado [i.39R38]: I would not say that, we identified more than 20 TGs and only that one is significantly altered so I wouldn't say that TG levels are higher in active. Probably just an exceptional case.

Comentado [VB40]: I would be less affirmative. Let's say that these results offer new research perspectives to explain facultative migration. sorry to break your enthusiasm! But I think that at this stage we have to be careful case

Comentado [i.41R40]: I agree with that! I use to be too ambitious in the first draft.

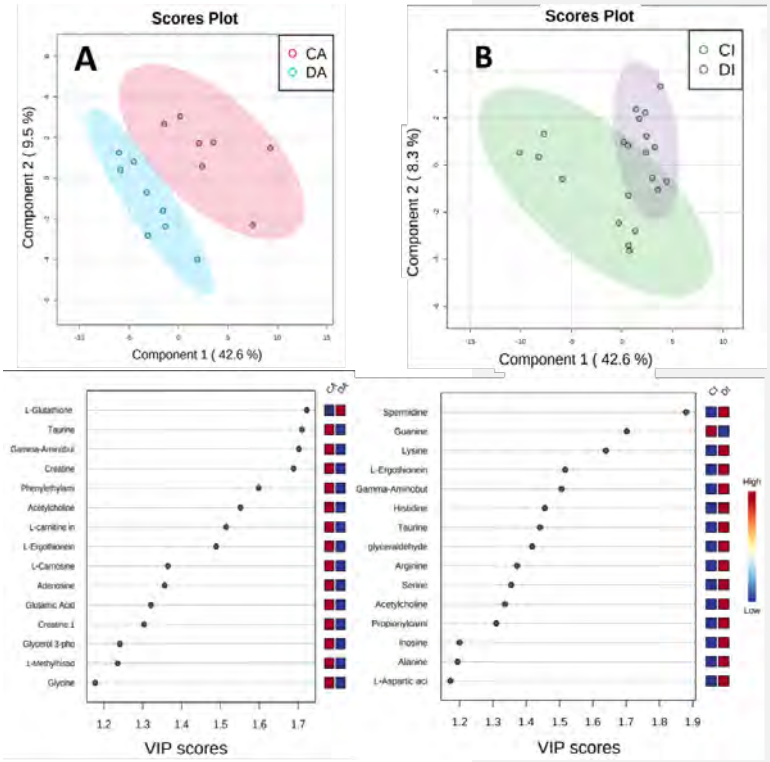


Figure 5.11: *PLS-DA scores plot (left) and VIP plot (right) of the polar metabolites identified in CA vs DA (A) and CI vs DI (B).*

Although the metabolites displayed are those relevant in the projection for the first LV, the same metabolites were found to be important for the second LV. The reduced form of glutathione (GSH), taurine, gamma-aminobutyric acid (GABA), creatine, phenylethylamine and acetylcholine were the top 6 compounds with greater relevance when discriminating between the two groups (Figure 5.12). These compounds were also the only compounds with significant ρ-values in the t-test with 95% confidence level (SI.5.12).

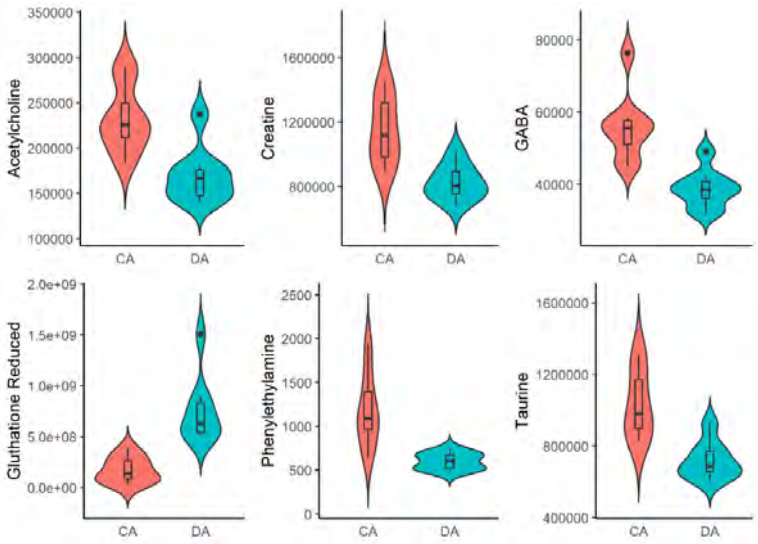


Figure 5.12: *Violin plots of the most relevant metabolites for DA vs CA discrimination. The shape of the "violin" shows the distribution of the samples.*

Glutathione is one of the most abundant antioxidants found in cells, but more specifically, the ratio between the reduced and oxidized (GSSG) forms of glutathione has been proven as a robust indicator of cellular health and oxidative stress in particular (Gu et al., 2015). Additionally, a reduced ratio of GSH/GSSG has been found in neurodegenerative diseases, such as Parkinson or Alzheimer (Owen and Butterfield, 2010), and neurotoxics may also produce disorders in this metabolic rate (Silva-Adaya et al., 2020). Despite we were not able to identify the oxidized form of glutathione, the significantly enhanced GSH levels might suggest a disorder in this metabolic ratio affecting the exposed eels. GSH, as some other stress proteins, is synthesized as a response of the antioxidant system to some stress factors, i.e., the presence of a contaminant, which might indicate that diazepam certainly has an impact on this group. Likewise, out of the 6 metabolites 4 of them are neurotransmitters or are related to the central nervous system. Diazepam actions at the benzodiazepine-GABA receptor promoting the binding of GABA. Our work indicates that the chronic exposure to diazepam decreases the GABA levels in glass eels. Since GABA is an inhibitory neurotransmitter and its effect is enhanced by the effect of diazepam, these results might suggest that glass eels develop an adaptive mechanism to diazepam involving a reduction in the levels of this neurotransmitter. In a similar way, diazepam also affects the neurotransmitters acetylcholine, involved in muscular contraction, and phenylethylamine, as well as taurine, which is not a classical neurotransmitter, but rather an agonist of the GABA receptor (as diazepam) that also has a main role as neurotransmitter among other functions including many cellular functions and antioxidant defence to stress, etc. (Ochoa-de la Paz et al., 2019; Wu and Prentice,

Comentado [VB42]: adaptative

Comentado [VB43]: the neurotransmitter for muscle contraction

2010). Thus, the impact of diazepam in the central nervous system of glass eels is hereby demonstrated.

On the other hand, the metabolic rate depression found in inactive glass eels can influence the effect of the exposure to diazepam. The PLS-DA model was built following the same approach (Figure 5.12.B) but, this time it was not suitable for the discrimination of the two groups, since a significant area of the 95% confidence ellipses overlapped. Furthermore, even if the VIP showed some of the neurotransmitters aforementioned lower in the list, and some compounds such as spermidine, guanine or lysine with high VIP scores, the statistical analysis indicated that levels of none of the metabolites had been significantly disturbed. In this manner, the thoughts were confirmed, both phenotypes respond differently to diazepam exposure. The reason behind this might be the metabolic rate depression observed in the inactive control group. This general decrease in metabolic levels can mask the effect of diazepam and the inactive status of the eels could also decrease the uptake and metabolization of the pharmaceutical. The analysis of the polar metabolome then suggests that during the glass eel stage, a one-week exposure to environmental concentrations of diazepam may affect the migration of active glass eels even one week after exposure. The analysis of the swimming activity of controlled and contaminated glass eels is in progress and will determine if their swimming ability is affected and if such neurotoxic effect may have major implications in the distribution and the fate of the European eel population. Finally, exposed active and inactive groups, DA and DI were also compared but the differences found between active and inactive controls were no longer observable (SI.5.13). Since diazepam does only affect the active phenotype, the contaminant seems to close the gap between these two groups

Data obtained from non-target screening of lipids was also used to explore the differences between exposed and control glass eels in both phenotypes. Unlike in the phenotype discrimination, the statistical analysis showed that the individual lipid content was not significantly different in one group or the other (SI.5.14). As for the fold change analysis, in the case of CA vs DA (SI.5.15), two lipids, PS 18:1/18:1 and O-PA 18:0/17:0 were up-regulated (fold-change threshold of 2.0) by the exposure to diazepam but since the t-test did not show any significancy and this trend was not repeated in any lipid within the same family, the null-hypothesis was accepted. The same was true for CI vs DI, where only PS 18:1/18:1 had a fold change > 2. Lipidomic analysis did not provide new insights into the different behaviour of active and inactive glass eels upon diazepam exposure.

5.4 CONCLUSIONS

Since glass eels are continuously exposed to contamination throughout their migratory journey through the estuaries, to a certain extent the fall in the population of this endangered species may be due to this exposure, which is especially acute in estuaries under high urban pressure. By means of metabolomic profiling, we have assessed the

effects of two of the most relevant contaminants released to the Adour estuary on glass eels. An exposure experiment to diazepam, irbesartan and their mixture was carried out over 7 days followed by 7 days of depuration phase. The metabolomic results showed that the mixture of both contaminants had the highest impact on glass eels, altering the metabolic levels of 11 metabolites. Between them, adenosine 5'-monophosphate, D-Glucose-6-phosphate, L-Citrulline and adenosine showed remarkably reduced concentrations after the exposure, which suggests that the energetic metabolism is sensitive to the contaminant mixture. The lipidomic analysis also revealed that the concentrations of seven lipids were also altered after the exposure to this mixture. In addition, metabolomics was also used in this work to find the differences in the metabolic profile of the two behavioural phenotypes of glass eels. Our findings suggest that the differences in the behaviour of glass eels are related to a metabolic rate depression in inactive glass eels. It was also demonstrated that these two phenotypes respond differently to diazepam exposure. These results stress the importance of monitoring the contaminants released to the glass eel habitat and the implications that may have in their health, and, finally, also confirms the suitability of metabolomics in attempting to explain the differences between migrant and non-migrant glass eels.

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5.6. APPENDIX STUDY 3

SI.5.6.1. Worksheets SI.5.1 to SI.5.15 (Link to same excel as Appendix Study 2)

SI.5.6.2. Setup used for the exposure experiments in Study 3

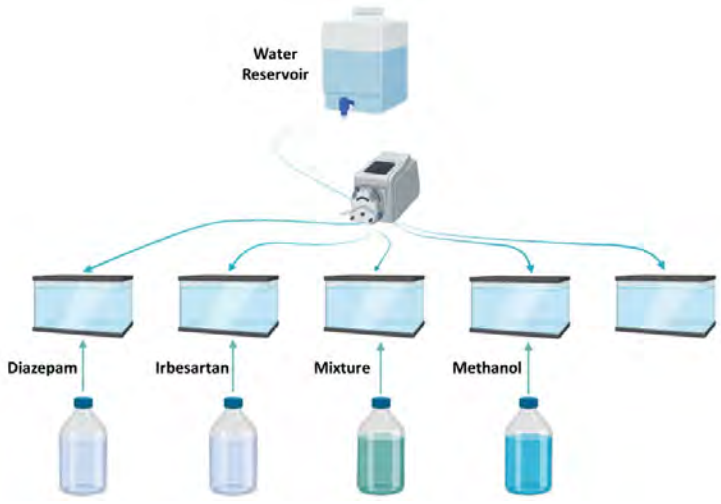

Figure SI.5.1: *Set-up used for the exposure experiments of Study 3. Note that for the second part of the study only two tanks were used since only the effect diazepam was assessed.*

SI.5.6.3. Metabolomics SNTS Workflow

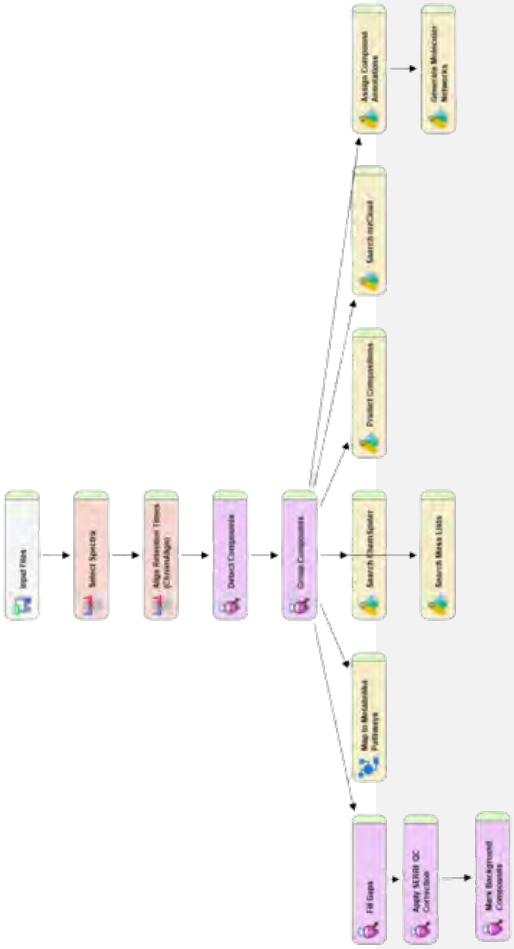

Figure SI.5.2: *Non-targeted screening workflow from Compound Discoverer 3.3 used in the Study 3.*

6. DISCUSSION AND MAIN CONCLUSIONS

Scientific Discussion – Pablo Picasso

To the best of our knowledge, this is the first time an integrative assessment of the occurrence of contaminants of emerging concern is carried out in the estuary of Adour and, above all, including the effects of the most relevant contaminants on the health and the behavioural pattern of glass eels.

If we focus on the works and studies carried out in the last ten years, we can say that this PhD goes far beyond the studies and data provided before. There are only two works that report a thorough description of organic and organometallic contaminants in the estuary (Cavalheiro et al., 2017; Miossec et al., 2019) and another two describing the behavioural patterns of glass eels as a consequence of the exposure to mercury (Bolliet et al., 2017; Claveau et al., 2015).

The underlying hypothesis that we deem not only plausible but also very likely is that the cocktail of emerging contaminants typically found in urban estuaries can severely modify the migration behaviour of glass eels. Since we can easily find many endocrine disruptive compounds, oxidative stressors and neurotoxins among the CECs, we can expect to observe modifications on the behavioural patterns that may affect the migration of glass eels and on the side effects in sex maturity. To address this big aim, we sketched a plan including the screening of CECs in the estuary in Bayonne, the prioritization of the contaminants found based on EDA and ERA approaches, and the monitoring of the glass eel metabolomic profile in controlled exposure experiments. Honestly, this plan is only a limited and partial approach to provide a full understanding of the main aim, but we are convinced that some of the results shown before and discussed here become a suitable springboard for uncovering new insights. We can start this general analysis using the SWOT approach, i.e., focusing on the strengths, weaknesses, opportunities and threats, just to offer a suitable frame for the discussion.

Regarding the strengths, we can highlight these three outcomes:

- The SETApp tool has shown to be very useful and not only within this PhD work. Though the suitability of the SET bioassay will be considered later, we are proud about the results obtained using this software but even prouder if we take into account the time and tedious work saved in the measurements.
- Thanks to the light EDA approach and the risk quotient analysis implemented in this PhD, we were able to focus our work on a reduced set of contaminants. Though this literal outcome could be also included among the weaknesses, we would like to enhance that with the exception of some works led by H. Budzinki (Gardia-Parège et al., 2022), this was the first time the EDA (as such) was applied in French estuaries.
- The metabolomics approach to study the effects of the exposure to the selected contaminants and to extend the behavioural study was risky, but to the fact that they run smoothly, we can enhance the different profiles found in active and non-active eels.

Regarding the weaknesses, we can admit these ones:

- As mentioned before, in parallel trials, SET bioassay has shown to be very demanding and highly uncertain when applied in a routine basis. Taking this implicit piece of information for granted, even with the developed automated method, *in vitro* bioassays would still be more suitable for tests with large numbers of samples.
- The risk assessment strategy used to prioritize the contaminants selected to assess the effects on glass eels was carried out using the available toxicity data for fish, which does not necessarily imply that these data are true to glass eels.
- The biggest weakness in the metabolomic approach is that we are still lacking a deep explanation to connect the analytical results and the actual physiological meaning.

Among the threats, we also admit these ones:

- The outcomes we obtained from the EDA and risk quotient analysis gives only a narrow scope of the environmental issue we want to understand. A more comprehensive study would have included a battery of bioassays to account for different modes of action.
- All the exposure studies could have been extended to a larger time span, surely more than one concentration and maybe to a different set of mixtures. In some cases, the results achieved in this PhD work would have required a more detailed experimental design.
- To confirm the differences in the metabolic profile of the two phenotypes studied, it would be necessary to analyse, at least, another set of samples.

Finally, in the case of opportunities, I think that:

- We have successfully enhanced the know-how in non-targeted and metabolomic analysis in a very complex issue and we have uncovered a new strategy to address a problem that has been a mystery for decades.
- The chances to implement a high-throughput EDA can overcome some of the difficulties we found when standard EDA was run. Nonetheless, the paradigm of the benefits of the double approach, through toxicity bioassays and nontarget screening, requires a lot of deeper research and work.

In the following sections I will complement this initial view with more deeper insights about the results achieved in the previous studies and the main scientific conclusions.

6.1. STUDY 1: Automation of the SET

In the first study of the thesis, the development of a novel predictive system able to reproduce the SET in an automatic way is described. First, an experiment was designed

to obtain larvae images covering a wide range of malformations. Four contaminants were chosen based on previous studies and/or mechanisms-of-action to expose the fertilized eggs and after 48h an image set of 272 larvae was obtained. An image analysis method based on pixel intensity thresholding was optimized and tested for the larvae belonging to different malformation levels to ensure that the morphology of the larvae does not affect to the algorithm. Then, this image set was used to build different classification methods and test their performance.

Since the ecotoxicological data for sea urchin is very limited, one of the difficulties that the development of the image classification model had to overcome was the selection of contaminants that could cause effects spanning the full range of malformations expected in real samples, which was not that straightforward. Although we knew about the toxicity of the chosen pollutants in sea urchin larvae, we did not know the sort of malformations they could generate. Moreover, it is not even fully known whether those malformations are specific to particular mechanisms of action or respond to any kind of chemical stressors and, so far, these malformations can only be correlated with a certain pollutant at a given concentration (Carballeira et al., 2012). Therefore, we had to check that the main morphological variations were included in the image set. For this reason, to minimize this sampling bias, the calibration set requires a continuous updated. Consequently, we have already considered a supervised integration of new measurements into the calibration process to update the model and make it more robust. It is worth stressing that although we say machine learning, the learning is supervised and based on parameters established by experts, or in other words, the app is nothing else than the automated version of the criteria of the experts who developed it.

In addition, the image analysis algorithm certainly worked, but, as usual, it worked under the specific conditions that are proposed in the work. This is very important too, since, as we have seen over time, some conditions in the image acquisition can hinder the correct treatment of the images and bias the analysis. One of the most important is the effect of the formalin. Formalin is used to fix the larvae, but, since it is added in a high concentration, it can degrade the larval tissue. This affects the proper classification and also the density of the larva, which has a major impact on the larva focus. With all this I do not mean to imply that the developed tool works badly, far from it, but I do want to make it understood that for a correct use it is necessary to follow the indications of the paper to the letter and to be careful with some conditions.

The suitability of image analysis and machine learning to address the automation are not in doubt. The classification method implemented in the SETApp was a HPLS-DA which yielded an ~85% classification accuracy in the validation. The performance of the developed tool was tested by comparing its results with those of two experts and comparable results were seen. It was also found that the error in the classification barely affects the construction of the dose-response curves, and the EC_{50} values were also comparable in both ways.

To achieve a correct classification, the problem was simplified by combining the two intermediate stages of malformation. It may seem that this is cheating, but the truth is that it was also found that using this approach there is hardly any difference between the toxicity values extrapolated from the dose-response curves. Where it does have a major impact is in understanding the mechanism of action of the pollutants, since this intermediate group is especially critical for understanding the sub-lethal effects. The app returns as a result the morphological group to which each larva belongs, but does not provide information on what type of malformation the larvae have among the different malformations that make up each group. Being so, if we could separate these two groups, we could at least have an idea of whether the larvae have in general an incorrect location of the skeletal rods or directly the absence of these (Figure 6.1). Still, imaging allows us to visualize the larvae whenever we want, and if a study of the modes of action is necessary, it can be done by checking the photos. The development of the app was not intended for such in-depth studies, but rather for implementation in routine analyses involving many samples. Even so, the differentiation of malformations of the intermediate group has been attempted, without success, but the problem, as always, is not the model, but the data. A breakthrough would be the implementation in our app of an approach similar to the ones that have been developed for zebrafish over the last years (Teixidó et al., 2019; Westhoff et al., 2013). This is the biggest advance that the app could have in the future, but for that many more images will be needed and new image parameters that are more specific for each malformation need to be found.

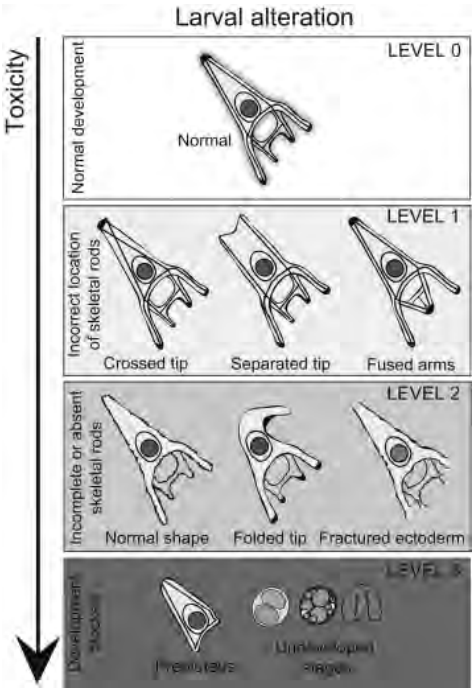

Figure 6.1: *Larval alteration levels proposed by Carballeira et. al. (Carballeira et al., 2012). Levels 1 and 2 collect the sub-lethal malformations of the larvae and their unification leads to the loss of information about the overall status of the skeletal rods.*

The chapter ends with the implementation of the app in a demanding scenario, the EDA of Bayonne's Pont de l'aveugle WWTP effluent. The app is used to estimate the toxicity of the raw sample and then to identify the toxic fractions, building the dose-response curves and obtaining the EC50 values from them. Additionally, from the toxic fractions the potential candidates to be contributors to toxicity were identified using suspect screening. Without a doubt, the effectiveness of the app was demonstrated and it was shown that the time required to run all the measurements could be reduced from days to hours. Nonetheless, to complete the full EDA work we should have calculated the toxic units of the identified contaminants to estimate their relative risk and to know exactly the contribution of each one to the total toxicity. However, although this is still pending, this was not under the scope of this thesis.

Beyond this fact, there are some drawbacks that should be considered as well. The first one is linked to the short reproductive period of sea urchins, which limits the bioassays to the months from March to June. A complete EDA study usually takes longer than three months if the contribution of the candidates is aimed, especially if more than one fractionation step is involved. In addition, the intrinsic variability of working with living

organisms complicates the reproducibility of bioassays, which is further accentuated if the study has to be extended over several years. Also, non-specific bioassays are generally more difficult to elucidate the individual contribution of candidates. Even with the "run-and-play" solution included in this PhD, bioassay preparation is laborious and many external factors can influence larval growth, which sometimes forces us to discard experiments and run them again. Maybe, the right context of sea urchin bioassay can be limited for routine ecotoxicological analysis and not so much for EDA, where *in vitro* bioassays play a more robust role (Jonkers et al., 2022; Lopez-Herguedas et al., 2022; Ma et al., 2022).

6.2. STUDY 2: Bioconcentration and biotransformation of contaminants in glass eels

In the second work we studied the bioconcentration and biotransformation of previously selected CECs in glass eels. The approach used for the selection of these contaminants was based on the ERA of the effluent of Bayonne's Pont de l'aveugle WWTP, which is directly released into the Adour estuary. First, the targeted analysis quantified the concentrations of many CECs included in our target list, for which the analytical standards are available. Then, RQs were calculated as the ratio between the measured concentration and the PNEC (calculated from the NOECs that were available in the literature) for three trophic levels (algae, invertebrates and fish) and the contaminants with the higher risk potential for fish were selected to carry out the exposure experiment on glass eels.

There are several assumptions that can cause controversy in this method for selecting the most relevant contaminants. On the one hand, there are insufficient experimental data to know the toxicity of each contaminant in fish and many of these data are obtained from *in silico* predictions. On the other hand, these toxicology data are usually calculated for model organisms or other isolated species, but none of those used in our work come from toxicity data in glass eels. Therefore, not only are we mixing data from different species that may not really be comparable, but also these data may not even provide a reliable toxicity in glass eels. This uncertainty is part of the game and we have to assume it with caution. Underestimation of toxicity can be a major problem when using predicted data and to avoid this in our work we adhere to the precautionary principle by using NOECs instead of EC_{50} when calculating PNECs. Although both types of data are often used in this type of approach, we opted for the more conservative method even though this may lead to overestimation of toxicity, for the sake of "*better safe than sorry*". In view of the results from both strategies (Tables 6.1 & 6.2), the first thing we can observe is that the results of the EDA with sea urchins alone would not have been useful as a method of prioritizing contaminants to assess the impact on fish, since none of the relevant contaminants in this study were found relevant for fish in the ERA. On the other hand, something very interesting that can be concluded from the combination of both studies is that, according to the bibliographic data and taking

into account that they are not data for sea urchins but for invertebrates, it seems that carbamazepine would be the main contributor to the toxicity found in fraction 10. Even so, we did not see fit to calculate the exact contribution based on these data, as it has been done for instance in Neale et. al., (Neale et al., 2015), precisely because this approach should always be done with data of the same species. Related to the contribution of carbamazepine, a very interesting study on its toxicity in invertebrates revealed that its metabolites could have up to 100 times more toxicity than the parent compound. The unfeasibility of identifying metabolites using the usual approaches is one of the drawbacks also highlighted by Jonkers et. al., (Jonkers et al., 2022), so for future work, we will propose a two-step approach for the characterization of the toxic fractions, where after the identification of the compounds, their metabolites are predicted by BioTransformer (see study 2) and a second screening is carried out including these TPs in the masslist.

***Table 6.1**: Overview of the seven identified contaminants in F10 (Study 1 table 2.3).*

Compound	Formula	ESI mode	m/z	t_R (min)	mzCloud Match	Level
Carbamazepine	$C_{15}H_{12}N_2O$	[M+H]	236.09496	7.80	99	1
Cetirizine	$C_{21}H_{27}Cl_3N_2O_3$	[M+H]	388.15537	7.40	89.1	1
Terbutryn	$C_{10}H_{19}N_5S$	[M+H]	241.13611	8.70	97.7	1
2-Hydroxybenzothiazole	C_7H_5NOS	[M+H]	151.00918	5.70	93.7	1
4-Methylbenzotriazole	$C_7H_7N_3$	[M+H]	133.06387	4.49	98.3	2a
Terbumeton	$C_{10}H_{19}N_5O$	[M+H]	225.1587	5.73	71.4	2a

***Table 6.2**: Results of the quantitative analysis and risk assessment of CECs in the WWTP effluent (Study 2 table 3.1). Coloured in green are the compounds selected for the exposure experiments and in blue the ones identified in fraction 10.*

Compound	Concentration (ng/L)			Family	RQ	min. PNEC
Sotalol	3053	±	54	β-blocker antihypertensive	0.01	Invertebrate
Diazepam	3005	±	74	Anxiolytic	**0.72**	Invertebrate**
Irbesartan	2676	±	38	Antihypertensive	**1.49**	Fish
Telmisartan	2033	±	52	Antihypertensive	**1.02**	Green Algae*
Azithromycin	650	±	6	Antibiotic	**34.2**	Green Algae
Carbamazepine	427	±	14	Antidepressant	**0.17**	Invertebrate
Cetirizine	377	±	6	Antihistaminic	0.03	Invertebrate
Propranolol	114	±	2	Anaesthetic	**0.18**	Fish
Terbutryn	35.1	±	0.3	Herbicide	**0.22**	Green Algae
2-Hydroxybenzothiazole	270	±	2.4	Industrial chemical	0	Invertebrate

The last thing I would like to add to this part of the criticism is that perhaps if the objective of the ERA had been only to assess the risk that our sample poses to the estuary, it would have been appropriate to give a total RQ for the sample, especially if comparison with other WWTP effluents were necessary. Although many papers use the sum of all RQs, I think that it is more convenient to calculate the sum of the RQs for each trophic level and choose the highest value (since the other option tends to unduly overestimate the risk), as described by Backhaus and Faust (Backhaus and Faust, 2012).

Three pharmaceuticals were selected for the exposure experiment on glass eels, diazepam, irbesartan and propranolol. Full organism concentrations were analysed and the BCFs were determined as the ratio between the glass eel concentration and the water concentration. In the case of diazepam and irbesartan a maximum BCF of 10 was obtained, whereas for propranolol, since the occurrence of this contaminant was observed at t_0 and t_{14}, it was confirmed that glass eels are contaminated with this compound in nature, which is not eliminated during the purification phase. Besides, the biotransformation of these contaminants was studied by the suspect screening of TPs but only one metabolite of diazepam was found, nordia

Comentado [NE44]: I still doubt about the steady state of the measurements. The BCF is based on a global partition approach, but it was clear that the system is dynamic and we fixed the maximum exposure time

This approach to the study of bioconcentration raises at lea
would be to ask why we have studied accumulation in the
different organs or tissues, which would give us more information on the distribution of the contaminant in the organism. In this particular case, even if the individual dissection of the organs was unfeasible, the sample treatment used by Figueiredo et. al., (Figueiredo et al., 2018) (i.e., dissection of the head, viscera and rest of the body) could have been an option, but still, due to the limits of quantification we would need to pool more glass eels (e.g., to have the necessary mass of head or viscera) and the number of animals was very limited being this an endangered species. The second question would be in relation to how the BCFs were calculated. Although, being an experiment where no feeding of animals is needed and thus, the bioconcentration equations for one compartment would fit well, again, the number of animals needed would not be feasible, especially with the strictness of animal ethics committees for protected animals. Both problems are related to the availability of glass eels which was also a limiting factor in reaching steady state, which we cannot be sure was achieved in our experiment. As for the biotransformation, I admit that knowing that glass eels have a very slow metabolism, perhaps this study was a bit risky, and for this reason, having found only one metabolism should not be a sign of defeat. In addition, after seeing the metabolomic results, where we can observe a great difference between the metabolism of the two phenotypes, it could be interesting to repeat the study differentiating these groups. The sensitivity to the contaminants was also different in these groups, so it could be interesting to study whether the different sensitivity to the contaminants of both phenotypes is related to their metabolization capacity.

In the last part of this chapter, the identification of CECs in control glass eels by suspect screening is addressed. The occurrence of propranolol in these samples was the trigger for this analysis where the presence of two more pharmaceuticals was confirmed, with a confidence level 1 and 11 more CECs were identified with a confidence level 2.

The fact that the glass eels caught are contaminated is something we could expect. However, having been caught at a remote point at the mouth of the estuary and being purified for 14 days, we would expect a full elimination of all the contaminants, and that is why the suspect screening of CECs was not carried out prior to any further analysis. This also underlines the fact that it is going to be increasingly difficult to find blank environmental samples for our analytical and toxicological procedures. Lastly, looking at the contaminants that appear in the glass eels it seems that some of them do not come from the effluent of the WWTP. For this reason, it would have been worthwhile to study the estuarine water itself and really see that these CECs are present in it by means of a suspect screening.

6. 3 STUDY 3: Metabolomic study of the impact of diazepam and irbesartan on glass eels and their migratory phenotypes

The impact of two of the contaminants selected previously in terms of the modification of the metabolic profile was shown in the study 3. Glass eels were exposed to diazepam and irbesartan at the levels found in the WWTP effluent, and their metabolic profiles were compared with a control group. One of the most challenging issues of these kind of experiments is the design of the exposure experiments because it is one of the keys to allow the minimization of confounding effects and the correct interpretation of the results. In our set-up, we had to use a minimum amount of methanol to prepare the stock solutions. Since this methanol could affect the glass eels, a second control group exposed to the same methanol concentration was added to the experiment. In the first section of the results, the differences between the clean control group and the methanol control group were studied and comparable results were obtained, which suggested that the amount of methanol used was safe for glass eels. In addition to this, the exposure time is also a rather misleading factor that can conceal background fluctuations of the metabolome, as it was highlighted in the study by Ziarrusta et., al., (Ziarrusta et al., 2019). In this particular study, the relevance of time was also studied with a parallel control tank along the whole exposure. In our case, it was observed that the metabolic profiles of the control group at the two times were significantly different, which would make it difficult to check the efficacy of the contaminant depuration later. Then, the metabolic profiles of the different exposure conditions were checked and the distances between groups in the PLS-DA scores plot was used to identify their dissimilarities with respect to the control group. The group exposed to the mixture of diazepam and irbesartan showed the greatest distance and was completely separated from the control group, suggesting that the alterations caused by this mix are the greatest among the groups. Additionally, the group of glass eels exposed to diazepam

Comentado [NE45]: Ziarrusta https://doi.org/10.1002/etc.4381

was completely overlapped with the control group, so the impact of this contaminant alone was negligible, and the one exposed to irbesartan was still slightly overlapped with the control group. The statistical analysis (t-test and fold change analysis) identified 5 significantly altered metabolites after the exposure to diazepam and irbesartan mixture. Regarding the lipidomic profile, the mixture of the two contaminants was also the most affected group and 7 lipids were found dysregulated.

Let's start with the two assumptions with which the paper begins. Unfortunately, given the low solubility of the chosen compounds it was completely necessary to add a solvent to improve the dissolution rate. This is something that could not be avoided, and it was therefore necessary to check that the metabolomics study was compatible with it (Maes et al., 2012). No metabolite was found to be significantly altered so, although it was risky, this assumption did not spoil the experiment.

Regarding the exposure time, we had no clue to think that this would hide an important variation in the metabolic profile, since the experiment was relatively short and the glass eels were not fed. Nonetheless, for these same reasons, we believe that the differences found in the two sampling times are not due to an intrinsic change in the glass eels (e.g., due to growth), but rather to the stress that the glass eels may have due to handling and tank. And this is a problem, since the effects we are observing would only be those that are not masked by this variability source. Once again, the sensitivity of metabolomics is evident and shows us that no matter how much we try to control the experimental conditions, any small factor can alter the results (Blanco et al., 2017; Stojiljkovic et al., 2019). For this reason, one of the conclusions of the study was that after interpretation of the results no worrisome outcome could be demonstrated from the exposure to the mixture of diazepam and irbesartan.

Apart from all this, I would also like to discuss the approach used in this work. Something that we have already tried in the past without making any progress, but that we should optimize, is the analysis of the most polar compounds. Metabolites such as pyruvate, citrate and other components of the Krebs cycle, coenzymes such as NAD, or nucleotides such as ATP are either impossible to detect or are detected with low sensitivity under the conditions we have used. Normally, their analysis involves the use of ion pair methods which are usually very aggressive with chromatographic instruments, or HILIC or mixed mode columns (Graven et al., 2014). Precisely related to sensitivity, the feeling I get from this metabolomics study is that the results of the suspect analysis do not favour group distinction as much as the targeted analysis. I mean, the manual review that we do metabolite by metabolite (adjusting the integration window, the baseline…) in the targeted analysis allows the integration to be much more reproducible from one sample to another, decreasing the group variability. As for the data analysis method, as indicated in the chapter, the aim is to use approaches that facilitate the interpretation of the results, in our case the PLS-DA, which through the information provided by the loadings allow us to make a selection of variables, the biomarkers in our case. Although it is true that these algorithms are more intuitive and easier to use than deep learning methods, they can also be misused

when they are not well known. For example, being Metaboanalyst one of the most used tools nowadays in metabolomics, its PLS-DA module offers by default an option that many people do not pay attention to, and that can lead to misinterpret the results. As you may know, in any regression tool, and PLS-DA is no less, you enter the Y block with the response you want to predict. In some cases, this block is a continuous response, but in the specific case of PLS-DA what is to be predicted are classes, usually categorical classes. These categorical classes must be translated into numbers to be used in the model, and the default way Metaboanalyst does this is to associate a number from 1 to ¨i¨ (where ¨i¨ is the number of classes). In this way, the difference between the first and the last group is greater than between the first and the second group, or in other words, not all the classes of Y block have the same weight. This could be useful when ordinal classes are used and the regression scale can be associated with the scale in our data, but, at the same time, it is a big problem when purely nominal data is used. And yet, the option comes by default (Figure 6.2). In contrast to our work, where the classes are completely nominal, a clear example with this type of ordinary classes where PLS-DA is applied in Metaboanalyst, is the study by Xu et. al., (Xu et al., 2022) where the increasing concentration of a single contaminant can be related to the increasing scale of the Y block. For purely nominal classes, Metaboanalyst allows to use PLS2-DA to create a dummy matrix of zeros and ones that is used to give the same weight to each class. What I want to emphasize with this is that using simpler algorithms does not free us from the need to understand them, and above all, that the next time you see a clear separation between classes, make sure that the classification model has been used correctly.

Finally, what I admit is the weak point of this work is the interpretation of the results. As far as we have gone, the identification of metabolites and altered metabolic pathways does not fully clarify the quantification of the effect of exposure to contaminants. We still need to do more work in this regard to be able to explain the physiological effect and thus be able to know in depth what is happening to the glass eels, what exactly these changes in metabolic levels translate into.

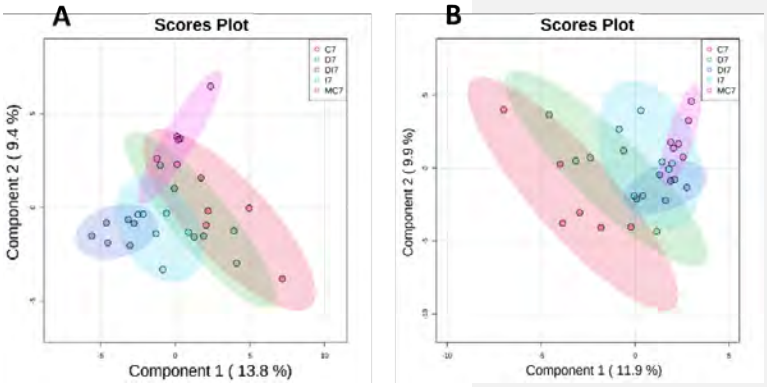

***Figure 6.2**: A = Original PLS-DA model of the different exposure groups in Study 3. B = PLS-DA model using the default settings of Metaboanalyst. Note that the software translates the names of the classes into numbers from 1 to 5 in alphabetic order, thus, these weights given to each class cause the groups to be arranged in the same order.*

The second part of the third study was focused on the differentiation of the two behavioural phenotypes of glass eels. The experiment carried out allowed the differentiation of glass eels that are able synchronize with the tide and migrate from those that settle in the substratum. Not only the metabolic profiles of the two groups were studied, but also the sensitivity of each of the groups to diazepam exposure. The results suggested that the metabolism of non-active glass eels was completely depressed in comparison to the active group. In addition, the lipidomic analysis found out that the levels of several cholesterol esters were dysregulated too. Furthermore, the effect of diazepam was noticeable in active eels, whereas for the non-active ones, the metabolic rate depression seemed to mask the effect of this contaminant.

I will not expand much more on this part as it shares much of the discussion with the previous one. The only thing to note is that in this part of the study, the usefulness of metabolomics shines much more brightly, since in this case we can safely defend that the results obtained do show the differences between the two phenotypes. As far as we can see, the difficult thing is not to find changes in the metabolome, but to relate them to the exposure to the pollutant, since you cannot be sure that they exist beforehand. It is much easier to search when you do know that there is a difference as in the case of the two phenotypes. To all this we must add that having studied the organism of the glass eels as a whole, the microbiome must also be considered in the interpretation, another factor that does not help to simplify the problem. Even so, we again have a problem similar to the one mentioned above, even if the difference between the groups is evident, without the physiological interpretation it is difficult to

know if the glass eels do not migrate because of metabolic rate depression, or if they enter this state precisely because they are unable to migrate.

Metabolomics is undoubtedly a growing field for the evaluation of the impact of pollutants, although many studies, including this one, still reveal the shortcomings of this field in terms of interpretation of the results. However, I do not believe that the day is far off when the standardization of a method for quantifying toxic effects by metabolomics will be achieved. In fact, there are already several articles that seek the association of metabolomic results with dose-response curves, with which to give an EC_{50} value (Yao et al., 2020). Just as I have already read some articles combining EDA with some omics (Guo et al., 2022), I look forward to the day when the two will be one, rather than having to move *From effect-directed analysis to metabolomic assessment.*

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7. POST SCRIPTUM

Scientia potentia est – Iker Alvarez-Mora

I feel like I finished the master 2 days ago. It has been 2 years and 10 months since I hit the jackpot with the cotutelle grant of the University of the Basque Country. Without wanting to take it as an excuse, I think that 3 years is not enough time to complete a thesis of this type and even less if a wild pandemic suddenly appears. It is true that it may be enough time if you decide to focus only on your own work and limit yourself to fulfil the activities directly related to your research plan, but I do not think that is what a PhD is all about. At least that is what I learned from my Jedi masters during the master year and that is the attitude I have taken during the thesis. I admit that my comfort zone is in front of the computer, I enjoy processing data more than preparing calibration solutions. However, it has been getting out of there, trying radically different things that has allowed me to find my preferences. Therefore, what you have read so far has only been my thesis, not my PhD. My PhD also had endless sampling days in water treatment plants, contaminant removal efficiency experiments in a pilot water treatment plant; it had some hyperspectral analysis of samples from Pompeii, a museum closed to us alone to analyse Goya's paintings; it offered me my first contact with teaching; it taught me how to work in a research team, and what a great team it was. What I appreciated most about these three years of doctoral studies is that they have helped me to become more independent (and I don't mean to leave my parents' house, which is precisely what the doctorate does not help you with). In that sense, I think that I have made good use of my doctorate, I feel that after these years I have fulfilled the objective of leaving as a completely independent researcher, capable of creating my own opinion on the topics on which I work.

One of the reasons for creating this post scriptum, apart from being disgustingly Mr. Wonderful, is precisely because I needed a space where I could open Pandora's box (since, luckily, Nestor didn't let me open it in the introduction). Suspect vs non-targeted screening. Where does one end and the other begin? In a first version of the introduction, I tried to be a non-target radical, but, as almost always in these cases, it did not work out well. By the very meaning of the word, something that has no target should not be biased. Therefore, any step that can direct even a little the identification of the unknowns, whether it is a sample treatment that does not cover as much as possible the extraction of the compounds (even the simple fact of having a sample treatment could be a reason to call it suspect and not non-targeted, that's how extreme I was...) or using a suspect list to name the compounds at the end of the filtering, was enough to reject the “non-target" term. However, Nestor was right when he told me that I was discussing the sex of angels. It is not a scientific problem but a semantic one, and I do not think it is worth debating about how close we are to making the purest non-target, I think that is all about doing well whatever we do. I have the feeling that the rush to get this ideal non-target may be counterproductive. But on the other hand, I understand it, we, the SNTS community, are little kids with a new toy. A toy that offers unlimited possibilities, and we think we can have them all. Can I close the box now?

In this previous radical version, I also went a bit too far in my criticism of deep learning. Which is ironic now since all the images preceding the different sections have been generated by an artificial intelligence that uses deep learning to learn from images on

the internet and generate completely new and unique images. You didn't really believe that Monet painted some WWTPs or that Van Gogh liked sea urchins, did you? Even so, it is true that I am not in favour of the use of these intelligences for specific purposes such as metabolomics, for example. One of the most important things in metabolomics is to understand what differentiates the distinguishing groups (i.e., the loadings in PLS-DA) so the use of completely black box algorithms does not help us at all with this. The mindset adopted in this regard throughout the thesis has been precisely to keep things easy and interpretable. Just 20 days before handing in this thesis I have read a new article that suggests that the use of PCA in genetics may not be as appropriate as it seemed, and that more than 50,000 studies that might be biased by using this method should be revaluated. If we can no longer trust even PCA, what is left? May the god of science have mercy on our souls.

I will have time later to thank all the colleagues that have been by my side in this journey but I would like to use this last part of the *post scriptum* to acknowledge all the people from the PiE-EHU and IBeA group for their help and for creating such an inspiring scientific environment. Of course, thank you Valérie and all the people from the UMR ECOBIOP group for all the help with the glass eels, as well as Dr Stéphane Panserat, Sergio Polakof and Geneviève Corraze for their help in the interpretation of the metabolomic results and Davide Ballabio for his help with the classification models built for the SETApp.

I would also like to give special thanks to UPV/EHU and UPPA universities for my predoctoral cotutelle scholarship as well as the Agencia Estatal de Investigación (AEI) of Spain and the European Regional Development Fund for the financial support through projects CTM2017–84763-C3–1-R and CTM2020-117686RB-C31, and also the Basque Government through the financial support as a consolidated group of the Basque Research System (IT1213–19).

I will take the license to end this thesis by thanking the people closest to me in my own languages.

Tesian zehar nirekin egon direnei esker onak ere, nire erara:

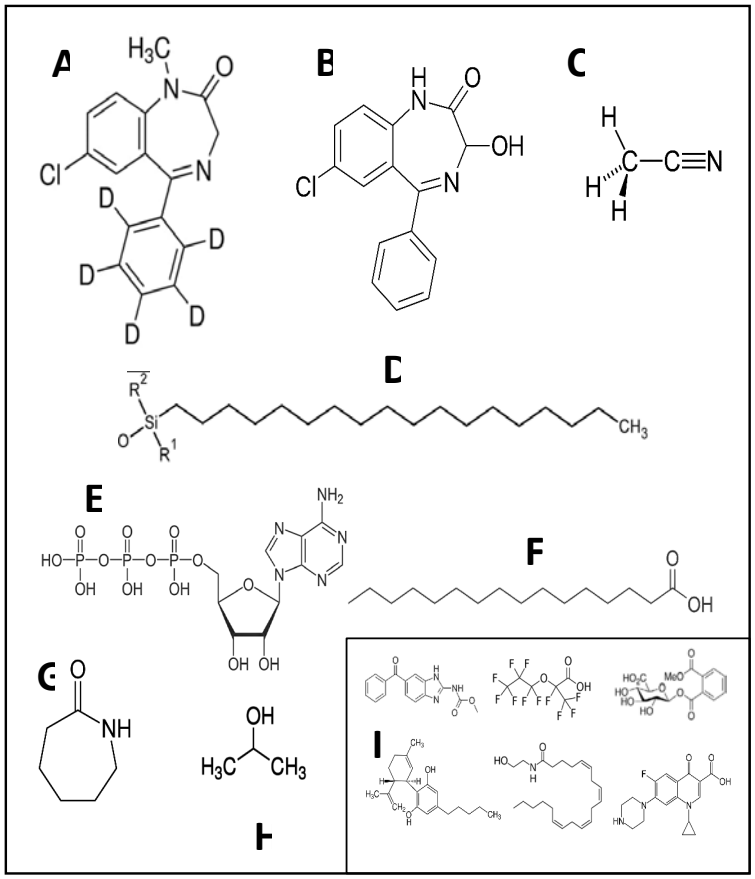

Figure <3: *A = Nestor; B = Mathilde; C = Maitane; D = Naroa; E = Mire; F = Olatz; G = Ailette; H = Jose; I = Mintegi*

Nestor: Diazepam. Tesian gertuen izan dudan konposatua, beharrezkoa dudan lasaitasuna emateko gai, aurrera egiteko bultzada, eta kasu honetan, gainera deuteratua, tesiko trazagarri ezin hobea.

Mathilde: Oxazepam. L'homologue du diazépam en France, merci de m'avoir donné l'opportunité de faire ma thèse avec vous et de m'avoir aidé quand j'en avais besoin.

Maitane: Azetonitriloa (0.1% formiko). Zuk erakutsi zenidan bidea, beraz zu gabe lortutako emaitzak (positiboak?) ezinezkoak lirateke.

Naroa: Nire C18-a. Bidean erretenituta gelditu naiz, eta badirudi nahiko ez-polarra naizela. Mila esker denagatik maiti!

Mire: ATP. Tesian zehar ez dut topatu nahiko nukeen beste, baina zuk transmititzen duzun energiagatik nago hemen.

Olatz: Azido Palmitikoa. Guztiz saturatuta zauden arren, burua da zure bereizgarria, eta espero dut urte askoz zure aholkuaren bila joan ahal izatea.

Ailette: Caprolactam. Beti gurekin, edozein momentutan hor zaude, filtroak ez dira zuretzat.

Jose: Isopropanol. Irtenbide onena, aurrera egitea kostatzen zaidanean, edozein oztoporen aurre, isopropanol apur bat eta easy-peasy.

Mintegia: Mebendazole, GenX, Monomethyl phthalate glucuronide, CBD, AEA, and Ciprofloxacin. Dena ez delako lan egitea izango. Bide hau erraztu didazue.

_____

Con mi familia no voy a ser tan friki.

Ama, saber que estás orgullosa ya me vale para seguir.

Aitatxu, tampoco tenías ninguna duda de que iba a poder con esto, una cosa más.

Nereeee, tres años de tesis no son nada comparados con el experimento de 21 años que tengo en casa, de momento está saliendo bien. Te toca mover ficha.

Tío, tía: Mis financiadores. Siempre me apoyáis en todo y no os lo agradezco lo suficiente.

Abuela: ¿Has visto que libro he escrito? Aunque yo creo que tú lo hubieses escrito mejor, pero por vieja, no por diablo, todavía me queda para ser el más listo de la familia. Quiero que sigas riéndote de mí muchos años. Te prometo que algún día empezaré a trabajar de verdad.

¡Os quiero family!