eman ta zabal zazu

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Estres faktore anitzen ekotoxikotasuna lurzoru organismoetan, paisaia aldakortasunaren araberako kutsatzaileen arrisku ebaluazioa eta bioerremediazio teknologien aplikazioa araztegi lokatzak jaso dituzten lurretan: *in vivo* eta *in silico* fokatzeak

(Ecotoxicity of multiple stressors on soil organisms, assessment of the risk of pollutants accounting for landscape variability and application of bioremediation technologies to sewage sludge deposition: in vivo *and* in silico *approaches)*

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Ecotoxicity of multiple stressors on soil organisms, assessment of the risk of pollutants accounting for landscape variability and application of bioremediation technologies to sewage sludge deposition: *in vivo* and *in silico* approaches

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CHAPTER 1

Lurzoru kutsatuen karakterizazio intentsiboa *in vivo* (test ekotoxikologiko) eta *in silico* fokatzeak erabiliz

(In vivo and in silico approaches for an integral characterization of polluted soils)

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ABSTRACT

The anthropic activities during the last decade are causing damages on ecosystems. In fact, animal husbandry, agriculture, and industrial activities have increased the proportion of soils contaminated with pesticides and metals. The presence of pollutants could affect soil organisms and the Ecosystem Services provided; thus, a proper risk evaluation is required in order to assess the final environmental impacts of pollutants. *In silico* models are tools designed to predict environmental concentrations of released pollutants that could assist on substances evaluation and regulation. Meanwhile, the health paradigm, understanding the natural environment as an inter-related compartment, has been spread within the scientific community. Hence, the measurement of toxicological effects produced by contaminants on soil organisms (using *in vivo* tests and biomarkers) gained a high reputation for evaluating environmental health. This work presents different approaches, techniques, software, guidelines and tests for assessing contaminated soils; crucial for an integrative soil assessment.

Keywords: Polluted soils, ecohealth, *in silico* models, ecotoxicology, soil organisms

LABURPENA

Azken hamarkadako jarduera antropikoek ekosistemetan desorekak sortzen hasiak dira. Horrela, abeltzaintzak, nekazaritzak eta aktibitate industrialek pestizida eta metalekin kutsaturiko lurzoruen proportzioa emendarazi dute. Kutsatzaileen presentziak, lurzoruetan bizi diren organismoak eta beraz, lurzoruak eskaintzen dituen Zerbitzu Ekosistemikoak erasatea ekarri dezake, arrisku ebaluazio egokien beharra eskatuz. *In silico* modeloak, kutsatzaile baten aplikazio osteko ingurumen kontzentrazioak aurreikusteko tresna egokiak dira, substantzien arrisku ebaluazioan eta erregulazioan lagun dezaketenak. Bestetik, kutsatzaileak lurzoruan duten inpaktua ebaluatze aldera, osasunaren paradigma ikuspuntua; hots, medio naturalak inter-erlazionatutako konpartimentu gisa ulertzen dituena, hedatzen hasi da komunitate zientifikoaren baitan. Era honetan, kutsatzaileek lurzoru organimoengan eragindako efektu toxikologikoen neurketak (*in vivo* test-ak eta biomarkatzaileak erabiltzen dituztenak) omen handia irabazi dute ingurumenaren osasuna ebaluatzerako orduan. Lan honetan, kutsatutako lurzoruak ebaluatzeko ikuspuntu, teknika, software, gidalerro eta test ezberdinak aurkezten dira; lurzoru ebaluazio integral bat burutzeko beharrezkoak direnak.

Hitz gakoak: Kutsatutako lurrak, ecohealth, *in silico* modeloak, ekotoxikologia, lurzoru organismoak

1-SARRERA OROKORRA

Bigarren mundu gudaz geroztik, biztanleria globala esponentzialki hazi izan zen gaur egungo 7700M-ak atzeman arte (UN, 2020). Estimazioen arabera, 2050 urtean, munduko biztanleria 9.7 bilioitara heltzea espero da (Garcia et al., 2019). Hazkuntza honetarako ezinbestekoak izan ziren, guda handien gabeziaz gain, osasun eta elikadura arloan emandako aurrerapausoak. Antiobiotiko eta txertoen aurkikuntzan emandako aurrerapausoek (Garcia et al., 2019), zein ugalketa arloan lortutakoek, bizi esperantza 69,11 (1960) urteetatik 83,5 (2018) urteetara hobetzea ahalbidetu izan dute (Espainian); umeen hilkortasuna izugarriki jaisteaz gain (%48.4a Europan azken 20 urteetan) (Eurostat, 2020). Bestalde, nekazaritza elikagaien industrializazioak, janaria kantitate altuetan produzitzea ahalbidetu zuten, neurri batean behintzat desnutrizio eta gosete arazoak ezabatuz; aurreko mendeetan sarri izandakoak.

Aldiz, aipatutako populazio hazkuntzak, mendebaldeko gizarteen dogma ekonomiko nagusiarekin batera; non, garapena hazkuntza ekonomikoaren baliokide den, baliabide naturalen xahutze etengabe bat bultzatu dute kultura kontsumista batekin batera. Era berean, ondasunen fabrikazio eta kontsumo etengabeak, hondakin eta emisio desberdinak sortzen dituzte; luzetara, medio lurtar, urtar edo aire konpartimenduan buka ditzaketenak; hauek kaltetuz. Izan ere, azken hamarkadetan gorakada eman duten jarduera antropikoek, desoreka handiak sortzen hasiak dira aipatutako ekosistemetan. Horiek horrela, komunitate zientifikoa beste garai geologiko batez hitz egiten hasia da jada: Antropozenoaz. Mundu mailako fenomeno honek, eredu produktibo eta energetikoen gaineko hainbat eztabaida sortu izan ditu, datozen hamarkadetarako ingurumen erronkak ezarriz.

Iragandako mendean eta oraingoan isuritako berotegi efektu gasek, atmosferan dauden gasen kontzentrazioa zeharo emendatu du; CO_2 aren kasu, industriaurreko garaitik gaur arteko %147ko igoerarekin; 407.8 ppm-ak gaindituz (WMO, 2018). Gas hauen hazkuntzak, lurrazaleko tenperatura gradu batean igotzea suposatu dute jada; eta, iragarpenek diotenez, 2100 urterako igoera hori 4°C-takoa izatera hel daiteke (Sherwood et al., 2014). Mundu mailako tenperatura igoerak fauna, flora, gizarte edo ekonomian sor ditzakeen efektuak ezezagunak dira oraindik; hala ere, simulazio

ezberdinen eta iragarpen modeloen bitartez posible da (ziurgabetasun jakin batekin) tenperaturaren emendioak sor ditzakeen efektu potentzialak aurreikustea.

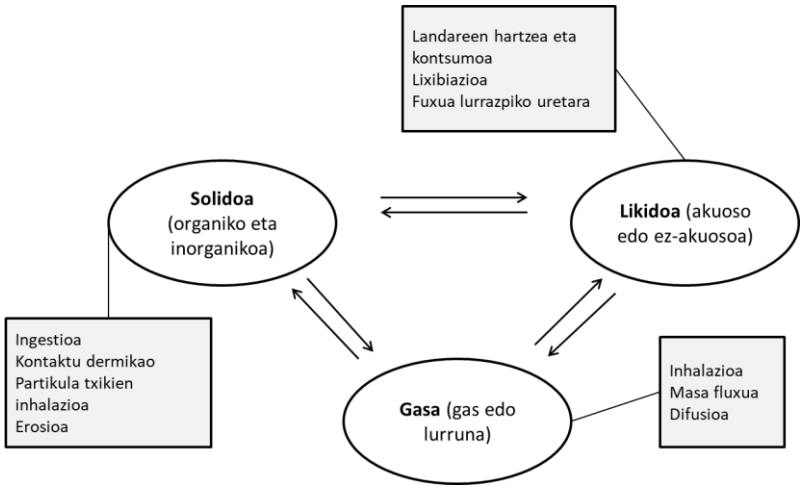
Industrializazio azeleratuak, elikagai beharren emendioak edo gero eta ugariagoak diren desplazamendu transozeanikoek, metal, pestizida, PAH, zein hidrokarburoen kontzentrazioa areagotzea eragin du munduko ozeano, laku, ibai, edo ur masa ezberdinetan. Era berean medio urtarra, bizi erabilgarriaren azken atalean dauden makroplastikoen degradaziotik sortutako mikroplastikoen kontzentrazio emendioari aurre egiten ari da. Estimazio ezberdinen arabera, urteko, 5-13 milioi tona plastikok (mundu mailako produkzioaren %1.5 eta %4-aren bitartean) itsasoan bukatzen dute (COM, 2018), jadanik, puntu batzuetan, 4000 partikula/m^3-ko kontzentrazioak antzemateraino (Andrady, 2011; Rochman et al., 2013; Ivar do Sul & Costa, 2014). Horrela, plastikoek epe labur eta luzera medio akuatikoan edo kate trofikoan sor ditzaketen efektuen gaineko eztabaidak sortu izan ditu komunitate zientifikoaren baitan.

Bestalde, eraldatu gabeko lurzorua gero eta baliabide urriagoa da. Elikadura eta Nekazaritza Erakundeak (FAO- *Food and Agriculture Organization* ingelesez) argitaratu duenez, abeltzaintzan erabiltzen da lurzoru kantitaterik handiena, basoen soiltze, lurzoru erosio, desertifikazio edo biodibertsitate begetalaren galera bidez ingurumena kaltetuz. Gainera, nekazal hedapenak, habitat naturalen nekazal eremuetarako aldaketak, eta hirien zabaltzeak, zeharo agudotu du lurzoru naturalen degradazioa (Pereira et al., 2017). Horrela, abeltzaintza, nekazaritza eta urbanizazio prozesuek (aktibitate industrialekin batera), fisikoki edo kimikoki kutsaturiko lurzoruen proportzioa zeharo emendarazi dute; lurzoru kutsatuen arazoa globalki hedatutako arazo izendatzea suposatzeraino (FAO 2015; Shi et al., 2017). Izan ere, Ingurumen Agentzia Europearraren (EEA- *European Environmental Agency* ingelesez) arabera, 2.5 milioi lursail aurki daitezke potentzialki kutsaturik Europear kontinentan; horien artean 340.000 eremu jada kutsatu bezala karakterizaturik izanik (EEA, 2014).

Lurzorua kutsatu dezaketen konposatu eta elementuak bi talde handitan bereizten dira: kutsatsaile organiko eta inorganikoetan (Cachada et al., 2018). Pestizidak, izurriteen zikloak aldatu, akatu edo kontrolatzeko substantzia (edo substantzia nahasketa) organiko edo inorganikoak dira (Castelo-Grande et al., 2010). Jatorri natural zein sintetikoa izan dezaketen substantzia hauen erabilera zeharo emendatu zen

bigarren mundu gerraz geroztik (Santos eta Galceran., 2002), elikadura espezieen produkzio eta hazkuntza hobetuaren bitartez nekazal ekoizpena bikoiztea ahalbidetuz. Bestetik, metal astunak, pisu atomiko eta dentsitate altudun (5 $g.cm^{-3}$ gutxienez) elementu inorganiko naturalak dira (Liu et al., 2020). Hauen artean animali eta landareentzat esentzialak diren elementuak: kobaltoa (Co), kobrea (Cu), kromoa (Cr), manganesoa (Mn) eta zinka (Zn); edo, esentzialak ez diren elementuak: kadmioa (Cd), beruna (Pb) eta merkurioa (Hg) aurki daitezke.

Konposatu hauek isuriak direnean medioan bertan edo medio ezberdinen artean migratu dezakete; izan ere, biosferaren euskarri diren, atmosfera, hidrosfera eta litosfera ez dira erlazionatu gabeko konpartimentu estankoak, zuzenki erlazionatuta dauden, fluxu konstanteko konpartimentuak baizik (honela Ekosfera sortuz)(1. Irudia). Hori dela eta, kutsadurak ,aldaketa klimatikoarekin batera, lurzoru emankortasuna kaltetu dezake solido/likido/gas oreka aldatuz eta lurzoruko karbono organiko kantitatea jaitsiz. Aldi berean, medio ezberdinetan gertatzen diren aldaketek, hauetan bizi diren espezieak kalte ditzakete; horrela Zerbitzu Ekosistemikoak kaltetuz.



1. irudia: Medio ezberdinen arteko fluxua eta medio bakoitzean bertako organismoenganako kutsatzaileek daukaten esposizio bidea. Scullion 2006-tik hartu eta eraldatua.

2-ZERBITZU EKOSISTEMIKOAK ETA LURZORUAREN OSASUNA

Zerbitzu ekosistemikoak giza ongizatean onura duten ondasun ekosistemikoak dira (Dominati et al., 2010; Jonsson et al., 2016; Pereira et al., 2017), gizakia hornitzen

duten maila ezberdinetako ondare eta zerbitzuak barneratuz (Costanza et al., 1997; MEA, 2005; Adhikari et al., 2016). Horrela, 2005. urtean, *Millennium Ecosystem Assessment*-ak 4 kategoria ezberdinetan taldekatu zituen zerbitzu ekosistemikoak: (1) hornitze zerbitzuetan (elikagai, ur, egur, zuntz eta erregai hornitze zuzen edo ez-zuzena), (2) erregulazio zerbitzuetan (ur eta gas, klima, uholde, erosio edota gaixotasunen erregulazioa), (3) zerbitzu kulturaletan (estetikoa, espirituala, hezkuntzazkoa eta aisialdikoa), eta (4) sostengu zerbitzuetan (habitaten sostenguan, nutrienteen zikloan, produkzioan eta biodibertsitatean), besteak beste (Adhikari et al., 2016; Jonsson et al., 2016).

Ekosistema lurtarren zerbitzuak, lurzoru propietateen eta hauen elkarrekintzen araberakoak dira; jatorri naturala edo antropikoa izan dezaketen prozesu fisiko-kimiko eta biologikoek baldintzatuta izanik batik bat. Erosioek, luiziek, karbono eta biodibertsitate beherakadek, edo elementu kutsatzaileek, lurzoruaren degradazioa bultzatzen dute; gaur egun, elikadura segurtasunean zein sostengarritasun arloetan aurre egin beharreko erronka izateraino (Oldeman, 1998; Godfray et al., 2010; Montgomery, 2010; Adhikari et al., 2016).

Lurzoruan bizi diren organismoek, ekosistemaren funtsezko zerbitzuetan laguntzeaz gain, sistemaren funtzionamendu jasangarria ahalbidetzen dute. Besteak beste, nutrienteen zikloa bultzatzen, ura purifikatzen, materia organiko dinamikak eta estrukturak erregulatzen, karbonoa lurrean bahitzen eta berotegi gasak mantentzen laguntzen dute. Zerbitzu hauek, nekazaritza jasangarrirako eta azpiegitura urbanorako baliabide garrantzitsuak izateaz aparte, ezinbestekoak dira ekosistema naturalen funtzionamendurako (Breure et al., 2012). Horregatik, lurzoruko populazioetan eman daitezkeen aldaketek sistema osoaren oreka asalda dezakete; garatutako zerbitzu ekonomikoak/ekosistemikoak eraginez.

Ekosistema baten kalitatea, helburu jakin baten garapenean erakusten duen erabilgarritasun edo efizientziaren bitartez interpretatzen da. Osasun kontzeptua, aldiz, funtzionaltasuna barneratzen duen kontzeptu zabalagoa da; hots, funtzioak modu jasangarriago batean garatzea jasotzen duena. Kalitate kontzeptuak problema bati kausa-efektu logika linealetik aurre egiten dion bitartean; osasun kontzeptuak,

EcoHealth eta *One Health* bezala ezaguna denak, ente bizi, konplexu eta interrelazionatu bat bezala ulertzen du ekosistema; bizitzaren garapenerako, bai giza zein animali bizitzarako, beharrezko dena. Biak, bai *Ecohealth* zein *One Health* kontzeptuak, izakera holistikodun fokatze sistemikoak dira; giza osasun, animali osasun eta ingurumen osasunaren arteko elkarrekintza konplexuan oinarritzen direnak (Harrison et al., 2019).

Hamarkadetan zehar, lurzoru baten egoera bere kalitatearen bitartez aztertu izan da; horretarako, kalitate edo onarpen atalaseak karakterizazio kimiko eta fisiko-kimikoekin erkatuz. Hala ere, azken urteotan agertutako kontzeptu holistikoek erabat aldatu izan dute fokatze hau. Modu honetan, lurzoruaren osasuna ezagutzeko parametro fisiko eta kimikoez gain lurzoruan bizi diren espezie adierazgarrietan kutsadura efektuak kontuan izatea lortu da arriskua ebaluatzerako orduan.

3-INGURUMEN-ARRISKUAREN EBALUAZIOA

Arriskua, akzio edo kondizio konkretu batek sor dezakeen/ditzakeen ondorio kaltegarri/ak gertatzeko probabilitate bezala definitzen da; arriskuen konbinazio eta esposizio ebaluazioa barneratuz (Muralikrishna et al., 2017). Ingurumen-arriskuaren ebaluazioa (*ERA, Evaluation Risk Assessment* ingelesez) agente arriskutsuek giza-osasunean eta kalitate ekologikoan sor ditzaketen elkarrekintzen/inpaktuen ikerketan oinarritzen da; hauetan, arriskua identifikatzea, arazoa formulatzea, arriskua analizatzea eta karakterizatzea bilatzen delarik (Muralikrishna et al., 2017). Ebaluazio hauek, arriskua kudeatzea eta euste zein erremediazio neurriak planteatzea ahalbidetzen dute. Izan ere, fokatze hau, lotuta daramatzan kudeaketa modeloekin batera, gero eta erabiliagoa da politika eta erregulazio maila ezberdinetan. Giza edo ingurumen osasunarentzat “onargarriak” diren arrisku mailak dituzten erregulazioak diseinatzea ahalbidetzen du, eta kokaleku erasokor/sentikorrak alterazio posibleen aurrean lehenesten laguntzen du.

Ingurumen agentzia, nazioarteko erakundeek eta komunitate zientifikoak zeharo onartu izan dute ezin daitezkeela Ingurumen-arrisku ebaluazioak karakterizazio kimiko batean bakarrik oinarritu. Izan ere, ikuspuntu honek ez du kutsatzaileek biotarengan eragiten dituzten efektuen gaineko informaziorik eskaintzen. Harira, kutsatzaileek eragindako efektu toxikologikoen neurketek omen handia irabazi dute ingurumenaren

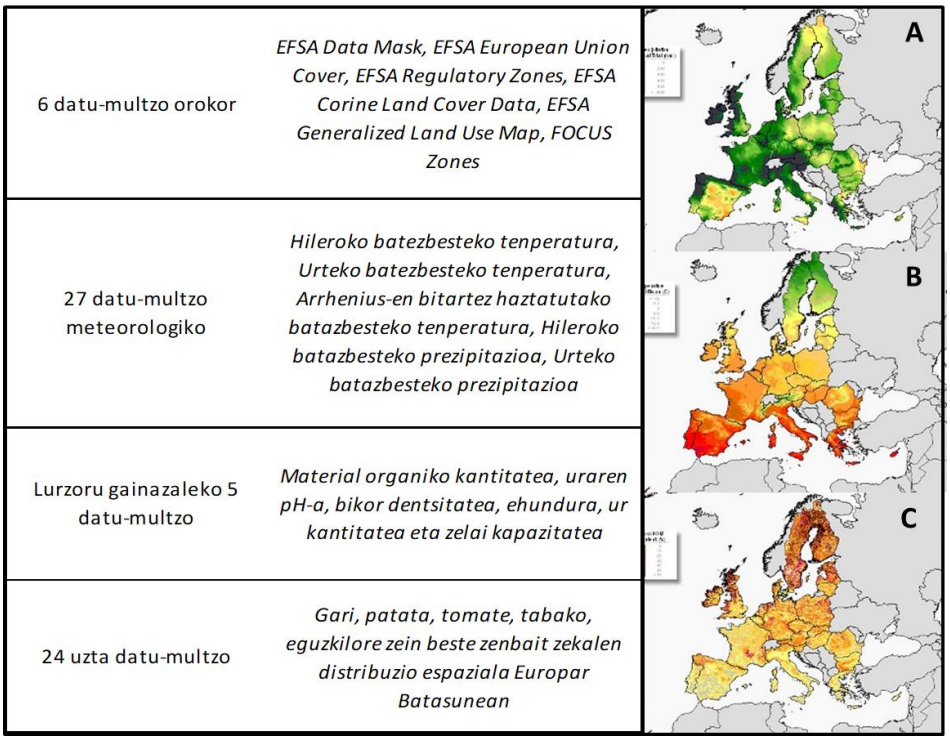
osasuna ebaluatzerako orduan (Bayne, 1989; Gray, 1992; Marigómez et al., 2004). Horretarako, kutsatzaileek maila ezberdinetan eragin ditzaketen efektuak ebaluatze aldera, neurketak burutzen dira konplexutasun biologikoko maila desberdinetan. Lurzoruan bizi diren organismoen gaineko arrisku ebaluazioa 91/414/EEC Kontseilu Direktibak garatutako "SANCO /10329/2002 346 *Terrestrial Ecotoxicology Guidance Document*"-aren gidaritzapean burutzen zen. Direktiba hau, 2011an indargabetuta gelditu zen 1107/2009 (CE) (produktu fitosanitarioen komertzializazioari dagokiona) araudia-ren bitartez. Era berean, Batzordearen (EB) 348 283/2013 (*REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals* ingelesez) eta 284/2013 araudiek, substantzia aktiboen eta landare-osasunerako produktuen (PPP, *Plant protection products* ingelesez) gaineko baldintza berri eta osagarriak ezarri zituzten.

Lurzoru organismoen gaineko arrisku ebaluazioak, arrisku ebaluazio orokorren printzipio berbera jarraitzen du; hots, (1) arrisku potentzialaren identifikazioa, (2) arrisku potentzialaren karakterizazioa, (3) esposizio ebaluazioa eta (4) arriskuaren karakterizazioa burutzen ditu. ERA burutzeko, substantzia toxiko ezberdinen inguruko *in silico* modeloak garatu izan dira. Hauen artean, zori modeloak eta efektu modeloak aurki daitezke; baita, biak konbinatzen dituzten FTE (*Fate Transport Effect* ingelesez) modeloak ere (Jorgensen., 2016). Zori modeloek, ingurumeneko konpartimentu jakin batean aurki daitezkeen kontzentrazio kimikoak iragartzen dituzte; hala nola, lurzoru edo erreka batean aurki genitzakeen konposatu kimikoen kontzentrazioak. Efektu modeloek aldiz, gorputz edo konpartimentu biologiko jakin bateko kontzentrazioa efektuetara itzultzen dute. Horretarako, estimatutako kontzentrazioen inpaktua, konplexutasun biologiko maila ezberdinetara estrapolatzen dute; hots, organismoaren hazkuntzatik, populazio edo komunitatearen garapenetik, ekosistema eta paisai aldaketetik, ekosfera osoa ikertu arte (Jorgensen., 2016). Hala ere, efektu modelizazio honetarako, zorizko aurreikuspen egoki bat ezinbesteko da (Tiktak et al., 2013).

3.1-*In silico* modeloen erabilera lurzoruen arriskuaren ebaluazioan

Ingurumen modelo kuantitatiboen erabilera oso hedatuta dago ERA-n eta ondorengo ingurumen erabaki hartze sistemetan (*EDSS: Environmental Decision Support Systems* ingelesez) (Bennet et al., 2013; Pinedo et al., 2014). Zorizko modeloen artean Elikagaien Babeserako Autoritate Europearrak (EFSA: *European Food Safety Authority*

ingelesez) garatutako PERSAM (*Persistence in Soil Analytical Model* ingelesez) softwarea aurki daiteke. Software hau, lurzoruan, zein lurzoruko ur fasean aurki daitezkeen produktuen, eta hauen azpiprodukturen kontzentrazio-aurreikuspenak (*PEC: Predicted Environmental Concentrations* ingelesez) kalkulatzeko garatuta dago. Kalkulurako, egitura espaziala daukaten 62 datu-multzo erabiltzen ditu softwareak: 6 datu-multzo orokor, 27 datu-multzo meteorologiko, lurzoru gainazaleko 5 datu-multzo eta 24 uzta datu-multzo erabiliz.



2. irudia: PERSAM softwareak PPPen kalkulurako erabiltzen dituen 64 datu multzoak eta kalkuluetarako gainjarritako mapa datuen 3 adibide: urteko prezipitazio totala (A), urteko bataz besteko tenperatura (B) eta gainazaleko materia organiko kantitatea (C)

Modeloak, 1x1 km^2-ko bereizmenarekin burutzen ditu kalkuluak, esparru horretako lur-erabilera nagusian oinarritzen delarik. Batik bat pestiziden patua aurreikusteko erabiltzen den software hau, gai da, produktuaren/pestizidaren ezaugarriak sartuta (kom zein koc adsortzio koefizienteak, pisu molekularra eta DegT50-Degradazio ratioa-) eta aplikazioaren maiztasunak sartuta (Urteko aplikazio kantitatea, lehenagoko aplikaziotik pasatako egunak eta fsoil-Lurzorura heltzen den frakzioa-): (a) *TIER-1* Aurreikusitako ingurumen kontzentrazioak (PEC) edo (b) *TIER-2* Aurreikusitako

ingurumen kontzentrazioen 95 pertzentila, lurzoru osoan eta lurzoruko fase likidoan, denbora (0, 7, 14, 21, 28 eta 56 egunetara) eta sakonera desberdinetara (1, 2.5, 5 eta 20 cm-tara) kalkulatzeko. Behin datu horiek izanda, esposizio aurreikuspen egoki bat izanda, posible litzateke efektu potentzialen azterketa edo modelizazio zehatz bat burutzea.

Bestalde, badira giza osasunaren gaineko arriskua kalkula dezaketen efektu modeloak (*HHRA: Human Health Risk Assessment* ingelesez). Hauek, substantzia jakin batek errezeptore potentzial batzuen gainean genera dezaketen efektu kaltegarrien karakterizazio eta kuantifikazioa burutzen dute (Pinedo et al., 2014). Arriskuen ebaluazio honek, esposizio kimikoen natura, magnitudea, denbora, garraio mekanismoa, esposizio bidea eta errezeptoreen sentikortasuna kontuan hartzen ditu (USEPA, 2012). Modelo hauen artean, Eusko Jaurlaritzak EAE-ko lurzoru kutsatuek gizakiengan sor dezaketen inpaktua ebaluatzeko RBCA (*Risk-Based Corrective Action* ingelesez) modeloa bermatua aurki daiteke. RBCA kimikoen askapenerako lanabes kit-a (*RBCA Tool Kit for chemical releases* ingelesez) Materialen Testerako Sozietate Amerikarrak (ASTM*: American Society for Testing Materials* inglesez) Estatu Batuetan argitaratutako modeloa da (Galan et al.,2014; Pinedo et al.,2014). Honek, esposizio bide bakoitzerako arrisku balio indibidualizatua zein metatua kalkulatzea ahalbidetzen du; ezarritako baldintza bakoitzean aurreikusitako atalase balioekiko konparazioa ahalbidetuz. Era honetan, eszenario bakoitzean lorturiko arrisku balioak atalaseekin konparatzea posible da; hortaz, arriskua edo arrisku-gabezia aurreikus daitekeelarik. Autore ezberdinek, RBCA modeloa arriskuen azterketarako gida estandarizatu eraginkor eta erabiliena bezala definitzen dute (Tsai et al., 2011). Izan ere, eredu hau munduan zeharreko hainbat puntu geografikotan aplikatua izan da autoritate erregulatzaile ugariren eskutik (Pinedo et al.,2014). Hala ere, eta erabakiak hartzeko sarri erabili izan den arren (lurzoru kutsatu baten deklarazioan, garbiketa/deskontaminazio ekintzetan, zigilatzeetan, traza-mailan kutsatutako lurzoruen ikerketetan), emaitz kontserbadoreegiak eta gain-estimatuak genera ditzake RBCA modeloak (Galan et al.,2014).

Hortara, ERAren sendotasun eta fidagarritasuna bermatze aldera, beharrezkoa da *in silico* frogekin batera eszenarioak esplizituki frogatzea. Horretarako, ikertu beharreko

lekuetan bizi diren espezie esangarriekin burututako toxikotasun bioentseguak erabili daitezke.

4-TEST EKOTOXIKOLOGIKOAK LURZORUKO ESPEZIEEKIN

Toxikotasun testetan, organismoak kutsatzaile kontzentrazio eta mota ezberdinen pean izaten dira medio ezberdinetan (ur, sedimentu edo lurrean), ondoren, organismoen hazkuntza, jarrera, eta biziraupena bezalako ezaugarriak ebaluatzeko (EPA, 1994). Horrela, test hauen bitartez, medio jakin batetan dauden kutsadura mailak organismoengan kalteak sortzeko adinakoak diren (edo ez) ebaluatu daiteke. OECD, ISO edo EPA bezalako erakundeek, nazioartean balioztatutako protokoloak garatu izan dituzte kimiko ezberdinen toxikotasunaren estimaziorako (Davies et al., 2003). Ekotoxikotasun test estandarizatu hauek, eskualde ezberdinetako laborategietan emaitza berdintsuak lortzea, konparazioak erraztea eta ezarritako toxikotasun neurketen fidagarritasuna areagotzea ahalbidetzen dute (Lopes Alves & Nogueira Cardoso, 2016).

Toxikotasun testen barruan, espezie, medio eta efektu desberdinak ikertzen dituzten entseguak daude. Test estandarren garapenerako espezie modeloak berauen garrantzia ekologikoagatik, laborategi mantenu errazagatik eta belaunaldi denbora laburragatik aukeratuak izan behar dira (Ronday & Houx, 1996; Fountain & Hopkin, 2005). Espezie modelo hauen artean: zizareak (*Eisenia andrei* eta *E. fetida*), enkitraideoak (*Enchytraeus albidus* eta *E. crypticus*), moluskoak (*Helix aspersa*), akaroak (*Hypoaspis aculeifer, Platynothrus peltifer*, eta *Oppia nitens*), isopodoak (*Porcellio scaber* eta *Porcellionides pruinosis*), kolenboloak (*Folsomia candida* eta *F. fimetaria*), Carabidae familiako intsektuak (*Pterostichus oblongpunctatus*, *Poecilus cupreus* eta *Oxythyrea funesta)* edo bestelakoak aurki daitezke (Van Gestel, 2012). Lurzoru ornogabeez aparte, espezie begetalak erabiltzen dituzten test estandarizatuak ere garatu izan dira kutsatzaile ezberdinen toxikotasuna aztertzeko. Test mota hauetan, hazi eta landareetan burutu daitezkeen neurketak erabiltzen dira toxikotasuna barematzeko; aipagarrienen artean, *Lactuca sativa* edo *Cucumis sativa* espeziekin burutu daitezkeen EPA-ren OPPTS 850.4225 (1996) eta OECD-ren 208 (2003) gidalerroak aurkituz. Aipatutako espezieen desberdintasun fisiologiko eta morfologikoei esker (elikatze eta portaera ohiturekin batera), kutsatzaileak hartzeko bide desberdinak aztertu daitezke; lurzoruaren, lurzoru poro-uraren edo airearen esposiziaren bitartez, zein elikaduraren bitartez (lurra jatean)

kutsatzaileak inkorporatzen baitituzte (Peijnenburg et al.,2012). Efektuei dagokienez, toxikotasun testek, parametro letal eta/edo subletalak neur ditzakete (EPA., 1994).

Zizareak (ISO, 2018 & 2018; OECD,1984 & 2016; Lopes Alves & Nogueira Cardoso, 2016), kolenboloak (ISO., 2014; OECD., 2009;), akaroak (Van Gestel et al, 1997) eta enkitraideoak (Jansch et al., 2005; ISO., 2004; OECD., 2004) dira lurzoru ekotoxikologi test baterietan gehien erabiltzen direnak. Izan ere, hirurogeigarren hamarkadaren bukaeran burutu ziren lurzoruetako lehen toxikotasun testak (Fox, 1964; Edwards, 1969); kolenbolo eta zizareak erabiltzen zituzten pestiziden toxikotasuna ebaluatzeko (Ghabour & Imam, 1967; Scopes & Liechtenstein, 1967). Zizareak, kutsaduraren aurrean modu sentikor eta neurgarrian erantzuten duten metal metatzaile efizienteak dira. Ondorioz, organismo behale hauen erabilpena zeharo zabaldu da lurzoruen kutsadura ikerketetan; bereiziki, *E. fetida/E. andrei* espeziak izanik erabilienak.

E. fetida (Savigny, 1826) lurrean deskonposatzen ari den materia organikoan, konpostean eta lizunean bizi den zizare epigeikoa da (Bilej et al.,2010). Organismo hauek, ezinbesteko zeregina burutzen dute lurzoru formazioan, MOaren deskonposaketan eta nutrienteen berziklapenean (Gaete et al., 2010). Ugalketa ziklo erlatiboki laburra dauka (Walker et al.,1996), arrautzen eklosiotik 21-30 egunetan (baldintza optimoetan) heldutasuna lortuz eta 45-51 egunetan hurrengo belaunaldia sortuz (Dominguez et al., 2004). Arrautzen lurreratzea, kopularen ondorengo 48 orduetan gertatzen da, oinordekotzaren produkzioa 0.35 eta 1.3 arrautza/egun-etakoa izanik. Hala ere, eta zizareek urte osoan arrautzak produzitu ditzaketen arren, urtaroen arteko hezetasun, tenperatura, janari eta bestelako faktoreen aldaketek, populazioen tamaina eta biomasetan eragin dezakete (Edwars & Bohlen, 1996; EFSA, 2017). Jaiotzen diren oinordekoen kantitateak 2.5 eta 3.8 bitartean oszila dezake; tenperaturaren ondorioz batik-bat (Dominguez et al., 2005).

Espezie hau kutsatzaile kimikoek eragindako toxikotasunaren erakusle ona izateaz gain, estres faktore anitzen efektuak (Hund-Rinke et al., 2001), lurzoruaren egoera fisikoa (konpaktazioa/trinkotzea, hidrologia, etab.) eta lurzoru erabilpenak ebaluatzeko adierazle sentikor eta egokia da (Lee, 1989). Guzti honek, organismo merke, egoki eta eztabaida etiko bakoa izatearekin batera, esperimentaziorako modelo organismo oso erabilia izatea bultzatu du (Bilej et al., 2010; Shi et al., 2017). *E. fetida*

zizareak kutsadurarekiko erakutsi izan duten sentikortasuna dela eta, zabalki erabiliak izan dira zenbait organismo internazionalek argitaratutako toxikotasun test estandarizatuetan (ISO 2018, 2018; OECD 1984, 2016) lurzoruen afekzioak ikertzeko (Ernst et al., 2008; Asensio, 2009; Irizar et al., 2014; Garcia-Velasco et al., 2016, 2017; Urionabarrenetxea et al., 2020).

4. 1- Toxikotasun testak *Eisenia fetida* zizarearekin

Kutsatzaileek *E. fetidan* eragin dezaketen arrisku potentzialak ebaluatzeko toxikotasun test akutuak eta kronikoak aurki daitezke. Test akutuen (OECD-207, 1984) artean, heriotza dosia (LC_{50}) bilatzen duten Paper kontaktuko testa eta Lurzoru artifizaleko testa aurki daitezke. Lehenengoan, ikertu nahi den sustantziaz hezetutako paperararekin kontaktuan jartzen dira zizareak bi egunetan zehar; efektu kimikoen behaketa arin bat egiteko eta kimikoen hartze eta biotransformazioen azterketa burutzeko teknika erabilgarria izanik (Van Gestel, 2012). Hala ere, test mota honek esposizio dermiko bidezko toxikotasuna antzematen du batik bat (Urionabarrenetxea et al., 2021); ingestio bidezko toxikoen barneratzea (fase solidoan egongo liratekeenak) ebaluatzeko, lurzoru bidezko esposizioaren beharra egonik (Belfroid et al., 1994; Contreras-Ramos et al., 2005; Garcia-Velasco, 2017). Lurzoru artifizialeko testek, kimikoek zizareengan eragiten dituzten toxikotasun efektuen datu adierazgarriagoak eskaintzen dituzte; horretarako, substratutzat %70 hare, %20 buztin eta %10 turbaz eginiko lurzoru artifizialak erabiliz (OECD, 1984). Azken urteotan, baldintza errealen adierazgarritasuna hobetzeko xedeaz, LUFA lurzoru natural eta estandarren erabilera hedatzen hasi da (Løkke & Van Gestel 1998). Izan ere, Alemanian (Speyer) komertzializatutako lurzoru hauen MO kantitatea (%1.25-5.36 bitartean) OECD lurzoruena baino baxuagoa da; nekazal lurzoruetara gehiago hurbilduz (%<10) (Van Gestel., 2012).

Behin kutsatzaileak organismo barnean daudela, kalte fisiologiko ezberdinak eragin ditzakete; hazkuntzan, elikagaien asimilazioan zein ugalketarako energi gastuan, azkenik, norbanakoen heriotza eragin arte (Garcia-Velasco et al. 2017). Hala ere, lurzoruko organismoenganako efektu toxikologikoak ikertzeko froga hedatuak diren arren, efektu subletal asko: portaera aldaketak, ugalketa aldaketak, inhibizio

entzimatikoa edo kalte genetikoa bezalakoak, ez dira erasotzen toxikotasun akuturako test hauetan.

Populazioen dinamikan daukan garrantzia dela eta, azpimarratzekoa da ugalketa azterketek toxikologian duten garrantzia (Joose & Verhoef, 1983; Kooijman & Metz, 1984; Spurgeon et al., 1994). OECD-222 testa adibidez, agente kimiko ezberdinek ugalketa errendimenduan sor zitzaketen efektuak aztertzeko diseinatutako testa da. Espresuki, hilkortasun eta hazkuntza parametroak neurtzen dira 4 astetan zehar (28 egun) esposiziopean izandako zizareetan; 56 egunetara ugalketaren gaineko efektuak (oinordekotza) ebaluatzen diren heinean. Modu honetan, eta entsegu hau erreprodukzio parametroak lortzeko diseinatuta dagoen arren, indikatzaile askoren lorpenerako (ECx, LCx, LOEC edo NOEC) test baliagarria da.

Aipatutako toxikotasun testetatik eratorritako heriotza eta ugalketa parametroak agente kimikoen arrisku ebaluazio eta erregulaziorako garrantzia handikoak dira (Spurgeon et al., 1994, 2004; Rodriguez-Ruiz et al., 2014). Hala ere, azken hamarkadetan, ikerkuntzaren esfortzuak azterketa hauen sakontzean jardun izan dira, ekotoxikologia modernoaren esparruan biomarkatzaile berriak garatu eta balidatuz (Garcia-Velasco, 2017).

4.2- Biomarkatzaileen neurketa *E. fetida* zizarean

Biomarkatzaileak, kutsatzaileenganako esposiziorik eta/edo efekturik izan den argituko duten parametro neurgarriak dira. Konplexutasun biologiko sinpleenetan (maila molekular, zelular, ehun maila) neurtzen diren arren, maila biologiko konplexuagoetan gerta daitezkeen (populazio, komunitate edo ekosistema mailan) aldaketak aurreikusteko duten ahalmenagatik (Spurgeon et al., 2005), diagnosi goiztiarrak burutzeko tresna eraginkor bezala erabiliak dira. Neurketa kimikoek ez bezala, biomarkatzaileek, kutsatzaileek eragindako inpaktu biologikoen garrantziari buruzko informazioa eskeintzen dute (Marigomez et al., 1996; Kammenga et al., 2000). Horregatik kutsatzeileek lurzoru ekosistemetan eragin ditzaketen efektuak kuantifikatzeko lurzoru ornogabeetan (*E. fetida* kasu) aplikaturiko biomarkatzaileen gaineko interesa asko hasi da azken urteotan (Hugget et al., 1992; Asensio et al., 2013; Irizar et al., 2015; Garcia-Velasco, 2017). *E. fetida* zizareetan gehien erabiltzen diren

biomarkatzaileen artean, DNA alterazioen behatzeak (maila molekularra), metalei elkartzen zaizkien proteina (MT- *Methallothioneins* - eta MBP- *Metal binding proteins*-ingelesez), kolinesterasa eta erantzun entzimatikoen neurketak, energia gordekinen kuantifikazioa (maila biokimikoa), lisosomen mintz egonkortasunaren azterketa (maila zelularra), espermaren kalitatea eta erantzun immunologiko, neurologiko, histologiko (ehun maila) eta portaerazkoen analisiak aurki daitezke (Scott-Fordsmand & Weeks, 2000; Sanchez-Hernandez, 2006; Irizar et al., 2014, 2015a, 2015b). Frogatua izan da gainera, metalekin kutsatutako lurzoruek mintz lisosomikoaren ezegonkortasuna (Asensio et al., 2007, 2013), aktibitate entzimatikoa eta geneen adierazpena (Spurgeon et al., 2006; Li et al., 2009; Zhang et al., 2009), estres oxidatiboa (Spurgeon et al., 2004; Berthelot et al., 2008; Zaltauskaite & Sodiene 2014) zein DNA-an kalteak (Fourie et al., 2007; Liang et al., 2011; Wu et al., 2012) eragin ditzaketela.

Azken hamarkadan, maila zelularreko biomarkatzaileen garapenean emendio argia gertatu da; hauen artean, zizareen zelula immunitarioetan (zelomozitoetan) aplikaturikoak nabarmenduz. Zelomozitoak barrunbe zelomikoko likido zelomikoan aske dauden zelula immunitarioak dira; gizakien leukozitoen homologoak (Hayashi et al., 2012). Likido zelomiko hau, esekiduran daramatzan zelomozitoekin batera, zizareen lakainetako nefridio pareetatik eta poro dortsaletatik kanporatzen da (Bilej et al., 2000; Cholewa et al., 2006), material ezezagunen errekonozimenduan eta eliminazioan zein zaurien koagulazio eta orbaitzean ezinbesteko rola izanik (Cooper et al., 2002; Kurek et al., 2007). Funtzio horietarako, entzima hemolitiko, proteolitiko eta zitotoxikoetaz baliatzen dira zelomozitoak (Kurek et al.,2007). Zelomozitoen artean, pigmentazio, ultraegitura, funtzio, konposizio granular zein portaera desberdina (atxikidura eta kimiostasi) duten (Hamed et al., 2002) bi subpopulazio nagusi bereiz daitezke: amebozitoak eta eleozitoak (Cooper et al., 1981; Engelmann et al., 2004, 2005;).

Amebozitoak (pikortsu edo hialinoak), zelomozito mota ugarienak dira (Adamowicz et al., 2005). Zelomaren estalki mesenkimatikoan jatorri duten zelula hauek (Hamed et al., 2002), aktibitate fagozitiko eta enkapsulatzaile handia duten immunozito eragileak dira (Engelmann et al.,2005; Hayashi et al., 2012). Eleozitoak aldiz, liseri-traktua estaltzen duten zelula kloragogenikoetatik askatzean sortzen diren zelulak dira (Affar et al., 1998). Osatzen dituzten bikor (kloragosomak) ezberdinek (Affar et al., 1998;

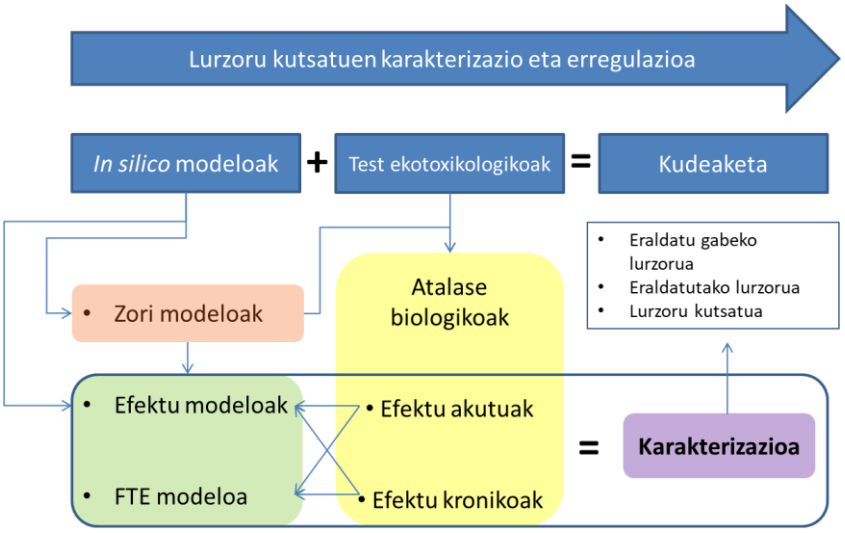
Peeters-Joris, 2000; Cholewa et al., 2006) eta erriboflabina metaketa selektiboek eragindako autofloreszentziak (Cholewa et al., 2006; Plytycz et al., 2011) oso bereizgarriak bilakatzen ditu. Zelomozito hauek ornogabeen zelula hepatikoen funtzio berdintsuak izango lituzkete (Cholewa et al., 2006) nutrizio funtzioa daukaten glikogeno eta lipidoen metabolismo, biltegiratze (Cholewa et al., 2006; Bilej et al., 2010) eta garraioan (Affar et al., 1998; Hamed et al., 2002) jardunez. Gainera, aktibitate fagozitikorik ez duten arren, zizareen homeostasi eta immunitate humoralean parte hartzen dute parte (Adamowicz et al., 2005; Engelmann et al., 2005; Cholewa et al.,2006; Garcia-Velasco, 2017); horretarako, peptido antimikrobianoak bezalako faktore humoralak ekoiztuz (Cholewa et al., 2006; Bilej et al., 2010).

Zizareetan neurtzen diren maila zelularreko biomarkatzaileak, zelomozitoen lisosoma mintzaren egonkortasunean zentratu izan dira batik bat. Lisosomen bakuola sistema, endozitosiz barneratutako kutsatzaileen (metalak edo elementu organikoak) edo zeluletako hondakinen metatze eta kanporatzean oinarritzen da (Moore, 1985; Viarengo, 1989). Hori dela eta, lisosomen patologia azterketa (mintz egonkortasunaren azterketa barne) oso erabilia izan da ingurumen inpaktuak aztertzeko orduan. Spurgeon-ek (2005) lisosoma mintzaren egonkortasun neurketa (biomarkatzaile molekular eta biokimikoekin batera) metalek sortutako estresa neurtzeko biomarkatzaile sentikorrenen artean sartu zuen. Horrek bultzatuta, ugariak izan dira azken hamarkadetan zelomozitoekin garatutako testak; mikroplaketarako garatutako MTT, XTT (zelulen aktibitate metabolikoa neurtzen dute), NRU (*Neutral Red Uptake*) edo Kaltzeina-AM zelula bideradarritasun testak kasu. Kaltzein AM testa, esterasen aktibitatean oinarritzen den (Kaneshiro et al., 2013) bideragarritasun zelularra eta zitotoxikotasuna neurtzeko metodo arin, sinple eta zehatza da (Garcia-Velasco 2017). Kaltzeina zetometil esterra (*Calcein AM*) zelula barnera sartzen da, behin barnean, esterasa intrazelularrek zitoplasman metatu den kaltzeina hidrolizatzen dute; zeina, konposatu hidrofiliko fluoreszentea bilakatzen den. Fluoreszentzia horren beherakadak, mintz zelularraren kaltea iragarriko luke; organismoa estres kimiko (metal astunek eragindakoa kasu) edo fisikopean dagoela iradokiz. Berriki, zelomozitoen bideragarritasunaz gain, zelomozito kontzentrazioa ere erabili izan da metalen inpaktua ebaluatzeko biomarkatzaile gisa (Kwak et al., 2014).

5- LURZORU KUTSATUEN DIAGNOSIA: IKUSPUNTU HOLISTIKOA

Aktibitate antropikoa dela eta kutsatua izan den lurzoru baten karakterizazioa burutze aldera, ezinbestekoa da afekzio kimiko eta biologikoak behar bezala aztertzea; batez ere, kutsatzaileek izaki bizidunengan eta ekosistemengan genera dezaketen arriskua barematze aldera.

In silico modeloak, kutsatzaile baten isuri/aplikazio baten osteko ingurumen kontzentrazioak aurreikusteko tresna egokiak dira; gainera, entsegu ekotoxikologikoetako dosien ezarpenean oso lagungarriak izanik. Aldiz, kutsatutako lurzoru bat kudeatze aldera, eta efektu biologikoak aurreikuste aldera, ezinbestekoa da kutsatutako lurzoruaren toxikotasuna aztertzea; zeregin horretan, toxikotasun testak eta biomarkatzaile biologikoak ezinbestekoak izanik. Hauen bitartez, konposatu jakinek, baldintza zehatzen pean organismoengan eragiten dituzten efektu biologikoak ikertzea bilatzen da; era honetan, LC_{50}, NOEC, LOEC edo EC_{50} bezalako hilgarritasun edo efektu parametroak eskuratuz. Era berean, parametro hauek, efektu modeloak eta FTE modeloak elikatzeko erabil daitezke; honela, lurzoruarekiko esposizio jakin batek edo lurzoru erabilera jakin batek suposatuko lukeen arriskuaren kuantifikazioan lagunduz.



3. irudia. Lurzoru kutsatuen karakterizazio eta erregulaziorako erreminten arteko erlazioak

Esan bezala, efektu modeloak eta FTE modeloak, ikertutako toxikotasun atalase biologikoetan oinarritzen dira arriskuaren kuantifikazioa burutzeko. Hala ere, test ekotoxikologiko hauek, laborategi baldintzatan eta kutsagai bakarrenpean burutakoak

izaten dira gehienetan; zelai baldintzekiko antzekotasun gutxi izanik. Laborategi baldintza optimoetan kalkulatutako atalaseak efektu modeloetan erabiltze aldera, estimazioak ziurgabetasun koefizienteekin doitzen dira; gehienetan, arreta-printzipioa kontutan izanik. Horrela, efektu modeloek aurreikusitako eszenarioak, zehaztasun gabekoak eta ziurgabetasun altukoak izaten dira gehienetan; sarri, inpaktuak gehiegi balioetsiz.

Hau esanda, eta efektu modeloak behar bezala elikatzeko, ezinbestekoa da ebaluatu beharreko lurzoruekin test ekotoxikologiko zehatzak burutzea. Horrela, estimatutako arriskua zehatzagoa izango da; eta horretara, ondorengo kudeaketa.

Behin lurzoru kutsatuen arriskua zehaztuta, lursailen itxiera, erabileraren aldaketa edo arrisku arintzea jorratuko dira; horretarako, betiere, lan ekotoxikologikoetan eta arrisku ebaluazioetan oinarrituz. Arriskuaren murrizketa, *in silico* eta ekotoxikologia lanen bitartez monitorizatutako bioerremediazio prozesuen bitartez, adibidez.

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CHAPTER 2

Kutsatutako lurzoruen erremediazioa teknologia biologikoen aldibereko konbinazioak erabiliz

(Simultaneous combination of biological strategies for the remediation of contaminated soils)

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ABSTRACT

Anthropogenic activities led to the proliferation of polluted sites or potentially polluted sites around the world; including Basque Country. Technologies to remediate or clean these soils, mainly polluted by heavy metals or organic pollutants are diverse and different nature. On the one hand, classic physico-chemical remediation techniques can be found; with high yields and short application times; but, high costs and high ecological impacts. On the other hand, biological techniques could be found. These technologies are cheaper and more environmental-friendly; but, they require long application times. Among most used bioremediation technologies plant-based phytoremediation, worm based vermiremediation, and mibrobia based bioremediation (or bioaugmentation) can be found. This paper deals with the main features of these techniques and their applicability; both as single remediadion techniques or combined.

Keywords: : soils, pollution, bioremediation, phytoremediation, vermiremediation

LABURPENA

Gaur egun ugariak dira mundu mailan zein Euskal Herri mailan aktibitate antropikoengatik kutsatuak dauden edo kutsatuta egon daitezkeen lurrak. Batik bat metal astun edo elementu organikoekin kutsatuta egoten diren dire lurzoru hauek erremediatzeko edo garbitzeko teknikak anitzak eta natura ezberdinetakoak dira. Alde batetik erremediazio teknika fisiko-kimiko klasikoak auki daitezke, errendimendu altu eta aplikazio denbora laburrak izan ohi dituztenak; baina, kontrara, kostu altu eta inpaktu ekologiko handiak eragiten dituztenak. Bestetik, teknika biologikoak aurkituko lirateke, merkeagoak eta ingurumenarekiko adeitsuagoak direnak; baina, aplikazio denbora luzeak behar dituztenak. Hauen artean, landareen bitartez burutzen den fitoerremediazioa, zizareekin burutzen den bermierremediazioa zein mibrobioekin burutzen den biorerremediazioa aurkitzen dira. Lan honetan teknika hauen ezaugarri nagusiak eta berauen aplikagarritasuna jorratzen dira; bakarkako erremediazio teknika zein erremediazio teknika konbinatu gisa.

Hitz gakoak: : lurzoruak, kutsadura, bioerremediazioa, fitoerremediazioa, bermierremediazioa

1-SARRERA

Lurzorua kutsatu dezaketen elementu kimiko ugarienen artean metalak (Cd, Cr, Cu, Hg, Mn, Ni, Pb, V, eta Zn), metaloideak (As, Bo, eta Sb), halogenatuak (F eta I), ez-metalak (Se) eta aktinoideak (U) daude (Hooda, 2010; Cachada et al., 2018). Metal astunekin kutsatutako lurzoruen arazoa mundu mailako problema bilakatu da berauen iraunkortasun eta natura ez-degradagarriagatik. Izan ere, izaera honek maila toxikoak izatera arte metatzea ahalbidetu izan du (Tang et al., 2019). Metal astunak, bide ezberdinetatik hel daitezke lurzoruetara; hala nola, pestiziden eta ongarrien erabilerarekin, produktu kimikoen sortzean, hondakin industrialen bitartez, meategietatik, hondakin-uren isuritik; hots, jarduera antropogenikoetatik (Gómez-Sagasti et al., 2019). Behin lurrean, bertako materia organikora atxikitzen dira, metalaren mugikortasun eta bioeskuragarritasuna murriztuz. Edonola ere, lotura hauek ekosistementzako esentzialak diren prozesuak ematen diren ataletan gertatzen dira; deskonposizio eta nutrienteen mineralizazioa etaginez eta ekosistemen funtzionamendua erasanez (Lukkari et al., 2004). Metal batzuk beharrezko traza elementuak diren arren, maila eta denbora jakin batzuen gaineko metalen esposizioak efektu toxikoak eragin ditzake lurzoru organismoengan; hauen ugaritasuna, dibertsitatea eta distribuzioa kaltetuz (Hopkin, 1989).

Lurzorua kutsatu dezaketen konposatu organikoen taldean aldiz, egituran karbonoa (talde funtzionalekin edo ez) daukaten konposatuak batzen dira; hala nola, pestizidak, hidrokarburoak, PAHak, PCBak, PBBak, PBDEak, PCDFak, PCDDak, farmazia produktuak edo surfaktanteak. Nekazal aktibitatearen areagotzeak pestiziden erabileraren emendioa ekarri du, gaur egun, 400 substantzia edo pestiziden erabilera onartua izanik Europako nekazal lurretan (Pelosi et al., 2014). Hala ere, argitaratutako ekotoxikologia ikerketa gehienak metalen toxikologian zentratu izan dira (Lowe & Butt, 2007), pestiziden efektuak gutxiago ikertu diren heinean (Pelosi et al., 2014). Gainera, ikertutako pestizida hauek, Europan erabilpena debekatuta daukaten pestizidak izaten dira gehien bat; hala nola, paraquat-a (1,1'-dimetil-4,4'-bipiridilo dikloruroa) dikloropropenoa (1,3-dikloropropenoa) edo cianamida. Metalak ez bezala, pestizida eta PAHak lurzoruan erraz degradatzen diren konpostauak dira (Brusseau, 1997). Lurzoruan

dauden kutsatzaileen degradazioa, bioeskuragarritasuna, eta toxikotasuna, lurzoruan pH-a eta MO kantitatea bezalako propietateekin erlazionatuta dago.

Konposatu/elementu kimiko hauek isuriak direnean edo medio ezberdinetara heltzen direnean, ingurumena degrada dezakete ekosistemen eta gizakiaren osasuna kaltetuz. Hori dela eta, lurzoruan dauden konposatuen kontzentrazioak eta hauek sor ditzaketen arrisku potentzialak ikertzea beharrezkoa da lurzoruaren sailkapen eta kudeaketa egoki bat egiteko.

2- LURZORUEN KUTSADURA EAE-N

Lurzoru baten egoera ikertzeko osasun ikuspegia gomendagarriagoa eta integralagoa den arren, gutxi dira oraindik ekotoxikologia testak legedian barneratu dituzten herrialdeak. Analisi kimikoetan baliatuta lurzoru bat kutsatuta dagoela onartu eta adierazten denean; hots, lurzoru kalitatearen adierazpena argitaratzen denean, neurri edo aukera ezberdinak plantea daitezke eszenarioaren arabera. Eusko Jaurlaritzako (EJ) Ingurumen Sailburuordetzaren arabera, lurzoru baten kalitatea, honen afekzioaren araberakoa izango da; hiru kategoria hauen artean egon daitekeena:

- **Eraldatu gabeko lurzorua**: kutsagarria izan litekeen jarduera bat jasan duen, baina bere kalitatean aldaketarik pairatu izan ez duen lurzorua.
- **Eraldatutako lurzorua**: Erreferentziazko balio adierazleak gainditzen dituzten substantzia kimikoak-kontzentrazioak aurki daitezkeen, baina giza osasunerako edo ekosistemetarako arriskurik ez sortzean, kutsatutzat hartzen ez den lurzorua. Erreferentzia balioen eta kontzentrazio balioen erkatzea egite aldera, EJ-ak Ebaluaziorako Balio Adierazleak (EBA) argitaratu zituen Otsailaren 4ko 1/2005 lurzorua ez kutsatzeko eta kutsatutakoa garbitzekoa legean (ondoren 4/2015 legeak, ekainaren 25ekoak ordeztua). Honen arabera, hiru maila ezartzen dira lur erasanduak sailkatzeko: EBA-A (substantzia jakin batek EAE-ko lurzoruetan era naturalean aurkitzen denean duen kontzentrazio-tartearen mailarik gorenarekin bat datorren estandarra), EBA-B (lurzoruaren erabilera desberdinen arabera definitzen den estandarra, arriskuaren

onargarritasun atalase baxuena ezartzen duena) eta EBA-C mailak (gehienezko arrisku onargarria, edo arriskuaren onargarritasun atalase altuena).

- **Lurzoru kutsatua**: Gizakiaren jardueren eraginez, ezaugarri kimikoak eraldatuta dauzkan lurzoru mota. Pertsonen edo/eta ekosistemen osasunentzako suposatzen duen arriskuagatik bateraezina egungo erabilera edo aurreikusitakoarekin.

EJ-ak erabiltzen duen irizpide honen arabera, kutsadura kontzeptua, arriskuarekin loturiko kontzeptua da; hau da, kutsadura, afekzio kimikoaren eta arriskuaren (erabilera jakin batean elementu kimikoa hartzaile batekin kontaktuan jartzerakoan, pertsonen osasunean edo ingurumen osasunean ondorio kaltegarriak izateko probabilitatea)) arteko elkarrekintzatik sortzen da.

Kutsatutako lurzoruekin kudeaketa aukera desberdinak daude. Horrela, kasurik txarrenean, lur esparrua itxi egin beharko litzateke, bertara sar zitekeen edozein erabiltzaileren osasuna babesteko xedez. Beste aukera bat, lurzoru horren erabilera aldatzea litzateke; erabilera berrien bitartez, arriskutsuak izan zitezkeen eszenario edo elkarrekintzak murriztuz edo mugatuz. Hala ere, Lurzoruen kutsatzea saihestu eta kutsatutakoa garbitzeko 4/2015 legeak (Ekainaren 25 ekoa), kutsatutako eta eraldatutako lurrak berreskuratzearen beharrizana ezartzen du. Legearen arabera, eraldatutako lurzoruak berreskuratzeko neurriek, lurzorua jatorrizko egoerara bueltatzea izango dute helburu; egoera ezagutzea ezinezkoa izango balitz, helburua lurzoruaren kalitate-estandar batzuk (teknologia onenak erabiliz) lortzea izango litzatekelarik: "*gutxienez EBA-B ebaluazio balio adierazleen parekoak edo petrolioaren guztizko hidrokarburoen baliokideak (TPH) eta, hala dagokionean, urarentzat ezarritakoen parekoak*" (Lurzoruen kutsatzea saihestu eta kutsatutakoa garbitzeko 4/2015 legea).

Lurzoru kutsatuak berreskuratzeko erremediazio teknologiak, kutsatzaileen toxikotasuna, bolumena edo mugikortasuna murriztuko duten prozesu fisiko-kimiko edota biologikoen multzoa dira (Betancur-Corredor, 2013; Aparicio, 2018). Teknologia hauen bitartez, lurzoru eta lurpeko ur kutsatzaileen mugikortasuna eragin dezaketen propietate fisiko eta kimikoak eraldatzea bilatzen da (gehienetan). Era berean, kutsatzaileen forma kimikoek eta lurzoru ezaugarriek zeharo baldintzatzen dute

erremediazio tratamenduaren aukeraketa. Lekuaren ezaugarri fisikoen eta kutsadura maila/motaren gaineko informazioa izatea beharrezkoa da eremuaren ebaluazio eta erremediazio egoki bat burutzeko. Horretarako, karakterizazio emaitzak, erregulazio domeinu konkretu batetako kalitate estandarrekin konparatzen dira, edo lekuaren arrisku azterketa espezifiko bat burutzen da. Karakterizazio emaitzak erregulazio konkretu batetako kalitate estandarrak baino altuagoak direnean edo arrisku ebaluazioa negatiboa denean; kudeaketa aukeretako bat, lurzoru horren erremediazioa burutzea da, lurzoru baldintzen zein beronen funtzionalitatea berreskuratzea ahalbidetuz.

3-LURZORU KUTSATUEN ERREMEDIAZIOA

3.1-Erremediazioa baldintza dezaketen lurzoruaren propietate kimikoak eta fisikoak

Erremediazioaren abiadura eta bideragarritasuna, lurzoru ezaugarriek baldintzatuta dago (Cerniglia, 1992; Mohan et al., 2006). Ezaugarri baldintzatzaileen artean, anioi organikoen presentzia, pH-a, katioiak trukatzeko gaitasuna eta MO kantitatea dira azpimarragarrienak.

Lurzoruko uretan, anioi inorganikoen presentziak (karbonatoak, fosfatoak, sulfuroak), lurzoruen metalak (kimikoki) konplexatzeko ahalmena eragin dezake. Izan ere, anioi hauek, konplexu disolbagaitzak sor ditzakete metal ioiekin; metalen desortzioa eta/edo prezipitazioa bultzatuz (Evanko & Dzombak, 1997).

Lurzoruen pH-a metalen disolbagarritasunean gehien eragiten duen faktorea da (Giller et al., 1998). Lurzoru pH balioak, 4 eta 8.5 barrutian izan ohi dira normalean, aluminioa izanik indargetzailea pH baxuetan, eta karbonato kaltzikoa ($CaCO_3$) pH altuetan (Wild, 1988). Metal katioiak mugikorragoak dira kondizio azidikoetan; kondizio hauetan, anioiak, oxido metaletan absorbitzera egiten duten heinean (Dzombak & Morel, 1987; Evanko & Dzombak, 1997). pH altuetan aldiz, katioiak prezipitatu edo mineralen gainazalean adsorbitzen dira; anioak, mobilizatu egiten direlarik. Burdin, aluminio eta manganeso metal oxido hidratuak, lurzoru metal kontzentrazioak zeharo baldintzatu ditzakete (Jacobson et al., 2005); izan ere, aldaketa ioniko, absortzio espezifiko eta gainazal prezipitazio bitartez, soluzioan dauden katioiak eta anioiak

baztertu ditzakete (Ellis & Fogg, 1985; Dzombak & Morel, 1987). Guzti hori, Katioiak Trukatzeko Gaitasunak (KTG, *CEC- Cation Exchange Capacity*-) eta Anioiak Trukatzeko Gaitasunak (ATG, *AEC- Anion Exchange Eapacity*) baldintzatzen dute. Lehenak mineral gainazal batean trukagai dauden katioi kontzentrazioari egiten dio erreferentzia; askotan, lurzoruek katioiak (metalak kasu) hartzeko duten gaitasuna edo afinitatea adierazteko erabilia izanik. ATG-ak aldiz, lurzoruak anioiak bereganatzeko duen gaitasunari egingo lioke erreferentzia; orokorrean, KTG baino baxuagoa izanik. Lurzoruan, ioi trukaketa ahalmen altua duten materialen artean, goian aipatutako oxido hidratuak eta buztinak bereziki aipatzekoak dira (Sposito, 1989).

Lurzoruko MO-a, pisu molekular altuko molekulak dauzkan eta deskonposaketaren aurrean erresistenteak diren konposatu koloidalen nahasturaz sortua dagoena (Nieder et al., 2008), hondar organikoen deskonposaketa biologiko eta kimiko bitartez sortzen da, aldatu gabeko material eta eraldatutako produktuen (humusa) artean desberdinduz (Garcia-Velasco, 2017). MO-ak ezinbesteko papera jokatzen du lurzoru funtzioen erregulazioan; besteak beste, gainazal kargen ekarpenean (KTG-a bezala adierazita) (Evanko & Dzombak, 1997), hezetasunaren erregulazioan, elikagai ziklapenean eta lurzoru estruktura mantenuan lagunduz (Kibblewhite et al., 2008; Garcia-Velasco, 2017). Gainera, MO-ak, eta bereziki talde karboxiliko eta fenolikoak dituzten material humikoek, positiboki kargatutako molekulak fixatzeko ahalmena daukate (katio metalikoak kasu); hauen eskuragarritasuna eta mugikortasuna jaitsiz (Ali & Dzombak, 1996).

USDA (1987, Estatu Batuetako Nekazaritza Saila) lurzoru taxonomia sailkapenaren arabera, lurzoru batetako partikula tamainen distribuzioa hiru azpitalde handitan sailkatzen da: hareetan (2 eta 0.05 mm bitartean), lohietan (0.05 eta 0.002 mm bitartean) eta buztinean (<0.002 mm). Tamaina handi eta ertaineko materialek (2 mm baino handiagoak) lurzoruaren eskeletoa osatzen dute, tamaina txikiko materialek (lohi eta batez ere buztinek) aldiz, lurzoruko osagai mineralen tarte aktiboena osatzen duten bitartean. Partikula finenak (<100 µm) erreaktiboagoak dira; batik-bat, material lodiagoak baino azalera/gainazal espezifiko handiagoa izaten baitute (Evanko & Dzombak, 1997). Honela, buztinean aberatsak diren lurzoruak, kimikoki aktiboenak izaten dira (Cornellis et al., 2012; Garcia-Velasco, 2017), partikula finenek, bizitzarako

beharrezkoak diren elikagaiak/nutrienteak edo kutsaduraren parterik handiena hartzen baitute. Gainera, partikulen tamaina distribuzioak, lurzoru estruktura, kolorea, porositatea, KTG-a eta Uraren erretentzio kapazitatea (WHC-*Water Holding Capacity*- -ingelesez) baldintzatzen ditu.

Hezetasuna ere, kontuan hartzeko faktorea da, lurzoruen ahalmen kimikoan duen eraginagatik batik-bat. Izan ere, lurzoruaren ur kantitateak, disolbatutako mineral kantitatean, pH-an eta erredox potentzialean eragiten du; era ez-zuzenean, kutsatzaileen egonkortasuna edo erreaktibotasuna eraginez.

3.2- Erremediazio teknologia konbentzionalak

Mende hasieran, kutsatutako lurren arazoa konpontzeko, kutsatutako lurra induskatu eta landa batean edo isolamenduan jartzen zen, kutsadura lekutik ateratzea eragozten zuten itxitura batean; honela, inguruko lur eremuak kutsatzea eragotziz (Scullion, 2006). Praktika hau, arriskuaren maneiu ikuspuntutik ikusita egokia zen arren, ez zen kutsadura edo kutsaduraren iturria erasotzen. Lur kutsatua zabor gisa kudeatzea litzateke nolabait. Gaur egun aldiz, batez ere arautze aldaketek bultzatuta (gero eta zorrotzagoak direnak), lurzoruen erremediazioaren gaineko paradigma aldatzen ari da, teknologia berri eta eraginkorragoak garatuz. Teknika hauek kutsatutako lurzoruan bertan aplika daitezke (*in situ*) edo kutsatutako eremutik atera eta hauetatik urrun tratatu daitezke, ondoren, jatorrizko eremura bueltatzeko (*ex situ*). Lehenengoak, kutsatutako lurrean edo ingurukoetan ematen ari diren ekintzen mantentzea ahalbidetzen du; bigarrenak aldiz, baldintzen maneiua, kutsatzaileen barreiatzea eta efizientzien optimizazioa ahalbidetzen duen bitartean.

Deskontaminazio teknikak, tratamenduaren izaeraren araberako talde ezberdinetan sailka daitezke: fisikoak (termikoak barne), kimikoak eta biologikoak nagusiki (Sparks & Corn, 1993). Egoera batzuetan, tratamendu eraginkor eta merkeena lortzera aldera, beharrezkoa izaten da teknika hauen bi- edo multi- konbinazioak burutzea (Armishaw et al., 1992; Scullion, 2006; Castelo-Grande et al., 2010).

Tratamendu fisikoen artean, teknologia termikoa (beiratzea kasu) (Taube et al., 2008), solidifikazioa (Shen et al., 2019), lurrun erauzketa, espaziazio aereoa (*Air Sparging* ingelesez), garbiketa (Mulligan, 2001), elektroremediazioa (Page & Page, 2012)

edo partikulen klasifikazioa aurki daitezke (Scullion, 2006). Kasu gehienetan, tratamendu hauek, kutsatzaileak lurzoru-ur konplexutik mugitzen dituzte; ondoren, tratatuak edo “isuriak” izateko. Kasu askotan, tratamendu fisikoek, degradazio biologikoen eraginkortasuna emenda dezakete, edo, era ez zuzenean, kutsatzaileen suntsitzea eragin dezakete. Bestalde, kutsatzaileen deuseztatze edo eliminazio hau, lurzoruen izaerak baldintzatua egongo da zeharo (erremediazio fisikoa eraginkorragoa da lurzoru marduletan).

Tratamendu kimikoak lurrazpiko ur kutsatuak tratatzeko erabiltzen dira gehien bat; hala ere, lixibatuen garbiketan ere erabiltzen dira (Armishaw et al., 1992). Tratamendu kimikoen bitartez, kutsatzaileak deuseztea, toxikotasun baxuagoko formetara transformatzea, erauztea edo immobilizatzea bilatzen da. Teknika ezagunenen artean oxidazioa, erredukzioa, hidrolisia, solubilizazioa, deklorinazioa eta pH manipulazioa aurkitzen dira; hala ere, ikerkuntza oxidazio/erredukzioan eta kutsatzaileen erauzketan zentratu izan da batik bat (Mulligan et al., 2001; Scullion, 2006). Tratamendu mota hauek, oso espezifikoak izan daitezke PCB edo alkano halogenatuak bezalako kutsatzaileentzat (Wood, 2001). Gainera, matrize ezberdinetan aplika daitezke baldin eta nahastura eraginkor bat bermatzen bada. Aldiz, lurreratutako kimikoek lurzorua kutsatu dezakete (baldin eta guztiz erreakzionatzen ez badute); eta kasu askotan, teknika hauek ez dira gai lurzoruko konposatu organikoen eta kutsatzaileen artean desberdintzeko; ondorioz, lurzoruko konposatuak degradatu edo kutsatzaileak tratatu gabe utz ditzaketelarik.

Teknologia fisiko-kimiko hauek, kutsatzaile askoren kontzentrazio mailak jaisteko teknika apropos eta eraginkorrak direla frogatu izan dute (Kastanek et al., 2016; Cai et al., 2019) azken hamarkadetan. Dena den, badituzte ere zenbait eragozpen; berauen konplexutasuna, inplementazio kostu altuak, aplikagarritasun eskasa eskala txikian edota gizartearen onespen eza besteak beste (Niti et al., 2013; Aparicio, 2017). Gainera, ez dute lurzoruaren funtzionalitatea berreskuratzen; lurzoruaren funtzio ekologikoak galduz. Aitzitik, tratamendu biologikoen gaineko arreta zeharo emendatu da azken urteotan, kutsatutako lurzoruetan dauden konposatuen degradazio, iraultze eta transformaziorako tresna ez-erasokor gisa (Salinas et al., 2015; Aparicio et al., 2017).

3.3- Erremediazio biologikoa

Ohiko teknikekin alderatuz, tratamendu biologikoak, kutsatzaileen degradazio osoa (edo substantzia ez toxikoetara aldaketa) ahalbidetzen duten prozesu naturalak dira. Aplikazio errez, merke eta malgukoak dira ingurumen baldintza ezberdinetan aplika daitezkeenak. Erremediazio mota hau, organismo (edo organismo multzo) ezberdinekin burutu daitekeen teknologia da; hots, mikroorganismo, landare edo/eta animaliekin. Bereziki aipagarriak dira bakteria, landare eta lurzoru ornogabeekin burutzen diren erremediazioak (Scullion, 2006); hauen artean, laborantza, konpostaia, biopilak, bioerreaktoreak, biolixibiazioa, fitoestabilizazioa, fitoerauzketa, fitodegradazioa edo bermierremediazioa izanik azpimarragarrienak (Gómez-Sagasti et al., 2019).

3.3.1-Bioerremediazioa landareak erabiliz: fitoerremediazioa

Fitoerremediazioa, lurzoru kutsatuak tratatzeko landareak erabiltzen dituen biorremediazio metodologia da. Bioerremediazio teknika gisa gero eta onartuago dagoen teknika hau (Chekol & Vough, 2001), kutsatzaileak landa eremu zabalak hartzen dituenean edo sustrai sakoneran aurkitzen direnean bereziki egokia da (Garbisu & Alkorta., 2003). Fitoremediazioan, emendio bat ikusten da kutsatzaileen degradazioetan inguruko tratatu gabeko lurrekin alderatzen bada. Izan ere, teknologia honen bitartez, mikroorganismo dentsitate eta aktibitate altuagoak lortzen dira errizoesferan (Cunningham et al., 1996; Liste & Alexander, 1999; Adam & Duncan, 2002; Fan et al., 2008). Gainera, landareen transpirazioak, sustrai inguruetan disolbatuta dauden kutsatzaileen kanporatzea ahalbidetzen du (Ferro et al., 1994; Liste & Alexander, 1999). Beraz, kutsatzaileak zenbat eta disolbagarriagoak izan, orduan eta errazagoa izango da landareentzat hauek erauztea (Nyer & Gatliff, 1996). Mekanismo ezberdinen bitartez lor daiteke fitoerremediazioa; horrela, fitoestabilizazioa, fitobolatilizazioa/fitodegradazioa eta fitoerauzketa aurki daitezke (1. Irudia).

Fitoestabilizazioan landareak erabiltzen dira lurzoruko kutsatzaileak immobilizatzeko (metal astunak kasu), berauen erosio edo lixibiazio bidezko bioeskuragarritasuna jaitsiz. Teknika hau, fitoerauzketa burutzea desiragarri ez denean (Chaney et al., 1997) edo kutsadura mailak erauzketa bidez jaisteko altuegiak direnean

erabiltzen da gehien bat (Marques et al., 2009), denbora luzeegia har dezakeelako edo kutsadura maila altuek landareen hazkuntza kaltetu dezaketelako (Jadia & Fulekar, 2008). Fitoestabilizazioa, kutsatzaileen prezipitazio, konplexaketa, sortzio edo metal balentziaren erredukzio (metalen kasuan) bitartez lortzen da batik bat (Adriano et al., 2004). Orokorrean, teknika oso baliagarria da kutsatzaileen immobilizazio azkar bat behar denean; hala ere, gehienetan medeapenen erabilera behar izaten dute.. Erabili beharreko medeapenen menpekotasun honek, erauzten ez den kutsaduraren beharrezko monitorizazioarekin batera, teknika zaurgarri bilakatzen dute.

Fitobolatilizazio bitartez, lurzoruko kutsatzaileak lurretik ateratzea bilatzen da. Horretarako, kutsatzaileak forma hegazkorretara transformatzen dira, jarraian atmosferara transpiratuak izateko (EPA, 2000). Merkurioarekin (batez ere) eta selenioarekin kutsatutako lurzoruak erremediatzeko erabiltzen da batik-bat teknika hau; konposatuak forma toxikoetatik, forma elementaletara edo toxikotasun gutxiagoetara transformatuz. Edonola ere, teknika honen arazo nagusia sortutako produktu berriak sor ditzaketen konplikazioak lirateke; hots, airean lurrundutako metal elementalak laku eta erreketara prezipitazio bitartez berrisurtzean sortuko liratekeenak. Izan ere, teknologia hau oso aproposa da transformazio horretan desagertu edo deusezten diren kutsatzaile organiko edo ez iraunkorrekin erabiltzen baldin bada. Kutsatzaileak iraunkorrak badira aldiz, teknologia honen bidez kutsatzaileen barreiadura eta translokazioa besterik ez da lortzen; arazoa lekuz aldatuz.

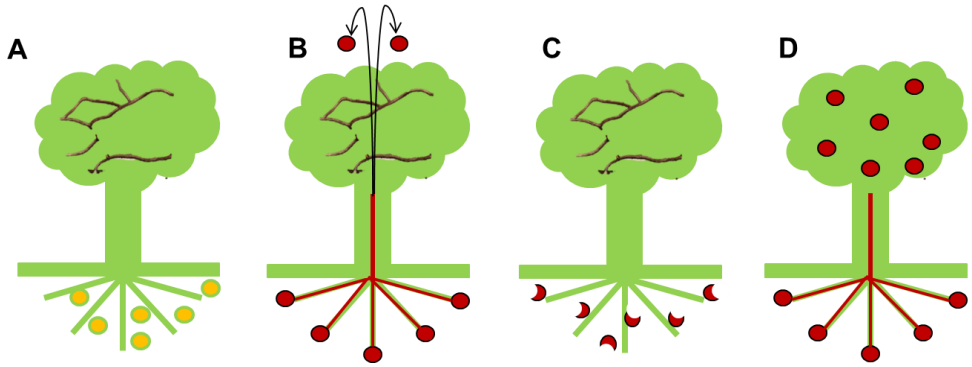
Fitoerauzketa ordea, fitoerremediazio formarik arruntena da. Honen bitartez, lurrean dauden kutsatzaileak, landareen hosto eta sustraietan metatzen dira (Chubuike et al., 2014). Modalitate honetan erabiltzen diren landareak, hazkuntza azkarrekoak, biomasa altukoak, sistema erradikular hedakorrekoak eta kutsatzaileak metatu zein toleratzeko jasateko gai izan ohi dira. Fitoerauzketaren arrakasta, kutsatzaile motaren, bioeskuragarritasunaren eta landareen egokitasunaren menpekoa izango da. Espezie hiperakumulatzaileek, landare arruntek baino 10-500 aldiz metal gehiago metatu dezakete (Chaney et al., 1997; Wenzel et al., 1999; Garbisu & Alkorta, 2003; Chubuike et al., 2014), fitoerremediaziorako ezpezie ezinhobeak izanik. Hipertoleranteak izan ohi diren espezie hauek (espezie arruntek baino kutsatzaile kontzentrazio altuagoak toleratzen dituzte), konpartimentu bakuolarretan edota zelula paretetan metatzen

dituzte kutsatzaileak; azken hauek, arnasketa edo banaketa zelularra bezalako ezinbesteko funtzioak burutzen diren konpartimentu zelularretatik urrun mantenduz (Salt et al., 1998; Garbisu & Alkorta, 2003). Hiperakumulatzaileekin burutzen den fitoerauzketaren limitazio handienetako bat, erauzitako metalak elikadura katean sartzea litzateke. Hala ere, hiperakumulatzailetzat katalogatuta dauden zenbait Brasikazea (*Brassicaceae* familiakoa) espeziek, tiozianatoak deritzen anioi kantitate handiak dituzte bere baitan (Navari-Izzo & Quartacci, 2001; Garbisu & Alkorta, 2003; Chubuike et al., 2014), animalientzat desatseginak eginez. Hiperakumulatzaileen beste eragozpen bat, hauek daukaten hazkuntza abiadura baxua eta biomasa txikia izango lirateke (Van Ginneken et al., 2007). Horri aurre egiteko xedez, alfalfa (*Mendicago sativa*), artoa (*Zea mays*) edo brasikazeak (*Brassica napus, B.juncea* eta *B. rapa*) bezalako hazkuntza eta biomasa handiko espezieak erabiltzen dira (Ebbs & Kochian, 1997).

Fabaceae familiako *Medicago* generoa, fitoerremediazioan gehien erabiltzen den generoetako bat da (Panchenko et al., 2017). 87 espeziek osatzen duten arren, gutxi batzuk izan dira fitoerremediazioan erabiltzeko testatuak. Espezie ezagunenen artean, alfalfa espeziea aurkitzen da; zeina, animalien bazkarako labore garrantzitsua den (Sengupta-Gopalan et al. 2007). Bazkalekuak edo larreek baino hobeto tolera ditzakete lehorteak, aldiz, ez daude lurzoru azidiko edo eskaski drainatuetara egokituak (Barnes & Shaeffer, 1995; Chekol & Vough, 2001). Kutsatzaileekiko erresistentea den eta bioakumulatzaile egokia den espezie hau (Carrillo & Cajuste, 1992; Wang et al., 2015) metal astunen eliminazio eta erremediaziorako biomaterial egokia dela frogatu da (Jadia & Fukerlar, 2008). Bestalde, zabalki jakinak dira petrolio hidrokarburoekin kutsatutako lurren erremediazioan alfalfak lor ditzakeen errendimenduak; hala nola, PAHen deuseztean (Reilley et al., 1996; Nichols et al. 1997; Criquet et al. 2000; Kirk et al. 2005; Schwab et al. 2006; Phillips et al. 2006, 2009; Fan et al., 2008; Panchenko et al., 2017), %64 (medagarririk gabeko lurzoruan) eta %54-eko (medagarridun lurzoruan) benzo(a)pireno jaitsierak eraginez (Hamdi et al., 2012).

Kasu gehienetan, landareek lurzoruan arinki eskuragarri dauden metalak absorbitzen dituzte. Beste batzuetan aldiz, metalak prezipitatu disolbaezin bezala egoten dira, landareentzat eskuraezin egonik. Kasu horietan, substantzia kelanteen

adizioak (bereziki EDTA eta/edo EDDS) metalen prezipitazioa saihesten eta metal kelatu konplexuen bidezko absortzioa hobetzen du; ondorioz, bioeskuragarritasuna emendatuz (Marques et al., 2009).



1 Irudia: Fitoerremediazio mekanismo ezberdinak;kutsatzaileen egonkortzea dakarren fitoestabilizazioa (egonkortutako kutsatzaileak horiz irudikatuta) (A), kutsatzaileen hegazkortzea dakarren fitobolatilizazioa (kutsatzaileen lurretik airerako fluxua irudikatuta) (B), kutsatzaileen deuseste totala edo partziala (ilargi itxurako metaketak) eragiten duen fitodegradazioa (C) eta fitoerauzketa (kutsatzaileen metatzea biomasan irudikatua) (D). Borobilak: kutsatzaileak.

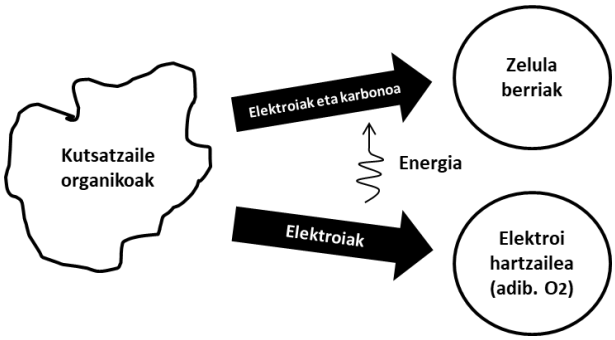
Erremediazio teknika hauek, kutsadura arriskuaren kontrol eta murrizketarako erabiltzen diren heinean merkatu balioa duten biomasa produzitzeko erabiltzen badira, fitomaneiu estrategiak aplikatzen direla kontsideratzen da (Robinson et al., 2009; Evangelou et al., 2015). Fitomaneiuaren fokatze honek, kutsatutako lurzoruak baliabide erabilgarri gisa ulertzen ditu; era jasangarrian ustia daitezkeenak. Munduan zeharreko lurzoru kutsatuak bioenergia, egurra, pulpa, bio-ikatza edo zuhainaren produkziorako erabil daitezke (Banuelos & Dhillon, 2011); kasu batzuetan, produktuari balioa gehituz, zink-ak biofortifikatutako materialen kasu. Fokatze honek, zoru emankorren gaineko presioa leuntzea eta kutsatutako lurren hobetzea (epe luzera) suposatzen du; era berean, sortutako irabazi ekonomikoek proiektuaren kostua (ko)finantziatu dezaketelarik (Robinson et al., 2009).

3.3.2- Bioerremediazioa mikroorganismoak erabiliz

Bakterioekin burutzen diren bioerremediazio estrategiak, indargabetze naturala, bioestimulazioa edo bioaumentazioa kasu (Mueller et al., 1996), *in situ* edo *ex situ* burutu daitezke. *Ex situ* tratamenduak, gehien erabiltzen direnak diren arren (Chubuike

et al., 2014), lurzorua induskatzea eta teknologiak aplikatuko direneko lan tokira garraiatzea suposatzen dute; *in situ* lanak, aplikagarriagoak eta erosoagoak diren bitartean (Aparicio, 2017).

Estatu Batuetako EPA-ren (1999) (*Environmental Protection Agency* ingelesez) arabera, indargabetze naturala prozesu naturalen bitarteko kutsadura isurien euste eta jaitsiera da. Prozesu natural horietan, degradazio biologikoa, lurrunketa, dispertsioa, diluzioa, desintegrazio erradioaktiboa eta MO zein kutsatzaile mineralen sortzioa barne hartzen dira (Mulligan & Yong, 2004). Beraz, indargabetze naturala, lekuko lurrazpi-geologiak, hidrologiak eta mikrobiologiak baldintzaturiko prozesua izango da. Gehien bat bentzenoak, toluenoak, etil bentzenoak, xilenoak eta hidrokarburoak degradatzeko erabiltzen den arren, konposatu inorganikoak eta pestizidak degradatzeko potentziala duen teknika da (Mulligan & Yong, 2004) (2. Irudia). Aitzitik, teknologia hau oso motela izatera hel daiteke; batez ere, biodegradazioa ingurumen faktore bat edo gehiagoz (hotza, lehorteak etb.) baldintzatuta dagoenean.



2 irudia: Kutsatzaile organikoek mikroorganismoen arteko elkarrekintza. Yeung et al., 2010 etik eraldatutako irudia. Kutsatzaile organikoek mikroorganismoak bi eratan hornitzen dituzte: (1) zelula berrien eraketarako karbono iturri gisa eta (2) mikroorganismoak energia lortzeko elektroiak emanez; izan ere, mikroorganismoek erreakzio kimikoak katalizatuz energia lortu ahal izango dute; lotura kimikoak apurtuz adibidez

Aktibitate mikrobiano hori mugatzen duten baldintzen manipulazioa egiten denean, konposatu kutsatzaileen metabolizazioa bultzatu nahian, **bioestimulazioaz** ari gara (Chubuike et al., 2014; Aparicio, 2017). Prozezu horrek, mikroorganismoek

karbono-iturri gisa erabiliko duten nutrienteen gehitzea barne hartzen du; gorotzen, medeagarri organikoen (Chubuike et al., 2014), egur txirbilen edo NPK (nitrogeno, fosforo, potasio) nutrienteen gehikuntza bitartez (Aparicio, 2017) lor daitekeena. Gehitutako nutrienteak erremediazio prozesuan gakoak diren mikroorganismoen hazkuntza eta aktibitateak emendatzen dituzte; ondorioz, eraginkortasuna handituz. Bioestimulazioa, batik bat PAHak bezalako kutsatzaile organikoen degradaziorako erabiltzen den arren (Lee et al., 2003; Abioye, 2011), metal astunen erremediaziorako ere erabili daiteke (Chubuike et al., 2014); adibidez, metalen bioeskuragarritasunean eragingo duen lurzoruaren pH-a eraldatuz.

Orain arte aipatutako teknikak lurzoruen erremediazioan oso erabiliak diren arren bioaumentazioa da bioerremediazio mikrobiano tresnen artean hedatuena. Bioaumentazioa, lurzoru kutsatzaileen degradazioa emendatuko duten ahalmen katalitikoak dituzten bakteria andui edo bakteria kontsortzioen inokulazioan datzan teknika da (Mrozik & Piotrowska-Seget, 2010; Lebeau, 2011; Agnello et al., 2016; Cycon et al., 2017). Autore askok frogatu izan dute bioaumentazioaren baliogarritasuna konposatu organiko ezberdinekin kutsatutako lurren bioerremediazioan (Abioye, 2011); kutsatzaileen degradazioa bultzatuz, kutsatzaileak mineralizatuz edo kaltegarriak ez diren konposatuetan transformatuz (Aparicio, 2017). Gainera, zabalki frogatu izan da mikroorganismoek burutu ditzaketen biometaketa, bioeraldaketa, biomineralizazio, kimiosortzio, biosortzio edo biometilazio prozesuek metal astunen bioeskuragarritasuna zein toxikotasuna mugatu dezaketela (Chen et al., 2017). Adibidez, Garbisu et al. (1995)-ek, *Bacillus subtilis* baziloa selenita toxikotasun baxuagoko Se elementalera transformatzeko gai zela ikusi zuen. Teknika honetan erabiliko diren mikoorganismoak kutsatzaileak erauzteko edo deusezteko kapazak izateaz gain, ingurumenera egokitzeko eta bertan bizi diren mikrobiota autoktonoarekin karbono-iturri eskasengatik lehiatzeko kapazak izan behar dute (Burmølle et al., 2006). Honen arrazoi, eta askotan organismo aloktonoak lurzoru komunitatean parte izatea lortzen ez dutela jakinda (Aparicio, 2017), gomendagarria izaten da mikroorganismo autoktonoak erabiltzea bioerremediazio tekniketan (Kieser et al., 2000).

Burkholderia xenovorans LB400 anduia (lehenago *Pseudomonas* sp. LB400, *Burkholderia* sp. LB400, *Burkholderia fungorum* LB400 deitua) graminis kladoaren parte

da; larre landareetako errizosferan sarritan aurkitu daitekeena (Salles et al., 2005). Nitrogeno fixatzailea den espezie hau (Caballero-Mellado et al., 2007) PCB-ekin New York-en kutsatutako lurzoru batetatik isolatu zen (Chain et al., 2006); gaur egun, ezagutzen den PCB degradatzailerik garratzitsuenetako bat izanik (20 PCB kongeneretik gora oxidatzeko gai dena) (Seeger et al., 1995, 1999; Maltseva et al., 1999). Gainera, LB400 anduiak konposatu aromatikoen degradaziorako moldakortasun kataboliko handia erakutsi izan du (Chain et al., 2006; Urtuvia et al., 2018), eta uzten hazkuntza ere bultzatu dezake (Caballero-Mellado et al., 2007). Izan ere, landareen hazkuntza bultzatzen duen nitrogenoa fixatzeaz gain, 1-amino-ziklopropano-1-karboxilato (ACC) deaminasa entzima bakterianoak ACC-a (etilenoaren aintzindaria) hidrolizatu eta etileno-mailak jaisten ditu, sustraien hazkuntza bultzatuz (Zhang et al.,2003; Caballero-Mellado et al., 2007) .

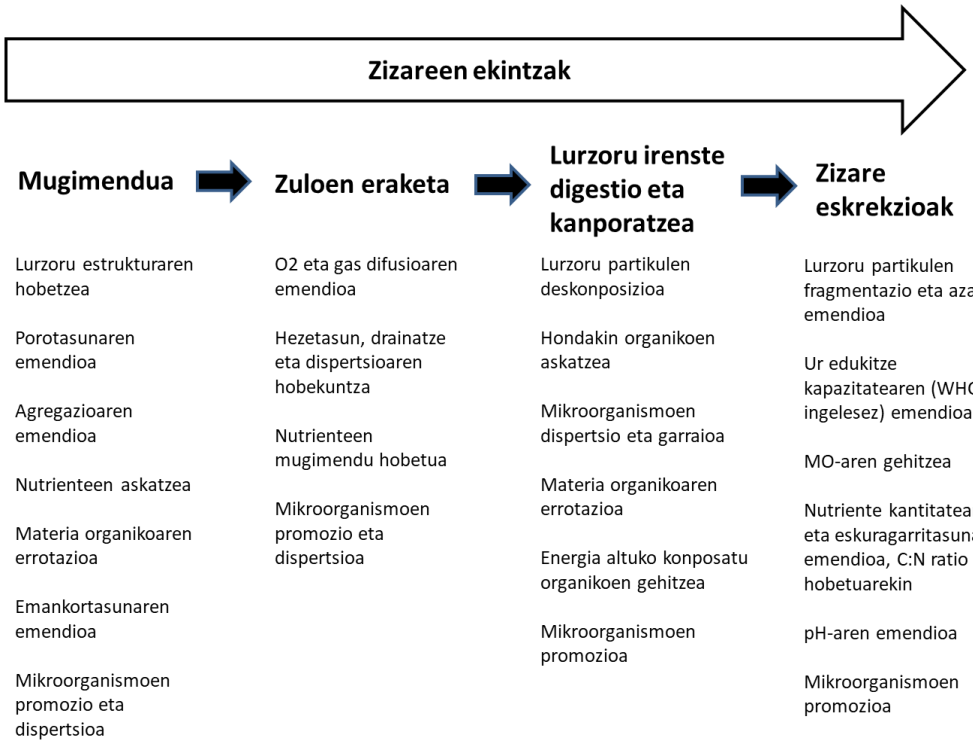
3.3.3- Bioerremediazioa zizareak erabiliz: bermierremediazioa

Bermierremediazioan, zizareak erabiltzen dira beraien aktibitatearen bitartez, MO bermi-konpost bilakatzeko (Sinha et al., 2008; Mohee et al., 2014; Kavehei et al., 2017). Beste erremediazio teknika batzuekin konbinazioan garatu daitekeen *in situ* teknika merkea da (Sinha et al., 2010; Kavehei et al., 2017). Bioerremediazio prozesuak irauten duten bitartean beharrezkoa izaten da lurzoruen hezetasun, oxigeno eta nutrienteen mailak homogeneoki mantentzea, batez ere, erremediatu beharreko lurrak sakonak, trinkoak edo buztinean aberatsak direnean (Hickman et al., 2008). Zizareak baldintza horiek lurzoruan mantentzen laguntzen dute; horretarako, ur, partikula, nutriente eta aireazio mugimenduak erraztuko dituzten tunelak eraikiz (Kretzshmar, 2004; Dominguez, 2004; Hickman et al., 2008). Horrela, lurzoruen porotasuna (Shipitalo & LeBayon, 2004; Hickmann et al., 2008), oxigenazioa, hezetasunaren erretentzioa/eustea/metaketa, lurzoruaren emankortasuna, eta nutrienteen eskuragarritasuna emendatzen dira (Edwards & Bohlen, 1996)(3. Irudia). Prozesu horiekin batera, lurzoru partikulen birrintze mekaniko bat gertatzen da gainera, partikula organiko eta lurzoru materialak txikituz (Kretzshmar, 2004). Horrela, partikula hauen gainazal azalera handitu egiten da (Edwards & Bohlen, 1996) material-mikrorganismo interakzioa bultzatuz (Dominguez, 2004).

Zizareen presentziak lurzoruetako faktore abiotikoak eragiteaz gain, baldintza biotikoak ere eragiten ditu; adibidez, mikroorganismoen aktibitatea emendatuz. Bestetik, zizareek barrunbe paretetan utzitako muki eta gorotzek, bertako karbono organikoarekin batera, mikroorganismoen hazkuntza eta barreiapena sustatzen dute (Farenhorst et al., 2001; Hickman et al., 2008); mukiek, gernuak eta glukosak mikrobio biomasa (eta aktibitate katabolikoa) emendatzen duten heinean. Lurzoru populazio mikrobianoen dibertsitate, kantitate eta aktibitateak, zizareen liseri-traktuko mikroorganismoen igarotze, promozio eta berpiztearekin erlazionatu izan dira (Brown & Doube, 2004; Hickman et al., 2008); hala ere, promozio hau, liseriketa denborak baldintzatua egongo da zeharo (Brown & Doube, 2004). Lurzoruan zeharreko mikroorganismoen barreiatzea ordea, zizareen azalarekiko adhesio bitartez (Edwards & Bohlen, 1996) eta barrunbeen bitarteko ur-fluxuen bitartez (Kretzshmar et al., 2004) gertatzen da.

Bermierremediazioa zabalki ikertua izan da kutsatzaile ezberdinen deuseztean, batez ere, kutsatzaile organikoen aurrean erakutsi izan duen eraginkortasun altuagatik. Horrela, intsektizida (Verma et al., 2006), herbizida (Binet et al., 2006), PAH (Ma et al., 1995; Eijsackers et al.,2001; Kersante et al., 2006; Contreras-Ramos et al., 2008; Tejada eta Masciandaro, 2011; Rodriguez-Campos; 2015; Rorat et al., 2017), PCB (Singer et al., 2001; Luepromchai et al., 2002) zein metal astunen (Goswami et al., 2014; Suthar et al., 2014; Sahariah et al., 2015; Rorat et al., 2017) biorremediazioan zizareak erabiltzen dituzten hainbat lan aurkitu daitezke.

Espeziaren aukeraketa, erremediatu beharreko lurzoru motaren, kutsatzaile motaren, zein elikagai eskuragarritasunaren araberakoa izan behar da (Edwards & Bohlen, 1996). Hala ere, erabilitako espezieen artean, *Eisenia fetida* espeziea da askogatik erabiliena (Hickman et al., 2008). Izan ere, maneiatzeko errazak diren zizare hauek, dominante bilakatzen dira beste zizare espezie batzuekin elkartutako sistemetan (Dominguez et al., 2005).

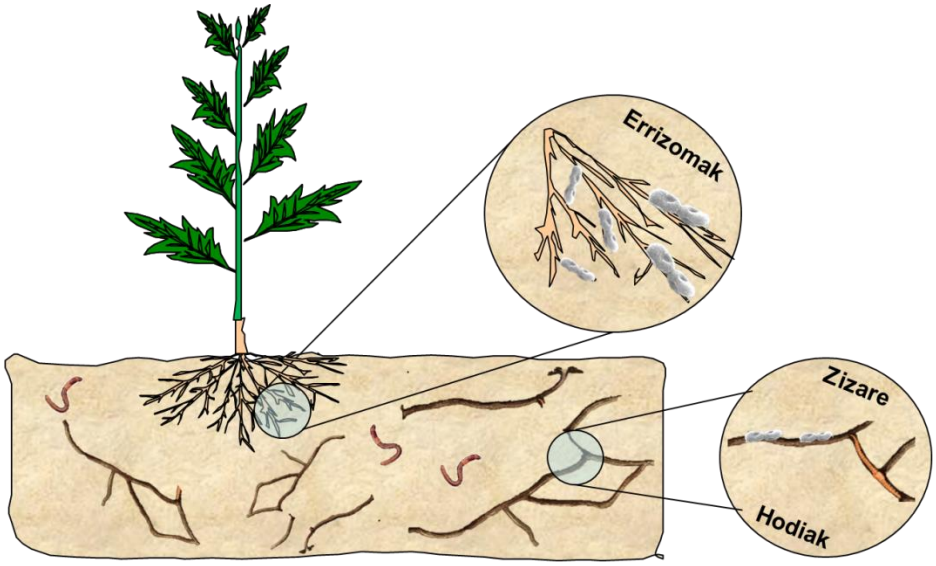


3 Irudia. Zizareek lurzoruan eragindako efektu biologiko, kimiko eta fisikoak. Hickman eta Reid, (2008)-en oinarrituta.

3.3.4- Bioerremediazio tekniken konbinazioaren bilaketa

Bioerremediazio teknologien erabilpen eta ikerkuntza zeharo hazten dabil. Hala ere, hauek, bakarkako konposatuen bioerremediazioa hobetzera bideratuta daude batik-bat (Nouha et al., 2016), kutsatzaile nahasketa duten lurzoruen gaineko ikerketak urriak izanik. Honen arrazoia, kutsadura anitzeko lurzoruen konplexutasunean eta bioerremediazio tratamenduen kutsatzaileenganako aldagarritasunean dago (Dong et al., 2013). Bioerremediazio prozesu orotan, elkarrekintza konplexuan dabiltzan faktore fisiko-kimiko eta biologikoek, lurrean dauden kutsatzaileekin interakzionatu dezakete hauen eskuragarritasuna eta mugikortasuna aldatuz. Era berean, biorerremediazio mota ezberdinetan diharduten organismoek hazkuntza eta metabolismoan aldaketak paira ditzakete; prozesuaren errendimendua erasanduz. Adibidez, konposatu inorganiko askok konposatu organikoak degradatzen dituzten mikroorganismoen aktibitatea inhibitu dezakete (Biswas et al., 2015). Bestalde, sor daitezkeen interakzioen artean, badira errendimendua bultzatu ditzaketen interakzioak ere. Mikroorganismo eta

landareen arteko erabilera konbinatua (4. Irudia), kutsatutako lurzoruak erremediatzeko teknika arinago eta efizienteagoa dela ikusi izan da (Joner & Leyval, 2001; Jamal et al., 2002; Marques et al., 2006; Weyens et al., 2009). Onura hauek, mikorriza elkarrekintzei esker gertatzen dira batik bat; kutsatutako lurretan landareen hazkuntza eta bizirautea ahalbidetzen dituztenak. Hala ere, badira errizosferan aurki daitezkeen, eta erremediazio prozesuetan oso erabiliak diren errizobakteriak ere (4. Irudia). Hauek, hainbat mekanismo erabil ditzakete landareen hazkuntza bultzatzeko: fitohormonak produzitu eta elikagaiak gehi ditzakete (Glick et al., 1995); sideroforoak edo beste agente kelante batzuk ekoiztu ditzakete (Kamnev & Van der Lelie, 2000); entzima espezifikoak ekoizi edo N-a fixatu dezakete (Khan, 2005); edo sustrai hazkuntza bultza dezakete etileno produkzioa jaitsiz (Glick et al., 1998; Chubuike et al., 2014).



4 irudia: Lurzoru kutsatuak erremediatzeko bermi-, mikro- eta fitoerremediazio tekniken elkarrekintza. Zizare eta landare sustraien arteko errizomak, zizare hodiak eta zizare hodietako bakterio komunitateak irudikatzen dira

Bestalde, zizare eta bakterioen arteko erlazioa interdependentea dela ikusi izan da; berauen arteko interakzioek populazio tamainak edo isuritako entzimak kontrola ditzaketelarik (Drake & Horn, 2007; Rodriguez-Campos et al., 2015) (4.Irudia). Harira, Hong et al (2011)-ek *E. fetida* zizareen liseri traktuetako komunitate bakterianoak

identifikatu eta sailkatu zituen; aerobioen artean *Aeromonas* (40%), *Bacillus* (37%), *Photobacterium* (10%), *Pseudomonas* (7%), eta *Shewanella* (6%) generoko bakterioak, eta anaerobioen artean *Aeromonas* (52%), *Bacillus* (27%), *Shewanella* (12%), *Paenibacillus* (5%), *Clostridium* (2%), eta *Cellulosimicrobium* (2%) generoko bakterioak aurkituz. Genero hauetako batzuk, *Bacillus* (Tiquia et al., 2010)*, Clostridium* (Tiquia et al., 2010)*, Pseudomonas* (Wu et al., 2011)*, Streptomyces* (Yee & Wood, 1997) *eta Shewanella* (Khalid et al., 2008) kasu, gai dira hainbat konposatu organiko degradatzeko (18 konposatutik gora: ziklohexanoak, bentzenoak, etilbentzenoak, kloroformoak, metanolak eta azetonak; Tiquia et al., 2010), eta metalen erredukzioan laguntzeko (Khalid et al., 2008; Wu et al., 2011).

Ohiko esperimentuetan, bioerremediazio errendimenduan eragina izan dezaketen faktoreak banan-banan ikertu izan dira, bakoitzaren efektua zenbatetsi nahian. Honek, denbora galera garrantzitsu bat suposatzeaz aparte, informazio asko galtzea eragiten du; aldagaien arteko interakzio ebentualei dagokiona (Urionabarrenetxea et al., 2021). Horretarako, ezinbestekoa da errealitatera gehien hurbiltzen diren eta berezko mugak saihesten dituzten teknikak edo konbinazioak erabiltzea. Era honetan bakarrik agerraraziko baitira, teknika bakoitzaren mugak eta teknika ezberdinen arteko sinergia, adizio edo kontrajartasunak: tekniken arteko erlazioak ulertzea erraztuz.

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HYPOTHESIS AND OBJECTIVES/ HIPOTESIA ETA HELBURUAK

HYPOTHESIS

Accurate soil health protection goals and recovery strategies can be achieved with the aid of *in vivo* and *in silico* approaches focused on assessing the biological effects of multiple stressors on soil organisms, the risk of pollutants accounting for landscape variability and the application of bioremediation technologies.

OBJECTIVES

In order to proof empirically this hypothesis, the following objectives were set:

1- To assess the joint impact of thermal and chemical stress on soils at different exposure times and levels of biological complexity in *Eisenia fetida* earthworms, using standard toxicity tests (OECD 207 and OECD 222) and biomarkers (viability of coelomocytes).

2- To integrate spatially explicit exposure and effect data of four Plant Protection Products (PPPs) active substances (esfenvalerate, cyclaniliprole, picoxystrobin and fenamidone) on non-target species (*E. fetida* and *Folsomia sp*.) accounting for European landscape and agricultural variability.

3- To predict the risk upon Ecosystem Services along European soils after four PPP (esfenvalerate, cyclaniliprole, picoxystrobin and fenamidone) worst case application into crops, considering impacts upon *E. fetida* and *Folsomia sp.,* pesticides mode of action, exposure, time-course effects and landscape variability.

4- To assess the toxicity of a natural soil after sewage sludges disposal to address potential future uses of Landfill 17 (Gernika-Lumo), applying standardized tests and biomarkers at different complexity levels (from cellular level to population) in different organisms (*E. fetida* and *Lactuca sativa*).

5- To apply different bioremediation technologies (phyto-, vermi- and microremediation) in soils polluted with sewage sludges and to find the treatment or the combination rendering the best remediation yield with the aid of chemical and ecotoxicological characterizations.

HIPOTESIA

Estres faktore anitzek lurzoru organismoetan eragiten dituzten efektu biologikoen azterketan, paisaia aldagarritasuna kontuan daukan kutsatzaileen arrisku ebaluazioan eta bioerremediazio teknologien aplikazioan oinarritutako *in vivo* eta *in siliko* fokatzeek ingurumen babeserako neurrien aukeraketa egokian eta kutsatutako lurzoruen berreskuratzean lagun dezakete.

HELBURUAK

Hipotesia enpirikoki frogatze aldera, ondorengo helburuak ezarri ziren:

1- Estres termikoaren eta kimikoaren inpaktu bateratua lurzoruan ikertzea denbora eta konplexutasun biologiko ezberdinetan *Eisenia fetida* zizareetan, test estandarizatuak (OECD-222 and OECD-207) eta biomarkatzaileak (zelomozitoen bideragarritasuna) erabiliz.

2- Landare-osasunerako Produktuen (PPP) lau substantzia aktiboren (esfenvalerate, cyclaniliprole, picoxystrobin eta fenamidone) esposizio datuak eta itu ez diren lurzoru espezieen (*E. fetida* and *Folsomia sp*.) gaineko efektuak integratzea, Europako paisaia eta nekazal aldagarritasunak kontuan izanik.

3- Lau pestiziden (esfenvalerate, cyclaniliprole, fenamidone eta picoxystrobin) kasurik txarreneko aplikazioek Europako lurzoruen Zerbitzu Ekosistemikoetan duten inpaktua aurreikustea, *E. fetida* and *Folsomia sp.* espezieen gaineko kalteak, pestiziden akzio mekanismoa, esposizioa, denboran zeharreko efektuak eta paisaia aldagarritasuna kontuan izanik.

4- Araztegi lokatz isurketek lurzoru naturaletan eragindako efektu toxikologikoen ebaluazioa 17 Zabortegiari (Gernika-Lumo) beste erabilera bat emate aldera, horretarako test estandarizatu eta biomarkatzaileak konplexutasun maila biologiko (zelula mailatik-populazio mailara) eta organismo desberdinetan (*E. fetida* and *Lactuca sativa*).

5- Araztegi lokatzekin kutsatutako lurzoruetan bioerremediazio teknologia ezberdinak (fito-, bermi- eta mikroerremediazioa) aplikatzea eta eraginkortasun altueneko teknologia edo teknologien konbinazioa bilatzea karakterizazio kimiko eta ekotoxikologikoetan oinarrituz.

RESULTS AND DISCUSSION

CHAPTER 3

Effects of elevated temperatures and cadmium exposure on stress biomarkers at different biological complexity levels in *Eisenia fetida* earthworms

This chapter has been published in:

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Urionabarrenetxea E.; Garcia-Velasco N.; Soto M. The influence of temperature upon cadmium induced toxicity at different levels of biological complexity in *Eisenia fetida* earthworms. 28 th Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC). Europe, Rome, 13–17 May 2018. Poster presentation

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ABSTRACT

Several ecotoxicological studies assessed metal toxicity upon soil biota and other communities but were mainly focused on the study of a single chemical and usually under optimal conditions of temperature. Meanwhile an increasing global warming is leading to new scenarios by combining different stress factors; chemical stress and thermal stress. Presently, this study aims to assess the joint effects produced by cadmium and elevated temperatures on earthworms different levels of biological complexity. *Eisenia fetida* earthworms were maintained at 19°C and 26°C and simultaneously exposed to four Cd concentrations (1.25, 2.5, 25 and 125 mg Cd/Kg soil) for 14 (Short term exposure) and 56 days (Reproduction test). Endpoints were addressed at different levels of biological complexity: reproductive impairment (cocoons and juvenile productions), Cd tissue accumulation, mortality of adults, weight loss and cytotoxic effects (coelomocyte viability). In the Short term exposure, increase in temperature produced a larger accumulation of Cd. Hence, earthworms exposed to 125 mg Cd/kg soil under heat stress (26°C) showed a two fold higher Cd accumulation comparing to those at 19°C. Earthworms exposed to moderate-high concentrations of Cd (2.5-125 mg Cd/kg) and maintained at 26°C showed severe weight loss and high mortality rates. The neutral red uptake capacity of coelomocytes extruded from earthworms exposed to the highest Cd concentration decreased after 14 d at 19°C, and more markedly at 26 °C. The reproduction impairment (decreased number of cocoons) was enhanced after exposure to concentrations higher than 2.5 mg Cd/kg at 26°C, and after exposure to 125 mg Cd/kg at 19°C. Earthworm reproduction capability is highly vulnerable to the effect of toxicants at elevated temperatures and sublethal concentrations.

Keywords: Temperature, metals, cytotoxicity, earthworms, reproductive impairment

LABURPENA

Metalen toxizitateak biotarengan eragiten dituen inpaktuak aztertzen dituzten ikerketa ekotoxikologikoak anitzak diren arren, gehienak kutsatzaile bakarren eragina ikertzen dute; orokorrean, tenperatura baldintza optimotan. Bitartean, beroketa globalak, estres faktore ezberdinak (kimiko eta fisikoak) konbinatzen dituen eszenario berrien emendioa eragin du. Harira, ikerketa honen bitartez kadmioak (Cd) eta tenperatura altuek zizareengan eragiten dituzten efektuak ikertzea bilatzen da. *Eisenia fetida* zizareak 19 eta 26°Ctan Cd kontzentrazio ezberdinenpean (1.25, 2.5, 25 and 125 mg Cd/Kg) mantendu ziren (14 eta 56 egunez; Epe laburreko esposizioa eta Ugalketa testa). Neurketak konplexutasun biologiko maila ezberdinetan burutu ziren: kalteak ugalkortasunean (cocoon eta jubenilen produkzioa), Cd metaketak ehunetan, organismo helduen heriotza, helduen pisu galera eta efektu zitotoxikoak (zelomozitoen bideragarritasuna). Epe laburreko esposizioan, tenperatura altuagoek Cd metaketeten emendioa eragin zuten. Izan ere, 125 mg Cd/kg lurzoru kontzentraziopean eta estres termikopean (26°C) izandako zizareek, kontzentrazio berdinpean 19°Ctara izandako zizareek baino bi bider Cd gehiago metatu zuten ehunetan. 26°tara Cd kontzentrazio ertain-altuenpean (2.5-125 mg Cd/kg lurzoru) izandako zizareek, pisu galera nabarmenak eta heriotza tasa altuak pairatu zituzten. 14 egunetan 19°C-pean Cd kontzentrazio altuenetan izandako zizareetatik ateratako zelomozitoek, gorri neutroa metatzeko ahalmenean beherakada nabaria erakutsi zuten; 26°Cpean fenomeno hau nabarmenagoa izanik. Ugalketa kalteak (beherakada cocoonen produkzioan) 2.5 mg Cd/kg lurzoru kontzentraziotik aurrera antzeman ziren 26°Cpean; 19°Cpean aldiz beranduago: 125 mg Cd/kg lurzoru kontzentraziotik aurrera. Zizareen ugalketa ahalmena toxikoekiko oso sentikorra suertatu da tenperatura altu eta kontzentrazio subletaletan.

Hitz gakoak: Temperature, metals, cytotoxicity, earthworms, reproductive impairment

1.- INTRODUCTION

There is a consensus on that the increase in temperature coming from the climate change is a global threat and a challenge for the 21st century. During last decades, greenhouse gas emissions have increased notably due to global industrialization, and this trend is predicted to continue rising accelerating climate change (Gonzalez-Alcaraz et al., 2016). Thus, air temperature is predicted to increase from 1.1°C to 6.4°C by the year 2100 (Nakicenovic et al., 2000) and as a consequence, shifts in the geographical distribution of species, phenotypical and phenological alterations, extinctions and, overall, deterioration of the ecosystems health are expected (Walther et al., 2002; Mugica et al., 2015). More precisely, the envisaged global change scenarios (increases in atmospheric CO_2, temperature, extreme events occurrence, ultraviolet radiation and precipitation) are likely to have significant consequences for the structure and function of soil ecosystems (Hochachka and Somero, 2002; Stacey and Fellowes, 2002). According to the IPCC, the most feasible effects to occur in soils are those related to temperature raises, changes in moisture content, acidification and hypoxia phenomena (AR5 Synthesis Report: Climate Change 2014 — IPCC).

Together with the climate crisis, the anthropic activity enhancement because of the population growth (according to United Nations global population has increased from 5.7 billion in 1994 to 7.6 billion in 2016) will carry with it the release of chemical compounds, which may pose a risk for human or the ecosystems health. Among the wide range of pollutants released, metals can be found at elevated concentrations in many parts of the world and due to their non-biodegradability and high toxicity (for instance As, Cd, Cu, Cr, Pb) being considered one of the most resistant pollutants affecting the environment (Spurgeon et al. 2005; Horvat et al., 2007; Chul Kong, 2013). Recently, large numbers of studies carried out on metal contaminated soils in Europe and other regions provided a wider global perspective on sources, dynamics and effects of metals in edaphic environments (Alloway, 2013). Nevertheless, these studies did not consider the co-occurrence of different stress sources in soils such as chemicals and increased temperatures related with global warming, which leads to new multi-stressor scenarios with unpredictable effects on soil health.

Soil organisms like decomposer earthworms are abundant, ubiquitous and important for soil functioning (Spurgeon et al., 2004). Due to their susceptibility to chemicals and other unique biological advantages (short life cycle, direct uptake of chemicals by their tegument and exposure via ingestion of soil, among others) much research has been done with earthworms as bioindicators of contamination and toxicity in soil (Paoletti, 1999; Schaefer, 2004; Spurgeon et al., 1994, 2004; Nahmani et al., 2007). Among epigeic species, *Eisenia fetida* is a model terrestrial organism broadly used in standard toxicity tests (OECD, ISO), due to its sensitivity to different toxicants providing early information on soil affection (Ernst et al, 2008; Asensio et al., 2009; Irizar et al. 2014a; García-Velasco et al., 2016, 2017). For instance, OECD developed the Acute Toxicity Test (OECD-207) and the Reproduction Test (OECD-222) in *Eisenia* species to evaluate biological effects generated by pollution. Apart from assessing effects on survival, body weight, reproduction, and accumulation and tissue distribution of metals (Huggett et al. 1992; Svendsen et al. 1996; 2007; Schlenk 1999; Van Gestel et al. 2009), measuring changes in the immune activity of earthworm coelomocytes can also be sensitive indicators (Asensio et al. 2007, Irizar et al. 2014a and 2014b). Recently, parameters such as the total number and viability of coelomocytes (membrane integrity by Neutral Red Uptake and Retention assays) have been used as cellular biomarkers to assess the impact of metals on annelids (Irizar et al. 2014a and 2015).

Most of the ecotoxicological studies regarding metal exposures and (sometimes) their posed effects are mainly focused on the analysis of single stressors and usually under optimal exposure conditions, obviating that environmental factors such as temperature, are in continuous change, and increasing in the era of global warming (Holmstrup et al., 2010). Elevated temperature in extreme scenarios can influence metal bioavailability. Therefore, the aim of this work is to study whether elevated temperatures can enhance the toxic effect of cadmium at different levels of biological complexity in *Eisenia fetida*. With that purpose, adult earthworms were maintained in LUFA 2.3 soils spiked with $CdCl_2$ (0-125 mg/kg) under optimal temperature (19°C) and thermal stress conditions (26°C). Standard toxicity tests (Short term exposure based on OECD 207- and Reproduction Soil Test -OECD 222-) were carried out, soil and tissue Cd concentrations were calculated, and effects on weight loss, mortality of adults, reproduction and coelomocyte viability were recorded.

2.- MATERIAL AND METHODS

2.1- Earthworms: *Eisenia fetida*

E. fetida earthworms were purchased from a commercial dealer (Lombricor SCA, Algallarín, Córdoba, Spain) and set as laboratory culture maintained in containers at 19 ± 2°C, in darkness and constant humidity (60%). As food source medication-free horse manure was provided when required. The earthworms used for the experiments were all healthy adults, clitellated and of similar size (300–600 mg individual weight).

2.2- Soil spiking procedure with cadmium and thermal stress conditions

Dry LUFA 2.3 standard soil (Speyer, Germany) was placed in glass containers and moistened to 40% of its water holding capacity (Table 1) to obtain 500 g wet weight. Soil samples were moistened with distilled water (control soils) or artificially contaminated with cadmium supplied as $CdCl_2$ (2.5 H_2O Sigma-Aldrich) to obtain four sublethal concentrations according to Irizar et al. (2015b): 1.25, 2.5, 25 and 125 mg Cd/Kg soil. After spiking, experimental soils were thoroughly mixed to ensure homogeneous distributions of the dissolutions and were left stabilizing during 7 days before the exposure of the earthworms.

Table 1. Main characteristics of LUFA 2.3 soils: Type, pH value (in dH_2O and 0.01 M $CaCl_2$), Cation exchange capacity (CEC , meq/100g), Water holding capacity (WHC , %) and clay, sand and Organic matter (OM, %) contents.

Soil type	Natural sandy loam
pH (dH_2O)	7.50
pH (0.01 M CaCl2)	6.80
CEC (meq/100g)	10.9
WHC (%)	37.3
Clay (%)	9#
Sand (%)	65#
OM (%)	1.88*

Particle size distribution according to United States Department of Agriculture (USDA)
*Organic matter calculated as 2 x percent organic carbon

In order to assess effects under thermal stress, the experiment was performed at different rooms acclimated to optimum temperature for earthworms (19 °C), and to a higher temperature (26 °C) calculated from the average of annual maximum

temperatures from local historical climatic series (1981-2010 period, Bilbao airport; © AEMET, Spanish meteorological agency). Earthworms were acclimated to LUFA 2.3 soil during 24 h at the selected temperatures before Cd exposure experiments.

2.3. Short-term exposure

Adult earthworms (clitellated, 0.3-0.6 g) were weighed in tens, introduced in a natural standard soil (500g LUFA 2.3 on each container) spiked with Cd (0, 1.25, 2.5, 25 and 125 mg Cd/Kg soil) and maintained at different temperatures (19 °C and 26 °C, same number of containers per each group) for 14 days. Exposures were carried out in absence of food, under continuous light (to guarantee continuous contact with soil) and constant humidity (periodically checked). Tissue metal accumulation (section 2.5) was recorded after 14 days and Neutral Red (NR) uptake in extruded coelomocytes (section 2.6) was quantified after 3 and 14 days of exposure.

2.4. Earthworm reproduction test (OECD-222, 2004)

Adult earthworms (clitellated, 0.3-0.6 g) were weighted in tens and exposed to a range of Cd concentrations (0, 1.25, 2.5, 25 and 125 mg Cd/kg soil) in LUFA 2.3 soil at 19°C and 26°C. According to the test, four replicates containing ten earthworms each were done per treatment. Test was carried out under controlled light-dark cycles (8/16 h) and 5 g of medication-free horse manure were provided weekly during the first 4 weeks (up to day 28). After this period, adults were removed from the soils and mortality was recorded. In order to determine the accumulation of Cd in tissues (section 2.5) and effects on weight loss only living animals were used. After the adult removal, their offsprings were kept for another 4 weeks (day 56) in the same experimental soils and conditions with the exception of the feeding. At day 56 effects on reproduction were assessed by counting cocoons and juveniles using the hand sorting technique twice in all samples (OECD, 2004).

2.5. Chemical and physicochemical analysis

At the end of the Short term exposure (day 14) and Earthworm Reproduction Tests (day 56), Cd concentration and pH were measured in experimental soils. The real concentration of Cd in soils was quantified following the EPA 3051A method. For that, soil samples (2 g) were acid digested (HNO_3: HCl, 3:1) in Teflon vessels in a microwave

oven, filtered after cooling (0.45 µm, 25 mm, PVDF) and analysed in Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7700-Agilent Technologies) in the Central Analysis Service of the UPV/EHU (SGIker).

Five earthworms per exposure treatment (Cd concentration and temperature) after 3 and 14 days (Short-term exposure; total: 100 individuals), and five earthworms per each of 4 replica after day 28 of Reproduction Test (total: 200 individuals), were placed in Petri dishes with moistened filter paper for 24 h voiding their gut content, and then, rinsed in distilled water. After, earthworms were dried in an oven at 120 °C (WTC Binder) for 48 h Dry samples were weighted (Sartorius CP225D) and rinsed in HNO_3 (69% Tracepur®). Once the concentrated acid was evaporated, pellets were resuspended in 0.01 M HNO_3 Tracepur® and Cd analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7700-Agilent Technologies).

For the measurements of the pH an adaptation of the ISO 10390: 2005 “Soil Quality Determination of pH in water” was followed. Soil samples from each replicate were mixed with distilled water (1:5), shaken during 1 min and left to settle for 45 min. The process was repeated twice and then the pH of the liquid phase was measured using a calibrated pH-meter (CRISON micropH 2001).

2.6. Coelomocyte viability (NR uptake assay) in exposed earthworms

After 3 and 14 days of exposure in the Short term exposure, 5 earthworms from each treatment (control, 1.25, 2.5, 25 and 125 mg Cd/kg soil at 19° and 26°C) were pooled together and their coelomocytes extruded to perform the NR uptake assay. First, earthworms were left avoiding their gut contents for 24 hours, then cleaned with distilled water by softly massaging their body in order to remove any soil particle attached to the tegument or in the posterior part of their digestive tract. Then pools of five individuals were immersed in extrusion solution (0.02% EDTA in PBS with 0.23% NaCl, 1 ml per worm) and were subjected to an electric stimulation with a 9 V battery to allow the release of coelomocytes through dorsal pores (Irizar et al. 2014a). The cell suspensions were transferred to tubes, centrifuged (530 g, 10 min, 10 °C) and resuspended in 5 ml of PBS for posterior cell counting under light microscope. Neubauer chamber was used to count and adjust the cell density of each pool to 10^6 cells per ml.

Then 2× 10^5 coelomocytes per well were seeded in a 96-well microplate (six well per treatment) and were left to stand at 18 °C in darkness for 30 min.

For the NR uptake assay, a working Neutral Red dilution (0.2% NR stock solution in PBS, pH 7.0-7.2) was prepared. After removing supernatant, 200 µl of the newly prepared NR working solution were added to each well. Simultaneously, negative controls (without cells) were introduced in order to test the removal of the dye in the wells. The microplate was incubated in the same previous conditions (darkness and 18°C) for 30 min to allow the uptake of the dye. Then, two washes were done (centrifugation: 530 x g, 5 min, 10°C; supernatant removal; addition of 200 µl PBS) to remove the dye completely and the presence of cells was checked through microscopical observations. Subsequently, PBS was removed and 100 µl of NR extraction solution (50% acetic acid, 1% ethanol, 49% distilled H_20) was added to the wells in order to withdraw the retained dye within lysosomes. Finally, absorbance was measured at 540 nm in a microplate reader Multiskan Spektrum Thermo Scientific spectrophotometer.

2.7. Statistical analysis

The statistical analysis of the data was carried out with the aid of the SPSS statistical package (IMB SPSS Statistics 23). To evaluate the normality of the data the Shapiro-Wilk test was carried out while the homogeneity of variance was assessed by using Levene test. Parametric data (number of cocoons and juveniles) was studied by one-way ANOVA. Furthermore, significant differences between samples were determined by Tukey test while differences between thermal treatments were explored with Student´s test. Non parametric data (weight loss at different days, chemical analysis, coelomocyte viability and mortality) was analyzed with Kruskal-Wallis followed by Dunn's post-hoc Test. Pearson's correlation was followed for the number of juveniles produced, in order to find the significance of the correlation coefficient (R) between thermal treatments. Statistically significant differences were established at $p < 0.05$ or $p < 0.01$.

3.- RESULTS

3.1- Cadmium concentration and pH of experimental soils

Soil pH did not change between treatments and remained very stable ranging from 7.26 to 7.61 after 14 days of Cd exposure at both thermal treatments (19°C and 26°C). These values were slightly (7.38 min.-8.14 max.) higher after 56 days of the reproduction test (Table 2). The real concentrations of Cd in experimental LUFA soils did not differ from the nominal concentrations in both Short term exposure and Reproduction tests (Table 2).

Table 2: Nominal and real cadmium concentrations (mg Cd/kg) and pH of soils at Short-term exposure (day 14) and after the Reproduction Test (day 56) at the two selected temperatures (19°C and 26°C). Cadmium accumulations in tissues after 3, 14 and 28 days in both temperatures are also represented. Asterisk reflects significant differences (*p<0.05*) between temperatures.

Short-term exposures	**19°C**					**26°C**				
Nominal (mg Cd/kg soil)	0	1.25	2.50	25	125	0	1.25	2.50	25	125
Real (mg Cd/kg soil)	0.19	1.50	2.40	23	117	0.13	1.10	2.54	22.30	116
pH	7.50	7.61	7.55	7.46	7.48	7.45	7.38	7.61	7.33	7.26
Cd in tissues 3d (µg Cd/mg earthworm)	0.00	0.01	0.01	0.04	0.08	0.00	0.01	0.01	0.04	0.08
Cd in tissues 14d (µg Cd/mg earthworm)	0.00	0.01	0.01	0.08	0.14	0.00	0.00	0.01	0.09	0.25

Reproduction test	**19°C**					**26°C**				
Nominal (mg Cd/kg soil)	0	1.25	2.50	25	125	0	1.25	2.50	25	125
Real (mg Cd/kg soil)	0.18±0.01	1.61±0.01	2.56±0.27	22.60±0.70	105.60±23.19	0.19±0.02	1.12±0.04	2.60±0.79	18.60±2.54	119±15.55
pH	8.14±0.09	7.65±0.01	7.95±0.06	7.96±0.02	7.64±0.01	7.64±0.24	7.76±0.13	7.47±0.14	7.38±0.36	7.44±0.02
Cd in tissues 28d (µg Cd/mg earthworm)	0.00±0.00	0.01±0.00	0.01±0.00	0.04±0.00	0.14±0.04 *	0.00±0.00	0.01±0.00	0.03±0.02	0.06±0.01	0.23±0.05 *

3.2- Short-term exposures (OECD-207, 1984)

3.2.1. Cd accumulation in earthworms

No cadmium was detected in tissues of control earthworms (Table 2). The accumulation of Cd in earthworms after 3 days of exposure showed a dose-dependant increase, being the maximum values (0.8 µg Cd/mg earthworm) reached after exposure to the 125 mg Cd/kg soil. Cd concentrations were similar at both temperature regimes

(Table 2). After 14 days of exposure Cd concentration in earthworms showed a similar dose-dependent trend with maximum values at the highest dose for both temperatures (Table 2). Earthworms accumulated more Cd after exposure at the highest temperature (26° C). Exposure to 125 mg Cd/kg soil under heat stress (26°C) showed a two fold higher accumulation comparing to those at 19°C (0.25 µg Cd/mg worm and 0.14 µg Cd/mg worm, respectively; significant differences at. $p<0.05$).

3.2.2- Coelomocyte viability (NR uptake assay) in exposed earthworms

The NR uptake assay performed in coelomocytes extruded from exposed earthworms showed no significant differences in cell retention capacity at both tempetarures after 3 and 14 d (Fig 1).

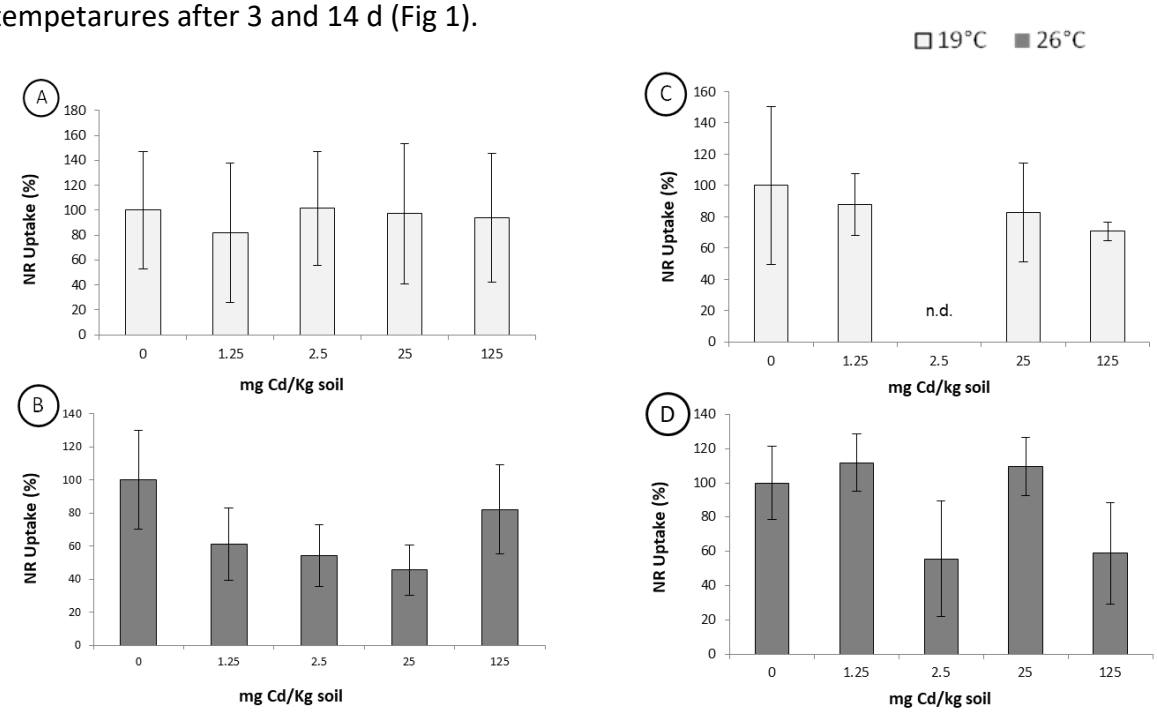

Figure 1.- NRU (% to the control) of coelomocytes extruded from *E. fetida* earthworms exposed to different cadmium concentrations: 0, 1.25, 2.5, 25 and 125, and maintained in a LUFA 2.3 soil at 19°C (A) and 26°C (B) for 3 days; 19°C (C) and 26°C (D) for 14 days (Short-term exposure). Means and standard deviations are shown. n.d means no available data.

The coelomocytes extracted from individuals exposed to 2.5 and 125 mg Cd/kg soil at thermal stress showed a lower capacity to retain Neutral red dye; 69% and 53% of control respectively (Fig. 1D).

3.3-Earthworm reproduction test (OECD-222, 2004)

3.3.1. Cd accumulation in earthworms

After 28 days of exposure of adult worms, a dose dependant Cd accumulation was observed in earthworm tissues (Table 1). Organisms under thermal stress (26°C) showed higher accumulation, mainly at the highest exposure concentrations (125 mg Cd/kg soil), nearly doubling the accumulation value at optimal temperature. In fact, statistically significant differences were observed between both thermal treatments for the highest dose. Although, organisms employed in the Reproduction Test were feeded with non treated horse manure the accumulation values remained very close to those quantified at day 14 of experiment in non feeded organisms (Short-term exposure).

3.3.2. Mortality and weight loss

The weight loss of adult worms after 28 d showed disimilar trends (Fig. 2). At 26°C the weight loss was more marked than at 19°C, and significant differences were found for all Cd treatments between both temperatures (Fig. 2).

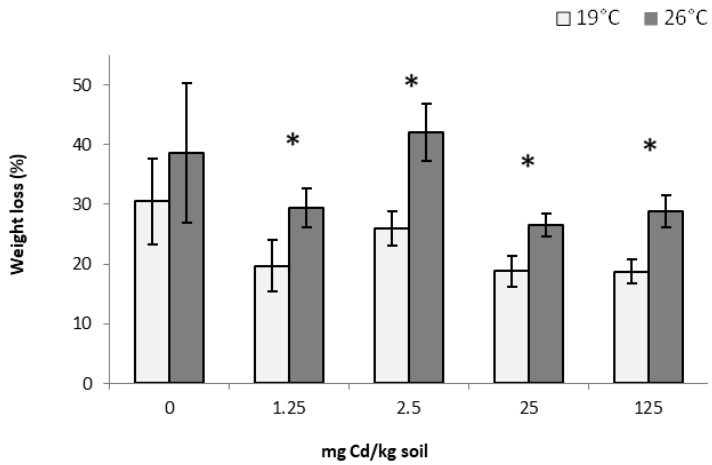

Figure 2.- Weight loss (% in relation to the initial weight) of *E. fetida* earthworms exposed to different Cd concentrations (0; 1.25; 2.5; 25 and 125 mg Cd/kg soil) in LUFA 2.3 soil under different temperatures (19°C and 26°C) for 28 days (Reproduction test). Means, standard deviations and significant differences between temperature treatments (*, $p \leq 0.05$) are shown.

Control organisms at both temperatures (19°C and 26°C) showed low mortality values after 28 days of treatment (<20%, Fig. 3). Exposure to all Cd treatments at 19°C did not produce significant mortality (0-20%). However, significantly higher mortality rates were recorded at 26°C for concentrations equal or higher than 2.5 mg Cd/kg (Fig. 3).

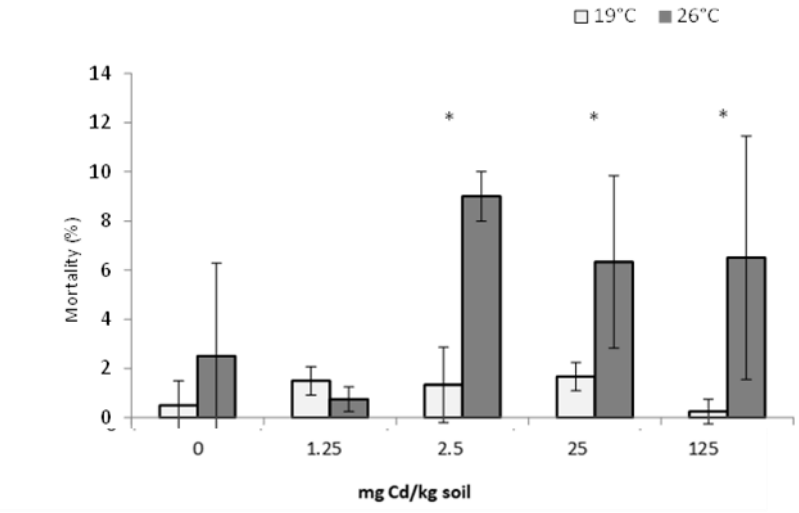

Figure 3.- Mortality (%) of *E. fetida* earthworms maintained at 19°C and 26°C and exposed to different cadmium concentrations: 0, 1.25, 2.5, 25 and 125, in a LUFA 2.3 soil for 28 days (Reproduction test). Means, standard deviations and significant differences between temperature treatments (*, $p \leq 0.05$) are shown.

3.4.3- Number of cocoons and juveniles

The number of cocoons did not change when increasing Cd concentrations at optimal temperature (19°C) although a slight trend to decrease ocurred at 125 mg Cd/kg (Fig. 4A). A reduction in the number of cocoons was observed at Cd concentrations higher than 2.5 mg Cd/kg (significant at 2.5 and 125 mg Cd/kg) under heat stress conditions (26°C).

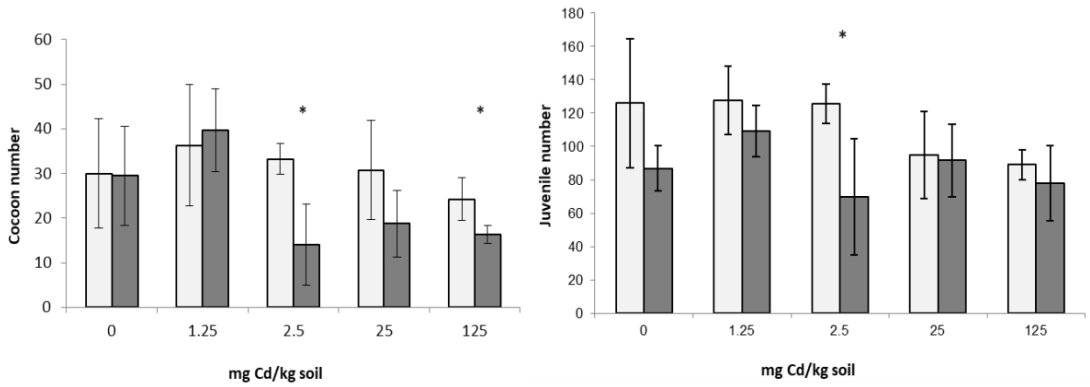

Figure 4.- Cocoons and juveniles counted at day 56 after exposing *E. fetida* earthworms to different cadmium concentrations: 0, 1.25, 2.5, 25 and 125, in LUFA 2.3 soil at 19°C and 26°C for 28 days (Reproduction test). Means, standard deviations and significant differences between temperature treatments (*, $p \leq 0.05$) are shown.

The number of juveniles produced by adult organisms exposed for 28 days to high concentrations of Cd (25 and 125 mg Cd / kg soil) at 19°C was significantly reduced according to the coefficient regression line (Fig. 4B, R=0.523**; $p \leq 0.01$). Although a similar trend was observed under heat stress (26°C) the number of juveniles produced was in all the cases lower than those exposed at 19°C. In fact, statistically significant differences were observed between both thermal treatments (19°C vs. 26°C) at 2.5 mg Cd/kg. In the case of cocoon production 2.5 and 125 mg Cd/kg exerted significant differences between both temperatures.

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4.- DISCUSSION

4.1- Soil characterization

Chemical bioavailability is affected by the physicochemical properties of the soil such as pH and OM, clay content and cation exchange capacity (Leduc et al, 2007; Smit and van Gestel. 1998; Spurgeon and Hopkin, 1995). All of them, and mainly pH and OM are in this sense directly related with the uptake, toxicity, degradability, and accumulation of pollutants (Kennette 1997; Nahmani et al. 2007). For instance, in acid pH, leaching capacity and metal availability are higher than in neutral and basic soils (Begum et al., 2012). Therefore, an adequate spiking and homogenizing procedure is one of the most crucial issues when characterizing the toxicity of contaminants in soils.

Presently, measured real cadmium concentrations in LUFA 2.3 soils at different temperatures (19°C and 26°C) for both tests experiments (Short term exposure and Reproduction Tests), did not differ from the nominal concentrations (1.25, 2.5, 25 and 125 mg Cd/kg ground). Similarly, the pH of the soils slightly changed between treatments. This slight increase in pH in some experimental soils after earthworm Reproduction test could be attributed to the excretion of intestinal Ca and NH_4-N which neutralize humic acids carboxylic and phenolic groups (Pramanic et al., 2007; Suleiman et al., 2017). In any case, an appropriate Cd homogenization procedure in LUFA 2.3 soils can be concluded at 19°C and 26°C, hence changes in metal bioavailability cannot be attributable to the experimental conditions.

4.2-Short term exposure

Earthworms are well known sentinel organisms and accumulate Cd in their tissues (Brewer & Barrett 1995; Lapinski & Rosciszewska 2008; van Gestel et al. 1993). Specifically, Cd tissue concentration in *E. fetida* increases in a dose dependent manner at exposure concentrations lower than 1000 mg Cd/kg soil dw (Lock & Janssen 2001). Accordingly, under present exposure conditions Cd concentrations in earthworms at 19 °C and 26°C for 3 and 14 days showed a dose dependent increase. However, at short exposure times (3 d) there were no significant differences with temperature, that were very clear after 14 days for the highest dose (125 mg Cd/kg). It seems that these dissimilar accumulation rates and their differences between temperature regimes were

more evident at longer exposure periods (Callahan et al. 1979), rendering larger accumulation rates in earthworms maintained at 26°C. Some authors pointed out that during short exposure periods, the pollutant can be absorbed through the skin (by metal absorption through the tegument) instead of via ingestion path, which is more predominant at longer exposure times (Honneycutt et al., 1994; Conder et al., 2002). However, García-Velasco et al. (2016) concluded that both, dermal (across body wall) and oral (via ingestion and absorption across the digestive gut epithelium) uptakes, can occur at short and long-exposure periods. Likely, similar conclusions could be obtained from present data (see accumulation at long-exposure periods, Section 4.3).

Temperature can be an "added" factor that plays a crucial role in enhanced metal accumulation in earthworms. Hence, under present exposure conditions, elevated temperatures increased Cd accumulation rates in *E. fetida* tissues, maybe due to explicit synergistic effects of metals while increasing temperature from 19°C to 26°C. Several authors concluded that Cd exposure leads to elevated energy costs as well as impaired aerobic energy production due to a progressive mismatch between oxygen demand increasing mitochondrial dysfunction with rising temperature (Cherkasov et al, 2006; Lanning et al, 2006; Cherkasov et al, 2007; Holmstrup et al. 2010).

Simultaneously, weight losses occurred, showing different trends in both thermal treatments after 14 days of exposure. Significant weight losses were enhanced at 26°C when temperature was the unique stressor. Interestingly, both stressors (temperature plus Cd exposure) acting jointly produced similar severe weight losses than in the case of control animals maintained at extreme temperatures. It seems that the effect on weight loss is masked by the fasting conditions required in this standard test. Sibly & Calow (1989) related this effect, in the case of exposed animals, to the increased energy expenditure due to the onset of detoxification processes. This response is not exclusive to earthworms and it has been previously described in other invertebrates (Holmstrup et al., 2010). The high weight loss in control samples (32% and 37%) maintained at 26°C could be related to the low organic matter content in the LUFA 2.3 soil (at most 2%). In fact, as previously concluded by Irizar et al (2014b), effects in *E. fetida* are produced not only by Cd bioaccumulation but also by the low OM content in soil. Furthermore, it cannot be discarded that the exposure to elevated temperatures and high Cd

concentrations can produce physical and functional disruption of the tegument as a whole (cuticle and epithelium), resulting in enhanced weight loss in earthworms as reported also for Ag NPs (García-Velasco et al., 2016). Further research is needed to clarify this possible toxic effect at tissue level and to link this alteration with the loss of weight at organism level, but it seems to be a general stress response not attributable to a unique sort of pollutants.

The reduction in the retention or uptake of NR dye in earthworm coelomocytes has been proposed as a reliable general stress biomarker indicative of sublethal toxicity in response to pollutants and environmental stressors (Scott-Fordsmand et al. 1998; Asensio et al. 2007; 2013; Plytycz et al., 2007; Irizar et al., 2014, 2015a, 2015b). Accordingly, in the present paper, different time-course responses were observed between Cd treatments and temperatures. For instance, after 3 days Cd exposure did not produce any significant damage at 19°C, however, a dose dependent effect on coelomocytes viability was observed for Cd doses ranging 1.25-25 mg Cd/kg at 26°C. Remarkably, an increase in NR uptake was observed after exposure to the highest dose (125 mg Cd/kg). Likewise, Irizar et al (2015a) found after *in vivo* exposure of *E. fetida* to 25 mg Cd/kg soil a significant decrease in NRU signal in coelomocytes followed by a significant increase after exposure to 62.5 mg Cd/kg soil, and a second decrease at 125 mg Cd/ kg soil. This so-called bimodal dose–response curve was previously described after *in vivo* and *in vitro* exposure of earthworm coelomocytes to a variety of metals (Irizar et al., 2014; Irizar et al., 2015a, 2015b). This effect could be related with alterations in the relative proportion of coelomocyte subpopulations, amoebocytes and eleocytes that behave differentially to stressors, being amoebocytes more resistant (Irizar et al., 2015a). However, García-Velasco et al (2016) pointed out that the absorbance values obtained after NRU assays did not entirely represent the dye retained within lysosomes, but the presence of dye deposits in the bottom of the wells that appeared to interfere with the absorbance measurements in NRU assay at the highest exposure concentrations. Regardless, it reflects the existence of a possible toxic response that was more marked after 14 days of exposure to Cd at 26°C, since the “increase” in NRU signal, indicator of possible cytotoxic response (Irizar et al., 2013;

Irizar et al., 2014b; Curieses et al., 2017), in coelomoytes was recorded at low doses (2.5 mg Cd/kg).

In summary, it can be concluded that multi stress exposure conditions (contaminant plus thermal stress -26°C-) can produce a toxic response at lower exposure doses (reduced toxicity threshold) than under optimal temperature (19°C) conditions, being this a potential risk for soil organisms in climate change scenarios.

4.3- Reproduction test with *E. fetida* (OECD-222)

Exposure to Cd for 28 days produced a dose dependent accumulation in earthworm tissues, being this accumulation significantly higher at 26°C than at 19°C, but only for the highest exposure dose (125 mg Cd/kg). This dissimilar accumulation rates, enhanced with temperature, might be related with a lower ingestion rate (remarkable weight loss) as previously mentioned in highly stressful situations in real soils (Hartenstein et al., 1981; Malecki et al., 1982; Neuhauser et al., 1984; Van Gestel et al., 1991; Spurgeon et al., 1994; Zaltaukaite and Sodiene 2014). As mentioned before, short-term accumulation of pollutants was related with dermal uptake, while long-term ones with ingestion (Honneycutt et al., 1994; Conder et al., 2002). Presently, accumulation of Cd at short (14 d) and long (28 d) exposure periods reached similar values suggesting that both mechanisms occur at the same time. Alike, García-Velasco et al. (2016) proposed the same intake pattern (dermal+ingestion) in earthworms exposed to Ag nanoparticles.

Once the first stage of the reproduction test ended (28 d), mortality was significatively remarkable (ranging from 63 to 90%) in those organisms exposed to high Cd concentrations (2.5 -125 mg Cd/kg) under 26°C. It can be concluded that elevated temperatures amplify the lethality of Cd, as mentioned before, probably due to the large energy costs and the progressive imbalance between oxygen demand and supply (Holmstrup et al, 2010). Previous works with *E. fetida* have concluded that the LC_{50} for Cd was around 300 mg Cd/kg in OECD soil (Asensio 2009; Neuhauser et al. 1984). Hence, no mortality should be expected under present exposure conditions, but even though, it was recorded. The explanation to this deleterious effect, more marked at extreme temperatures for a given Cd concentration, might be related with the very low amount of OM in LUFA soils (2%) in comparison with OECD soils (10%). In the same way, Irizar et

al (2015) pointed out that OM content in soil affects both Cd bioaccumulation and toxicity in *E. fetida*, and very low values might represent another stress factor *per se*.

Effects exerted by environmental factors (including pollutants) at high levels of biological organization have the advantage of being ecologically relevant and are widely used tools for the overall assessment of soil health (Lionetto et al. 2012). For instance, effects on reproduction are one of the most sensitive toxicological parameters because even small changes in reproduction can severely affect the survival of the population (Scott-Fordsmand et al. 2008).

Presently, reproduction was a very sensitive endpoint together with mortality and weight loss, due to the net accumulation of Cd in earthworms mainly exposed to Cd at elevated temperatures. Hence, cocoon production was only slightly affected by Cd exposure at optimal temperature (19°C). However, in poikilotherm animals temperature is a key environmental factor that affect their physiological state, and therefore, reproduction effects at extreme temperatures were more marked. Consequently, the joint effect exerted by elevated temperatures together with pollutants can affect the development time and reproduction rate in earthworms (Spurgeon et al., 1994; Svendsen et al., 2007; Zaltauskaite & Sodiene, 2014), and the production of cocoons was strongly limited at exposure doses higher than 2.5 mg Cd/kg at 26°C. Effects on reproduction have been recorded after exposure to concentrations as low as 18 mg Cd/kg (van Gestel et al. 1992). Therefore, our results suggest that combined effect of Cd exposure and heat stress can drastically reduce *cocoon* production.

As mentioned before a low OM content can pose also a direct effect on reproduction. In fact, Irizar et al (2015a) suggested that Cd toxicity increases extremely when OM content is below 6 %, and the OM content of presently used LUFA soils is around 2%. In addition, and regarding the number of juveniles, different trends were observed between both temperatures. Under 19°C at 25 and 125 mg Cd/kg soil treatments a slight decrease on juveniles number occurred. This effect was magnified as result of thermal stress. Moreover, if the results of the number of juveniles at 26°C are compared with weight loss after 28 days results, a clear inverse relation between the two parameters is distinguished. This inverse relation suggests that weight loss is in some way related with the reproductive strategy carried out.

In summary, the effects of cadmium exposure in earthworms were enhanced at increasing temperature (from 19°C to 26°C). These effects were more marked at longer exposure periods (14 d vs. 28 d) and linked with higher accumulation rates due to dermal and digestive incorporation of Cd. Moreover, high mortality rates and reproductive impairment occurred at longer exposure periods at 26°C. It can be concluded that elevated temperatures (in a possible future scenario of global warming) can enhance metal bioavailability and produce more marked toxic effects as a function of time at different levels of biological organization in soil macroinvertebrates.

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CHAPTER 4

Predicting environmental concentrations and the potential risk of Plant Protection Products on non-target soil organisms accounting for regional and landscape ecological variability in European soils

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ABSTRACT

The concern about Plant Protection Products (PPP) is related with their effect on some soil organisms considered non-target species which could be highly sensitive to these products. The European Food and Safety Authority (EFSA) has developed *in silico* models to assess environmental risk of pesticides covering different non-target organisms and tools. Accordingly, the software tool Persistence in Soil Analytical Model (PERSAM) has been used for calculating PPPs predicted environmental concentrations (PECs); and further potential risk (by means of Toxic Exposure ratio, TER) regarding agricultural authorisation. However, soil characteristics and environmental variables change along a latitudinal axis through the European continent; influencing the availability of PPP, their toxicity, and hence, their risk. Therefore, there is a need to develop landscape based environmental risk assessment methods addressing PPP regional variability within the EU. The main objective of this work was to integrate spatially explicit exposure data (PECs) and effect data (TER) of four PPP active substances (esfenvalerate, cyclaniliprole, picoxystrobin and fenamidone) on non-target species accounting European landscape and agricultural variability. The study was focused on the effects produced by the above-mentioned pesticides on two soil organisms: *E. fetida* earthworms and *Folsomia sp.* collembolans. Soil variability and climatic differences (environmental variability) among European soils divided in three large Euroregions along a latitudinal transect (Northern -N-, Central -C- and Southern-S- Europe) were analysed.

Keywords: Landscape, variability, PPP, non-target, ERA, agricultural-soils

LABURPENA

Landare-osasun produktuek (PPP-*Plant protection product*- ingelesez) itu espezieak ez diren organismoengan eragiten dituzten efektuen inguruko ardura zeharo emendatu da azken hamarkadetan. Harira, elikadura segurtasunerako autoritate europearrak (EFSA-*European food safety authority*- ingelesez), itu espezieak ez diren organismoak arriku ebaluazioetan barneratzen dituen proposamen eta tresna ezberdinak argitaratu ditu azken urteotan. PERSAM sofwarea (*Persistence in Soil Analytical Model*- ingelesez), PPPen ingurumen kontzentrazioak kalkulatzeko (PEC-*Predicted environmental concentrations*- ingelesez), eta hortaz PPPek organismoengan eragindako arriskua (TER- *Toxic exposure ratio*- ingelesez) kalkulatzeko erabiltzen den *in silico* modeloa da; produktuen nekazal erabilpena onartzeko (edo ez) beharrezkoa. Aldiz, PERSAMeko databaseetan aurkitzen diren lurzoru ezaugarriak eta ingurumen aldagaiak asko aldatzen dira Europear kontinenteko ardatz latitudinalean zehar; PPPen eskuragarritasuna, eta hortaz arriskua, baldintzatuz. Honela, beharrezkoa da Europear batasuneko paisaia aldakortasuna barneratzen duten ingurumen arrisku ebaluazioak garatzea. Lan honen helburua, 4 PPPren exposizio eta efektu datuak integratzea da. Bereiziki, bi intsektizidek (esfenvalerate eta cyclaniliprole) eta bi fungizidek (picoxystrobin eta fenamidone), itu ez diren bi organimorengan (*Eisenia fetida* zizarea *eta Folsomia candida* kolenboloa) duten inpaktua ebaluatuko da; horretarako, Europar eskualdeen arteko aldakortasuna (Europa Iparralde, erdialde eta hegoaldea) kontutan izanik.

Hitz gakoak: Paisaia, aldakortasuna, PPP, ez-itu, ERA, nekazal-lurrak

1-INTRODUCTION

Population growth over recent decades has increased the challenges associated with food supply. As a result, food production is one of the greatest challenges among the objectives of the 2030 agenda for sustainable development (UN, 2015). At the same time, the activities related to food production are associated with complex socioeconomic and environmental impacts to be considered for sustainability (Sala et al., 2017). For instance, landscape homogenization and agricultural expansion significantly degrades the soil, while, climate change, rural lands transformation into urban areas and over-use of chemical products on crops might increase soil health problems (Pereira et al., 2017).

Every year, 2.4 milion a.s (active substance) tons are released into the environment (US EPA, 2011), being Europe the largest pesticide consumer worldwide (Enserink et al., 2013; Storck et al., 2017). Applied pesticides can enter soil or surface waters via leaching and run-off, thus affecting non-target species in both terrestrial and aquatic ecosystems (Schreiner et al., 2021). These species are those directly and/or indirectly exposed to PPP without being primary targets around or in the crops. Consequently, these substances can contribute to natural biodiversity and soil property losses while enhance crop production (Li et al., 2016; Pereira et al, 2017). Hence, testing effects on earthworms and on non-target foliar arthropods is an integral part of the ecotoxicological risk assessment needed for the authorization of PPP (Kohlschmid & Ruf, 2016). For instance, earthworm toxicity test with *Eisenia fetida* based on acute (mortality and weight loss; OECD-207, 1984) and chronic (reproductive, growth, and behavioural effects; OECD-222, 2016) endpoints are conducted routinely to assess PPP (Kohlschmid & Ruf, 2016). Additional testing (OECD-232, 2016) on soil arthropods (i.e. *Folsomia candida*, *Hyposaspis aculeifer*) is required if the product is applied directly on or into the soil (Romeis et al., 2013; Kohlschmid & Ruf, 2016).

Currently, the environmental risk assessment of pesticides using *in silico* models takes into account predefined scenarios, and is assumed to represent realistic worst-case conditions. Scenarios and models are mostly applied to the exposure assessment (environmental fate and behaviour of the substance) where environmental concentrations are estimated after pesticide disposal into crops. Afterwards, based on

the obtained concentrations, an effect assessment must be conducted in order to evaluate risk upon soil living organisms (non-target). The software tool "Persistence in Soil Analytical Model" (PERSAM) was developed as the result of collaboration between JRC and EFSA (EFSA, 2015) for calculating the predicted environmental concentrations (PECs) of substances and their transformation products in soil. For this calculation, the software uses 62 data sets: 6 on general information on European landscape, 27 on meteorological features, 5 on soil characteristics and 24 on crops. This software tool, along with the EFSA guidance document, is an accurate approach for supporting risk management decisions in the authorisation of active substances or the final PPP (EFSA, 2015). However, environmental variables and agricultural practices vary in a latitudinal axis along the European continent influencing PPP availability and toxicity (Bonmatin et al., 2015; Ogungbemi and van Gestel., 2018). Among this influencing environmental variables, pH, organic matter, the presence of colloid particles or even topography (influences water content, soil development and erosion) can be found (Tarbuck & Lutgens, 2005; Shroder et al., 2008; Zou et al., 2018). Moreover, local climate characteristics determines soil weathering and have influence in leaching processes, conditioning soil fertility along the latitudinal gradient. Therefore, there is a need to develop landscape based environmental risk assessment methods for PPP addressing regional variability within Euroregions.

The main objective of this work was to integrate spatially explicit exposure data (PEC) and effect data (through TER- Toxicity Exposure Ratio) of four PPP active subtances (esfenvalerate, cyclaniliprole, picoxystrobin and fenamidone) on non-target species accounting European landscape and agricultural variability. The study is focused on the effects produced by the above-mentioned pesticides on two soil organisms: *E. fetida* earthworms and *Folsomia sp.* collembolans. These model species are representative of different exposure scenarios and routes for toxicants: solid phase (earthworms) and pore water (both) (Belfroid et al., 1996; Fountain and Hopkin, 2005; Ogungbemi and van Gestel, 2018). Soil variability and climatic differences (environmental variability) among European soils divided in three large Euroregions along a latitudinal transect (Northern -N-, Central -C- and Southern-S- Europe) will be analysed.

2-MATERIALS AND METHODS

2.1- Selection of PPP active substances and non-target organisms

Four well-known pesticide active substances (two from insecticides - esfenvalerate and cyclaniliprole - and two from fungicides -picoxystrobin and fenamidone-) were carefully selected due to their extensive use and their high ecotoxicological risk (identified in previous risk assessments) for earthworms and non-target arthropods. Information regarding good agricultural practices (GAP), chemical properties, and toxicity data was retrieved from available EFSA databases (references in Table 1). The crops onto which the selected PPPs are usually applied as well as target pests have been included in Table 2. Briefly, esfenvalerate (S)-cyano-(3-phenoxyphenyl) methyl] (2S)-2-(4-chlorophenyl)-3-methylbutanoate) is mainly administered to control pests in wheat, barley and potato crops such as moths, beetles, flies, and other insects, inducing a stomach action (Bal-Price et al., 2017). Cyclaniliprole or 5-bromo-N-[2-bromo-4-chloro-6-(1-cyclopropylethylcarbamoyl) phenyl]-2-(3-chloropyridin-2-yl) pyrazole-3-carboxamide for its part, is normally sprayed during growth stage in stone fruits, pome fruits, grapes, eggplants, peppers, potatoes and tomatoes, to control *Cydia pomonella* (pome fruits); *Leptinotarsa decemlineata* (potatoes) or *Spodoptera spp* (tomatoes, eggplants and peppers) among others. This insecticide belongs to the diamide groups acting on the ryanodine receptors located in the endoplasmic reticulum of insects, inducing muscle paralysis (Troczka et al., 2017). Picoxystrobin ($C_{18}H_{16}F_3NO_4$) is a preventive and curative fungicide called methyl (E)-3-methoxy-2-[2-[[6-(trifluoromethyl) pyridin-2-yl] oxymethyl] phenyl] prop-2-enoate belonging to strobilurin group of chemicals. This hazardous substance for aquatic organisms is commonly sprayed on wheat, barley, oats, rye and triticale in winter, late winter, spring and summer. Fenamidone (C_{17} H_{17} N_3 O S) is a systemic foliar fungicide called (5S)-5-methyl-2-methylthio-5-phenyl-3-phenylamino-3,5-dihydro-4H-imidazol-4-one. Hazardous for aquatic environment, is generally sprayed and used on tomato and potato plants for the control of fungal pathogens such as *Phytophthora infestans* and early blight *Alternaria solani*.

Table 1.- Summary of the bibliographical sources used to assess the effects of PPPs.

PPPs	Source
Esfenvalerate	Report and Proposed Decision of the United Kingdom made to the European Commission, from July 2013. Volume 3, Annex B.9: Ecotoxicology. Pages 390 to 399.
Cyclaniliprole	Volume 3, Annex B9, pages 167 to 174 of the "Draft Assessment Report prepared to support the approval of the following active substance according to Regulation (EC) 1107/2009 and the subsequent inclusion into Annex B of Regulation (EC) 540/2011", year '15.
Picoxystrobin	Volume 3, Annex B.9 of the "Draft Report and Proposed Decision", from July 2015. Pages 123 to 127 and 219-222.
Fenamidone	Volume 3, Annex B.9, pages 160 to 181 from the Renewal Assessment Report under Regulation (EC), year of 2015

Eisenia fetida earthworms and *Folsomia sp.* collembolans were the selected non-target organisms to assess effects of PPP. Different bibliographical sources, toxicological studies and official guidelines made by and /or approved by EFSA, were analysed to obtain toxicity endpoints (LC_{50}, NOEC; See chapter 5). The majority of the chronic toxicity tests on *E. fetida* followed the OECD Guideline No. 222 (OECD, 2016), and the ones with *Folsomia sp.* collembolans followed OECD Guideline No. 232 (OECD, 2016). Apart from toxicity endpoints, test conditions (soil, pH, and temperature), the purity of the compound, and the effects observed were considered in all the analysed studies.

Table 2. Summary of the PPP active substances, crops of application and target pests.

Type of PPP	Crops	Target pests	References
Esfenvalerate Insecticide (pyrethroid)	wheat, barley and potato (spring, winter)	moths, beetles, flies, and other insects	Bal-Price et al. (2017), Casida et al. (2013), Abreu-Villaça & 2017
Cyclaniliprole Insecticide (diamide)	stone fruits, grapes	*Cydia pomonella* (pome fruits); *Leptinotarsa decemlineata* or *Spodoptera spp*	EFSA, 2015a Troczka et al. (2017) Opper et al. (2010)
Picoxystrobin fungicide (strobilurin)	wheat, barley, oats rye and triticale (in winter, late winter, spring and summer)	*Phytophthora infestans* and early blight *Alternaria solani*	Paramasivam & Chandrasekaran (2013); Tentu & Tentu (2016)
Fenamidone fungicide	Tomato and potato plants		

2.2- Predicted environmental concentrations (PECs) of PPPs in crop soils along a gradient of European regions

The PERSAM software tool (Persistence in Soil Analytical Model; version V2.0.1) was used to calculate PECs of PPPs active substances. Simulations with numerical models allowed estimating PECs in crop soils; for that, calculations in 1x1 km surface squares were performed along European landscape. 95^{th}-percentile PECs were selected as representative exposure scenario for each Euroregion (N, C, S). The data extraction for fenamidone and cyclaniliprole was carried out simulating applications on tomatoes and potatoes due to the large information available and their spread usage (widely described in the peer reviews by EFSA). Tomatoes and spring cereals were the selected crops for picoxystrobin while only potatoes were selected for esfenvalerate due to agricultural practices. The pesticide input in soil was set according to good agricultural practices (GAP) recommended for each pesticide. For all the cases, the worst-case scenario/practice was selected; so soil PECs after dosing pesticide in the crops (time= 0) and at 1 cm depth in soil (to ensure the effect on non-target organisms) were considered.

Additionally, the ratio between PECs in different soil compartments (pore water-pw- and total soil-tc-) was obtained in order to understand PPP soil distribution:

Thus,

$$PEC_{ratio} = PECpw/PECtc$$

was obtained.

Gathering together PEC_{ratios} regarding all the studied compounds, crops and GAPs, an integrated PEC_{ratio} was obtained in order to address the influence of landscape variability among Europe.

2.3- Determination of PPP risk scenarios for non-target organisms: TER calculations

On the basis of the relevant worst case PECs and toxicity endpoints for PPP active substances, toxicity exposure ratios (TERs) for acute and chronic exposure of *E. fetida* and *Folsomia sp.* were calculated for both compartments (pw and tc). The estimated TER values were used to indicate potential risks arising non-target soil organism along a gradient of European soils. Toxicity was based on experimentally determined acute (LC_{50}) and sublethal/chronic endpoints (NOEC) for the tested

compounds (published in EFSAs technical reports). These toxicity values were then related to PECs at 2.5 cm depth: worst-case scenario depth most likely to be inhabited by the aforementioned organisms (Sulivan et al., 2009).

TER (acute or chronic) was calculated according to the following equation:

(Eqn. 1)

$$TER = \frac{LC50/NOEC\ (mg/kg)}{PEC\ (mg/kg)}$$

In TER calculations for compounds with log Pow >2 (partition coefficient regarding bioaccumulation potential), toxicity endpoints were divided by a factor of 2 (Van Gestel, 1992).

Moreover, following 91/414/EEC directive, critical trigger TER values were established for regulation purposes: 10 for acute and 5 for chronic TER for terrestrial vertebrates, earthworms and arthropods (EC 1991; Commission Regulation 2011). If TER values are lower than these thresholds, studied scenarios can be considered as risky or hazardous; field specific studies being required.

The lack of toxicity endpoints for pore water exposure was adjusted with the formula to calculate PECs in liquid phase used by PERSAM tool. Thus, biological endpoints for pore water were estimated through the endpoint for total soil. This formula estimates the concentration on pore water by relating the concentration on total soil, the volumetric water content, the dry bulk density, the organic matter (OM) content, the OM/water distribution coefficient and the maximum liquid concentration in soil.

$$C_{L,peak} = \frac{C_{T,peak}}{\theta / \rho + f_{om} K_{om}}$$

(Eqn. 2): formula to calculate maximum concentrations in liquid phase used by PERSAM

Where:

$C_{T,peak}$: Maximum total concentration in soil

θ_{fc}: Volumetric water content

ρ: Dry bulk density

F_{om}: Organic matter content

K_{om}: Organic matter/water distribution coefficient

$C_{L,peak}$:Maximum liquid concentration in soil

Data regarding European soils physicochemical characteristics were obtained from PERSAM (Decorte et al., 2016).

Apart from the above mentioned TERs for each soil compartment, an additional ratio between the TER calculated in total soil and pore water was estimated in order to address the most restrictive compartment.

So,

If, $TER_{tc/pw} = \frac{TERtc}{TERpw} < 1$ (0, 1); risk on TC would be more restrictive; meaning that risk for soil depending organisms is higher.

If, $TER_{tc/pw} = \frac{TERtc}{TERpw} > 1$ (1 ,∞); risk on PW would be more restrictive; meaning that risk for pore water living organisms is higher.

3- RESULTS AND DISCUSSION

3.1. PECs of PPP in crop soils along a gradient of European regions

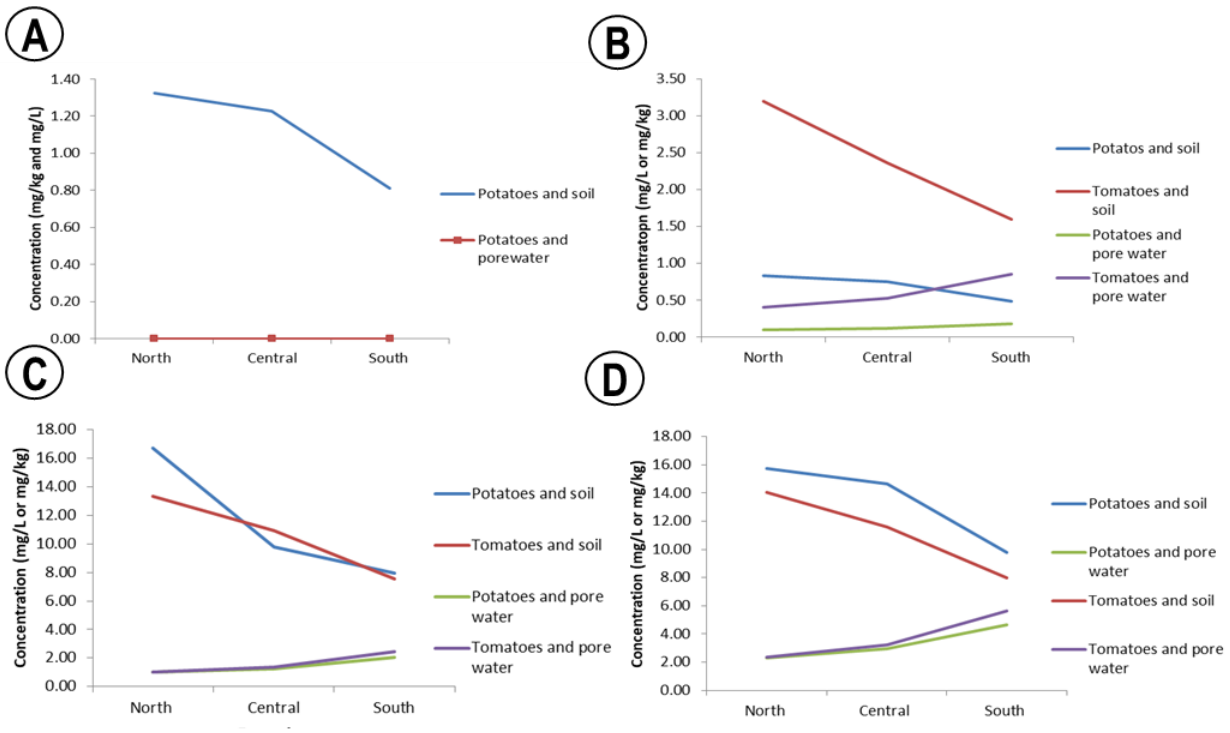

Figure 1. PEC in soil (mg/kg) and pore water (mg/L), after a recommended worst case (T0 and 1 cm) application of esfenvalerate (A), cyclaniliprole (B), picoxystrobin (C) and fenamidone (D) on potatoes, tomatoes and spring cereals in northern, central and south European agricultural soils.

3.1.1. Esfenvalerate concentrations in potato crops

The highest concentration of esfenvalerate in total soil was found in north Europe, with values ranging 3.876 - 5.565 mg/kg. The rest of European soils exhibited values lower than 1.34 mg/kg. (Figure 1SM, Supplementary Material). The PEC of esfenvalerate in total soil followed the gradient N>C>S (Fig. 1A) for all exposure times and depths (Table 1SM). Concentrations in the liquid phase were very low for all the soils.

3.1.2 Cyclaniliprole concentrations in potato and tomato crops

The highest concentration of cyclaniliprole in potato crops (total soil) was around 3.83 mg/kg in the N while in the rest of the continent ranged 0.86 - 1.45 mg/kg

(Fig. 2SM). In the liquid phase, the highest values were about 0.237 mg/L in southern countries, while the rest of the continent showed values between 0.128 and 0.164 mg/L. (Fig. 2SM).

The PECs of cyclaniliprole in tomato crop soils were higher in total soil than in liquid phase, reaching 14.82 mg/kg in the N region. The rest of Europe exhibited concentrations around 3.38 mg/kg. For the liquid phase, the range of concentration varied from 0.221 to 0.956 mg/L (in the S). Overall values for the rest of Europe ranged between 0.51 and 0.66 mg/L (Fig. 2SM; Tables 3SM). An increasing cyclaniliprole concentration gradient N>C>S was observed for total soil in both crops (Fig. 1B).

3.1.3 Picoxystrobin concentrations in spring cereal and tomato crops

The PECs of picoxystrobin in both crops were higher for total soil than for liquid phase, reaching values of around 60 mg/kg in the N. The rest of European soils showed very low concentration in total soil (around 15 mg/kg) in a clear N to S decreasing gradient (Fig. 3SM). For liquid phase, an opposite trend was observed (N<C<S; Fig. 1C), with high values in the S (up to 2.61 mg/L in cereal crop soils and 0.99-1.40 mg/L in tomato crop soils) (Fig. 3SM; Tables 4-5SM).

3.1.4. Fenamidone concentrations in potato and tomato crops

In potato crop soils, total soil exhibited higher concentrations comparing to liquid phase with values between 15.96 mg/kg (S) and 65.05 mg/kg (N) (N>S gradient; Fig. 4SM, Fig. 1D). For liquid phase, the highest concentration was found in the S (around 6.11 mg/kg) while most of the continent showed concentrations between 3.29 and 5.17 mg/L in this phase; following N<S trend (Fig. 4SM, Tables 6-7SM).

The highest concentrations in N tomato crops were found in total soil (65.02 mg/kg) while for liquid phase the lowest values were achieved (1.39 mg/L). A decreasing gradient in total soil from north to south region was observed; the opposite happened for liquid phase (Fig. 4SM, Tables 6-7SM).

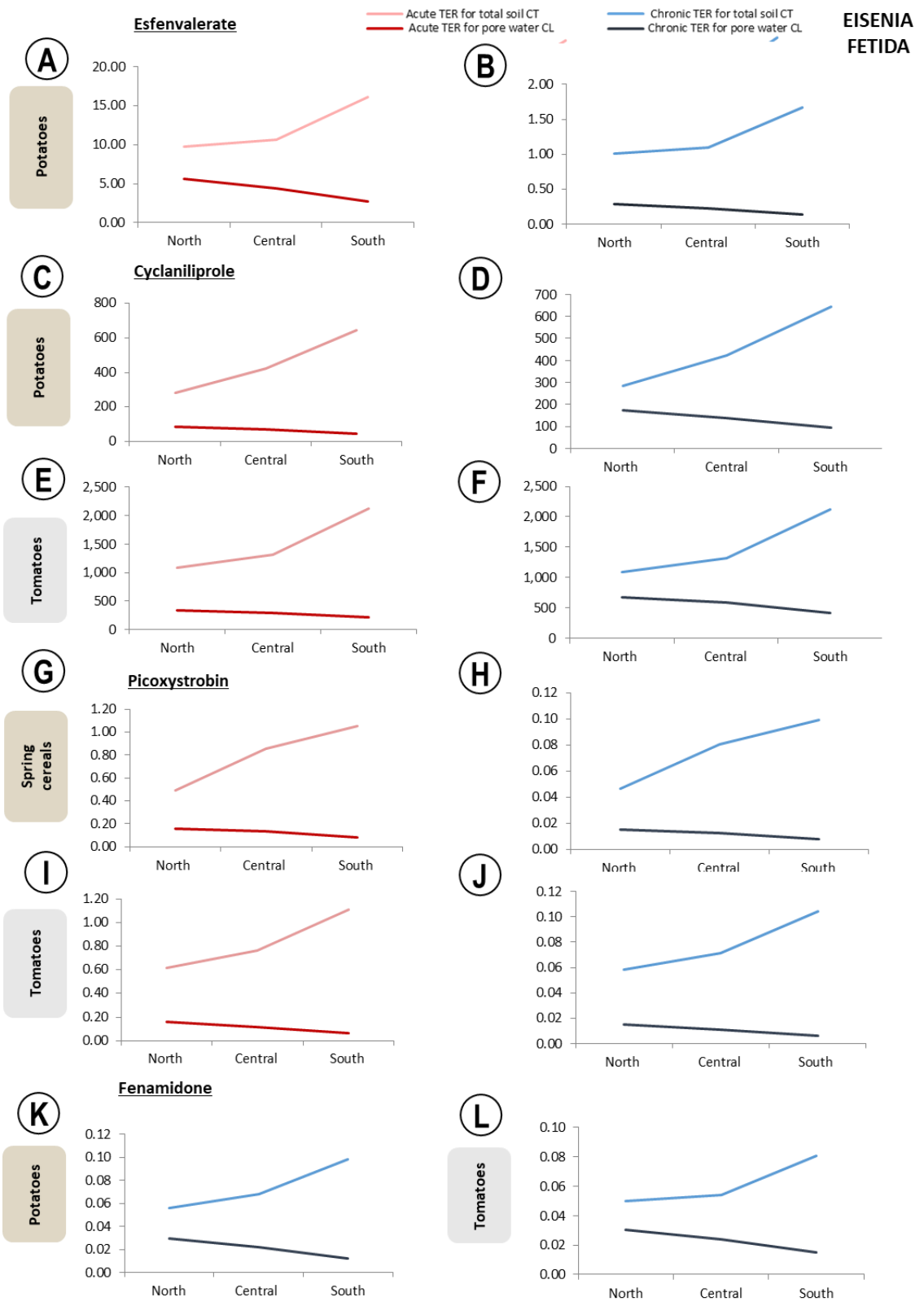

Figure 2. Acute and chronic TER for *Eisenia fetida* earthworms in total soil (TS) and pore water (PW) at upper layers (2.5 cm) after PPP application in north, central, and south European agricultural soils.

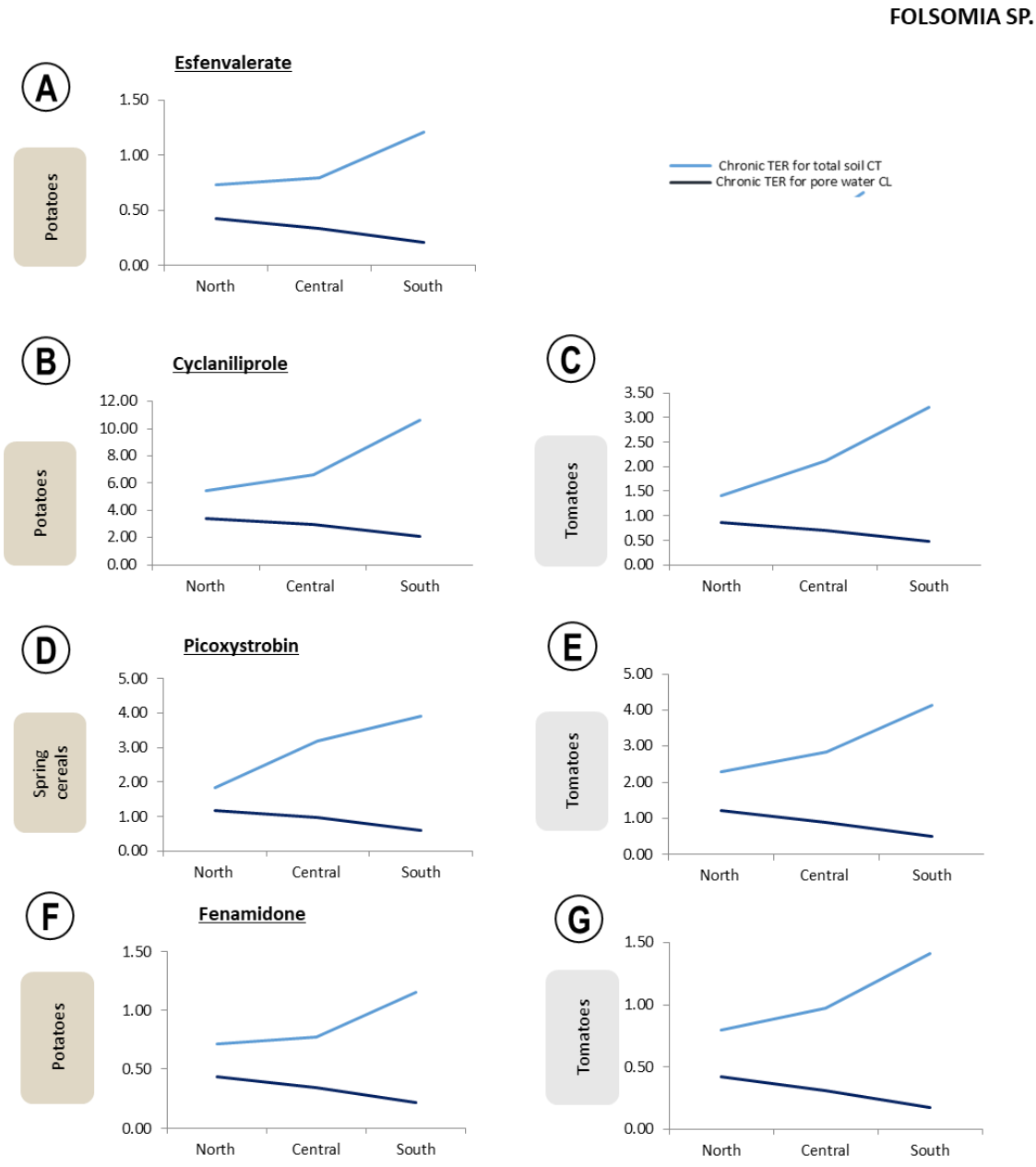

Figure 3. Chronic TER values for *Folsomia sp*. collembolla in total soil (TS) and pore water (PW) at upper layers (2.5 cm) after PPP application in north, central, and south European agricultural soils.

3.2. PPP RISK SCENARIOS FOR NON-TARGET ORGANISMS: TER CALCULATIONS

The risk was determined for selected non-target species (*E. fetida*, *Folsomia sp*) after esfenvalerate, cyaniliprole, picoxystrobin and fenamidone application to crops; for that toxicity exposure ratios (TER) were calculated from PECs obtained by PERSAM model.

3.2.1. *Eisenia fetida*

3.2.1.1. Esfenvalerate

TER values for esfenvalerate in total soil of potato crops showed values over 10 in C and S soils, suggesting no acute risk for *E. fetida* earthworm in these regions. However, values below 10 were estimated for northern soils. Meanwhile chronic risk was concluded (<5) for the three areas (N, C, S). Acute and chronic risks in total soil for *E. fetida* were addressed with N to S risk decreasing trend (Figs. 2A, 2B). These results might be related with the higher OM content usually present in northern soils (Decorte et al., 2016; Xu et al., 2019) and also by the high partition coefficient of the esfenvalerate (kom: 145997 L/kg). Both factors together could lead to bindings between soil OM and pesticide, consequently an increased pesticide uptake by soil ingestion could occur on earthworms (Belfroid, 1994; Chung and Alexander, 1998; Contreras-Ramos et al., 2006, 2008; Wang et al., 2018). TER values in pore water (TER_{pw}) were under TER critical values for both: acute and chronic. $TER_{TC/PW}$ showed an increasing trend from north to south, however, values over 1 indicated a higher risk for soil living organisms in pore water comparing to total soil (Figs 4A). The lower precipitation rates and higher temperatures in southern soils (Xu et al., 2019), which makes soil water evaporate, may lead to pesticide concentrations increase in pw. Moreover, the low OM content in southern soils (Xu et al., 2019) together with the Kom of the pesticide might enhance this risk.

3.2.1.2- Cyclaniliprole

Acute and chronic TER were far from the legal thresholds established; therefore the application of the above mentioned cyclaniliprole concentrations would not cause any remarkable effect on earthworms in potato crops (Figs. 2C, 2D). Comparing between crops, slightly lower TER values could be observed in potato crops, suggesting

that risk was slightly higher than in tomatoes (Figs. 2E, 2F). Acute and chronic $TER_{TC/PW}$ increased from north to south for potato and tomato crops. In both cases $TER_{TC/PW}$ values were always over 1 suggesting that acute and chronic risks for *E. fetida* in pore water is more critical than in total soil fraction of potato (Figs. 4B) and tomato crops (Figs. 4C). Moreover, this risk is enhanced in southern soils comparing to northern; and also in potato crops comparing to tomato (Figs. 2C-F). The environmental factors behind this critical risk in pore water from southern soil might be related to higher temperatures and lower precipitation rates and OM contents. The higher risk under tomato crops might be due to dissimilar agricultural practices (e.g. tillage, irrigation, shade, soil characteristics of the crop) (Asfary et al., 1982; Romero-Aranda et al., 2001; Zhou et al., 2018).

3.2.1.3-Picoxystrobin

Acute and chronic risks were found for earthworms after picoxystrobin application on spring cereal and tomato crops in north, central or south Europe (Figures 2G-J). While the risk on pore water increased from north to south, the opposite happened on total soil. Comparing both crops, slightly higher acute and chronic risks were observed for spring cereals. $TER_{TC/PW}$ values were always over 1 for both crops (Figs. 4D, 4E) suggesting that acute and chronic risks for *E. fetida* in pore water were more critical. However, higher $TER_{TC/PW}$ values were registered in southern soils than in northern soils; being enhanced in spring cereal crops.

3.2.1.4-Fenamidone

Chronic TER for both, pore water and total soil, showed values below the legal threshold (5), suggesting a risky scenario for earthworms living in potato and tomato crops in northern, central or southern soils (Figs. 2K, 2L). As already mentioned for the rest of compounds, the risk for earthworms on pore water increased from N to S; while the opposite trend was observed for risk in total soil. However, risk for earthworms on potatoes showed to be higher than on a tomato crops. Similarly, $TER_{TC/PW}$ showed values upper than 1 for both crops, suggesting that also for the case of fenamidone, the risk/exposure through pore water was more critical than via total soil; and more significant in the south than in the north (Figs. 4F, 4G).

3.2.2- *Folsomia sp.*

3.2.2.1-Esfenvalerate

In all European regions TERs were below the established legal threshold (5) for both soil compartments (total soil and pore water), suggesting that esfenvalerate could pose chronic risk to collembolans in potato crops (Fig. 3A). The risk in total soil decreases from north to south while the risk in pore water increases in the same direction. $TER_{TC/PW}$ showed values over 1 indicating more critical risk in pore water than in total soil, mainly in the S (Fig. 5A).

3.2.2.2- Cyclaniliprole

Chronic TER values were above legal threshold (5) for total soil indicating no risk to collembolan communities in potato crops, while cyclaniliprole could exert risk (TER<5; Fig. 3B) through pore water, mainly in the S. In tomato crops, potential risk was observed in total soil and pore water, being the risk higher at N and S, respectively (Fig. 3C). It seems that risk for *F. candida* is higher in tomato crops than potato crops (Fig. 3B,3C). $TER_{TC/PW}$ showed values higher than 1 for both crops, so risk in pore water appeared to be more critical than in total soil (Figs. 5B, 5C). When comparing European regions, risk on pore water was more critical in southern soils.

3.2.2.3- Picoxystrobin

Chronic TER for picoxystrobin and collembola in spring cereals and tomato crops showed risky scenarios in both soil compartments (TER<5) (Figs. 3D, 3E). TER values for both crops showed an increasing trend from north to south in total soil; and a decreasing trend in pore water. Therefore, risk for *Folsomia sp* exposed thought pore water increases from northern soils to southern soils; while risk in total soil decreases. $TER_{TC/PW}$ was over 1 for all the regions and both crops, confirming that risk in pore water is more critical than in total soil (Figs. 5D, 5E). $TER_{TC/PW}$ values were higher in S than in N, and higher in tomato crops than in spring cereals.

3.2.2.4- Fenamidone

Fenamidone chronic TER for potato and tomato crops exhibited inadmissible risks (TER <5) for collembolans in both soil compartments (in total soil and pore water)

and regions (Figs. 3F, 3G). Chronic TER values in pore water for both crops showed higher risk in southern soils than in northern ones. The risk for total soil exhibited the opposite trend; higher in the N. $TER_{TC/PW}$ were over 1 in all the regions and both crops, confirming that the risk of this fungicide in pore water is more critical than in total soil; especially in southern soils (Figs. 5F, 5G). $TER_{TC/PW}$ was higher in tomato crops than in potato crops; demonstrating higher risk in the former crop.

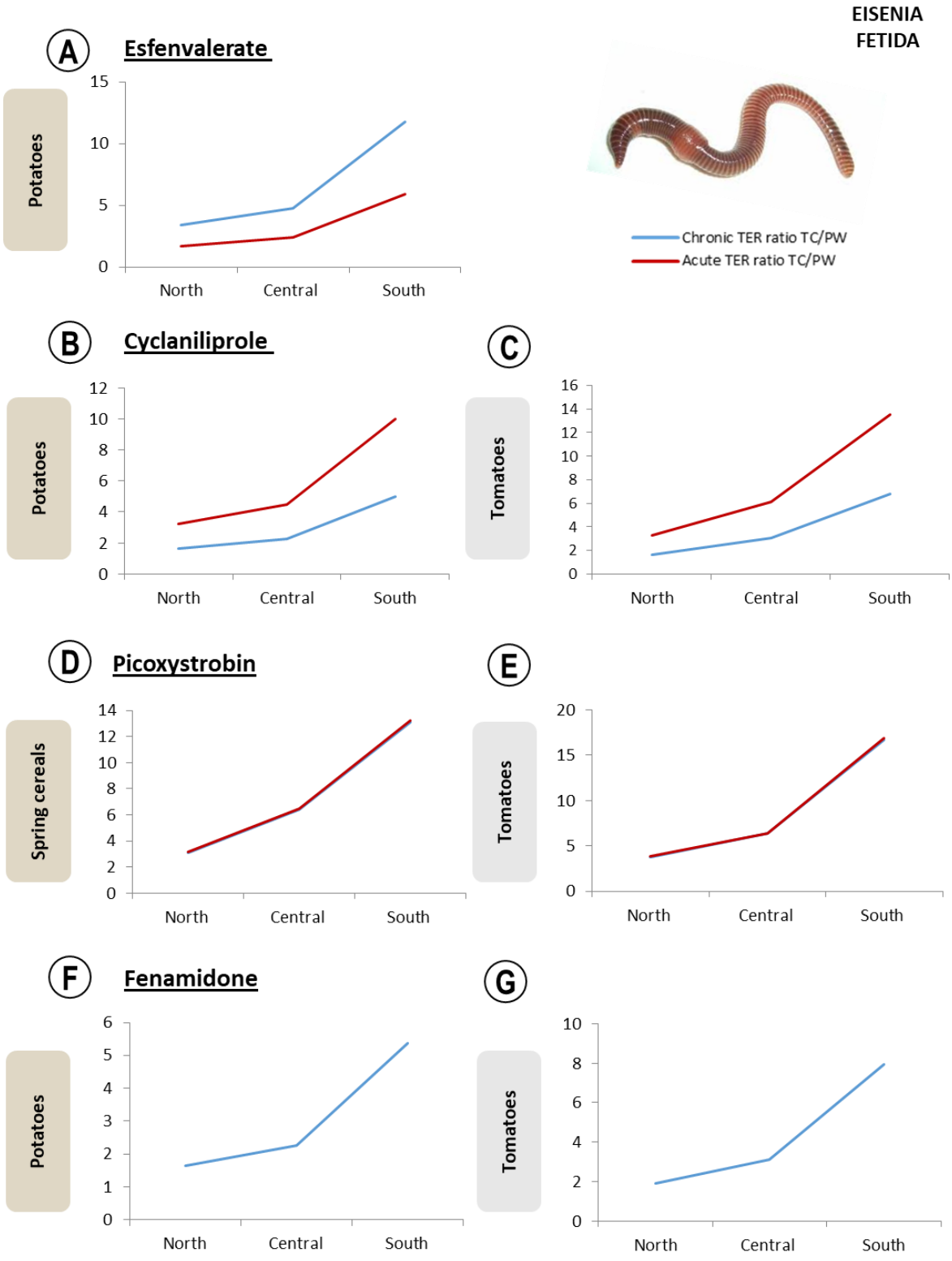

Figure 4. Acute and chronic $TER_{TC/PW}$ for *Eisenia fetida* earthworms at upper layers (2.5 cm) after esfenvalerate (A), cyclaniliprole (B,C), picoxystrobin (D, E) and fenamidone (F,G) application in north, central, and south European agricultural soils.

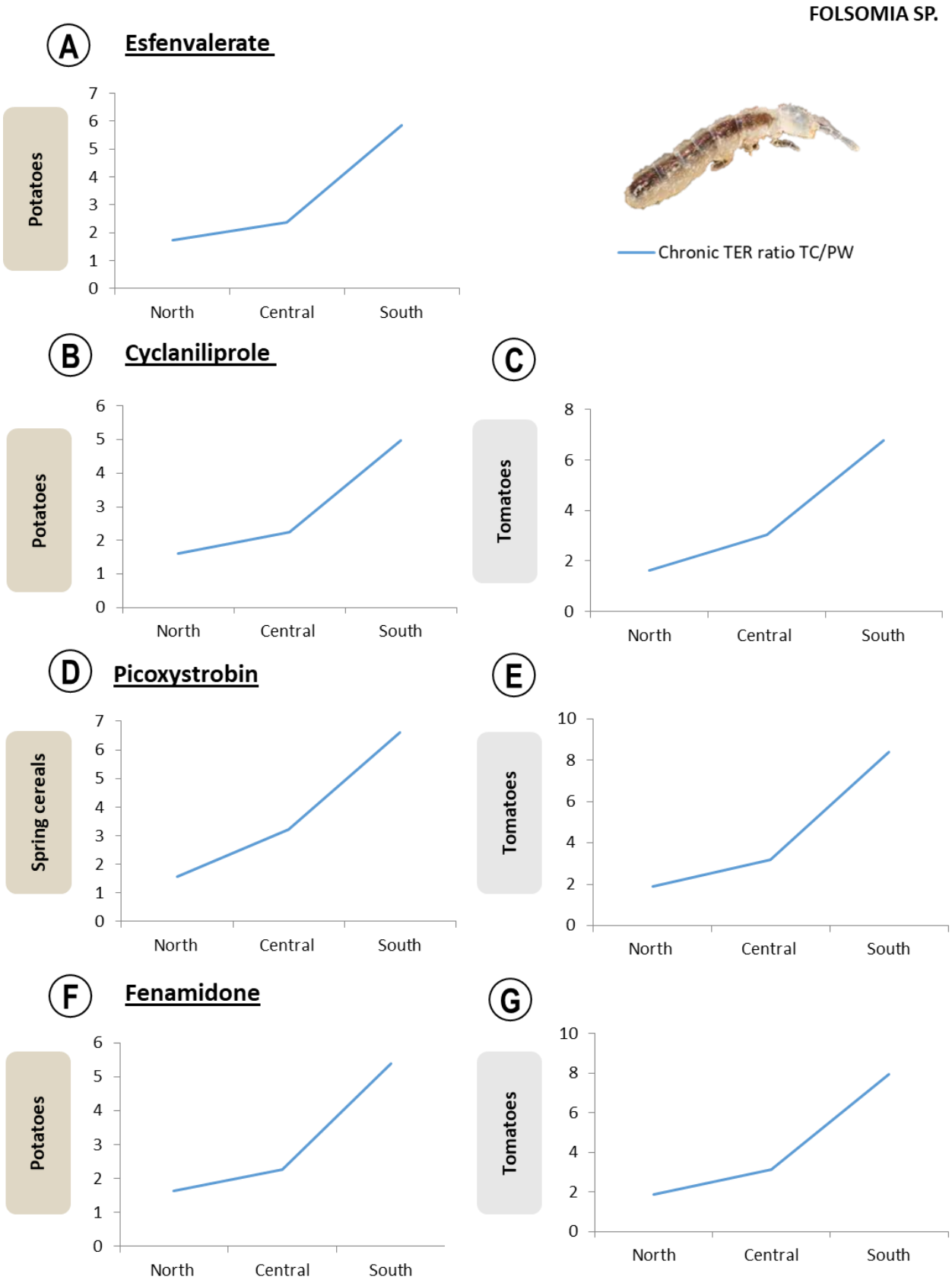

Figure 5. Chronic $TER_{TC/PW}$ for *Folsomia candida* collembolans at upper layers (2.5 cm) after esfenvalerate (A), cyclaniliprole (B,C), picoxystrobin (D, E) and fenamidone (F,G) application in north, central, and south European agricultural soils.

3.2.3- Risk estimations along European landscapes based on PPP soil distribution

The lack of toxicity endpoints for pore water specific exposure in the selected organisms and pesticides, made impossible the estimation of the risk on pore water accurately. However, by studying PEC ratio (PECs for pw respect to PECs for tc) and assuming that the higher PEC is, the higher the risk; different patterns were observed when comparing Euroregions. The integrated PEC_{ratio} (Fig. 6) showed the influence of landscape variability on the PPPs, which could affect non-target organisms such as *E. fetida* earhworms and *F. candida* collembolans. The index confirmed a north to south potential risk increase. This suggest that earthworms and collembolans exposed through pore water would be in higher risk in southern soils than in northern soils. This pattern can also be seen in the Figure 7, where clear increases from north to south were observed on PEC_{ratio} for fenamidone and cyclalinliprole in potato and tomato crops. The different variations observed along the European latitudinal axis can be explained principally by climate and landscape variability. It is known that pH varies in a latitudinal axis along the European continent (Xu et al., 2019) influencing the availability of some pollutants such as pesticides in soil. In the case of European soils, pH varies between 4 and 8.5 being more acidic in northern and northeastern soils comparing to southern ones (Xu et al., 2019). Some compounds are more retained at higher pH, and are more available at low pH values (Martinez and McBride, 2001; Shroder et al., 2008) enhancing their toxicity (Zou et al., 2018). Thus, in the present study major toxicities in total soil can be expected for a same pesticide application in the north in comparison to the south. Apart of pH, the OM plays also an important role on the partitioning of elements present in soil (Visioli et al., 2013). The highest contents of OM are in the northeast of the continent: Finland, Estonia and Lithuania (0.85 Kg /Kg of soil), followed by Poland, Belarus and Denmark (0.23 - 0.49 kg / kg of soil) (Decorte et al., 2016) probably due to higher precipitations and lower temperatures that may decrease OM decomposition (Jenny, 1980; Xu et al., 2019). In those regions, colloid particles could facilitate the retention of PPPs in soil, since the humus is formed by acidic compounds of high molecular weight with different functional groups (Tarbuck & Lutgens, 2005).

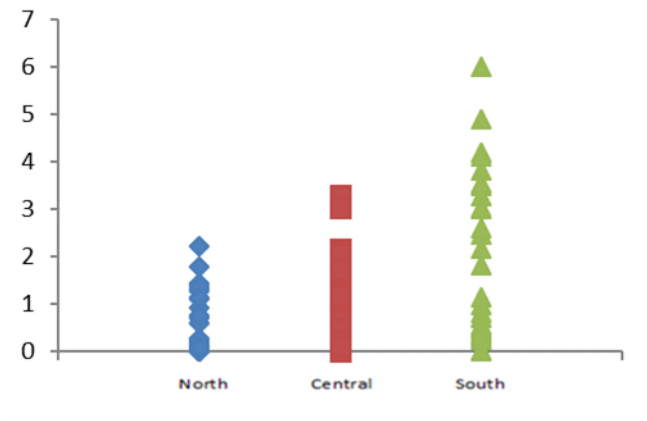

Figure 6. Integrated PECratio values (all compounds, crops and GAPs) among European regions (North, Central and South).

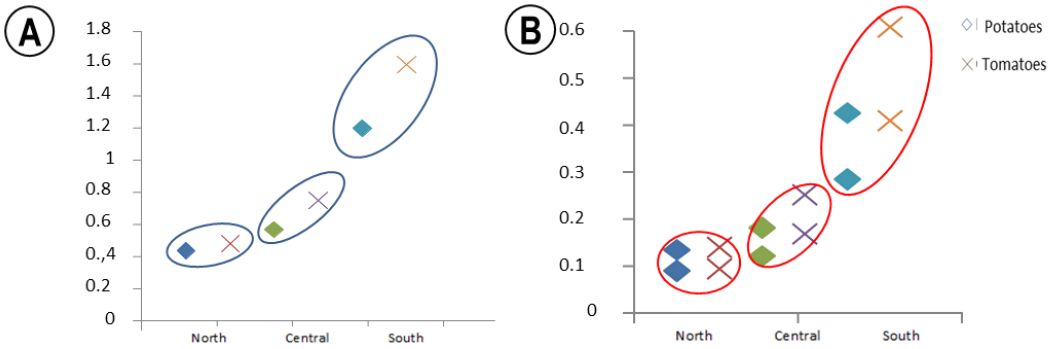

Figure 7. PECratio for fenamidone (A), cyclaniliprole (B) taking into account the crop and the landscape variability. For each crop and region, the lowest and highest PEC values are shown.

In southern soils opposite spatial distribution patterns between OM and pH were described (Xu et al,. 2019): lower OM contents and significantly higher pH values. The leaching of dissolved organic carbon is accelerated by high pH values resulting in the reduction of OM in the surface of the soil (Andersson and Nilsson, 2001). It is evident that temperature and precipitation is correlated with OM and pH (Fabian et al., 2014). Soil characteristic together with high temperatures and low precipitation rates could lead to lower soil retention, and hence, to a greater migration of the pesticide to the pore water in the south. Hence, all these factors could drive to a pore water decrease (due to a higher evapotranspiration) and therefore, to a pesticide concentration and risk increase.

A different PEC_{ratio} was observed depending on the crop where the PPP was sprayed (Fig. 7). As a general trend, a higher PEC_{ratio} was recorded in tomato crops than in potato crops. Thus, it can be stated that in tomato crops organisms will be exposed to higher concentrations of PPP assuming higher risk impairments in pore water. Moreover, this increasing trend in risk was very clear from north to south. Irrigation regimes might be behind this dissimilar behaviour together with landscape variability. It is known that due to their shallow root system (Asfary et al., 1982), potato crops are hardly influenced by drought severity and duration (Onder et al., 2005, Ünlü et al., 2006; Zhou et al., 2018). In fact, some European potato arable lands (Denmark) are dominated by low water capacity coarse-soils, requiring high irrigation regimes in order to maintain quality and yield (Shock et al., 2007). This higher water regimes could increase soil pore water quantity and thus reduce PPP concentration. Similarly, a greater gloom which increases the shade and therefore reduces evapotranspiration (Kendy et al., 2006) (affecting pesticide concentration on pore water) could modify the partition and the impact of the pesticides.

Apart from the environmental factors, properties of each pesticide may affect also pollutants availability in soil. Among the data on pesticide properties introduced on PERSAM, Kom coefficient was the most influencing factor for pollutants concentration and risk distribution among regions. As a general trend, higher pesticide concentrations were observed on pore water when the kom of pesticides was low. This way, fenamidone (Kom: 225 L/kg) was the PPP with higher PEC_{ratio} while esfenvalerate (Kom: 145997 L/kg) showed the lowest PEC_{ratio} (Fenamidone > cyclaniliprole (Kom: 280 L/kg) > picoxystrobin (Kom: 520 L/kg) > esfenvalerate). Highly lipophilic compounds are spected to be adsorbed in soil matrix (Ogungbemi and van Gestel, 2018); thus, compounds like esfenvalerate with high Kom will have a high affinity to the OM on soil, being bound to it. Moreover, Belfroid (1994) and Jager et al. (2003) demonstrated that oral uptake is enhanced in OM rich soils with highly lipophilic or hydrophobic compunds (Ogungbemi and van Gestel, 2018). Meanwhile, compounds like fenamidone with lower Kom values will have less affinity to the OM on soil and will migrate more easily to other soil compartments such as pore water, being more easily

incorporated by dermal uptake (Fountain and Hopkin, 2005; Ogungbemi and van Gestel, 2018).

5.-CONCLUSIONS

The predicted concentrations of esfenvalerate, cyclaniliprole, picoxystrobin and fenamidone on crop soils varied depending on the soil compartment and spatial variability along Europe. The combination of low temperatures, low pHs and high organic matter contents in total soil could enhance pesticides risk for non-target organisms; especially in northern soils. By the contrary, high pesticide concentrations in pore water could exert toxic responses in non-target species; mainly in southern areas. Apart from the landscape variability, a strong relation was found between the estimated risk values and the treated crop type or the characteristics of PPPs. Thus, a higher risk for pore water living organisms was noted on tomato crops comparing to potato crops; even more in southern soils. Similarly, pesticides with low Kom showed higher PECs on pore water; and therefore higher risks. In a context of proper soil management, soil characteristics have be taken into account to make accurate PPP exposure and effect estimations.

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SUPPLEMENTARY MATERIAL

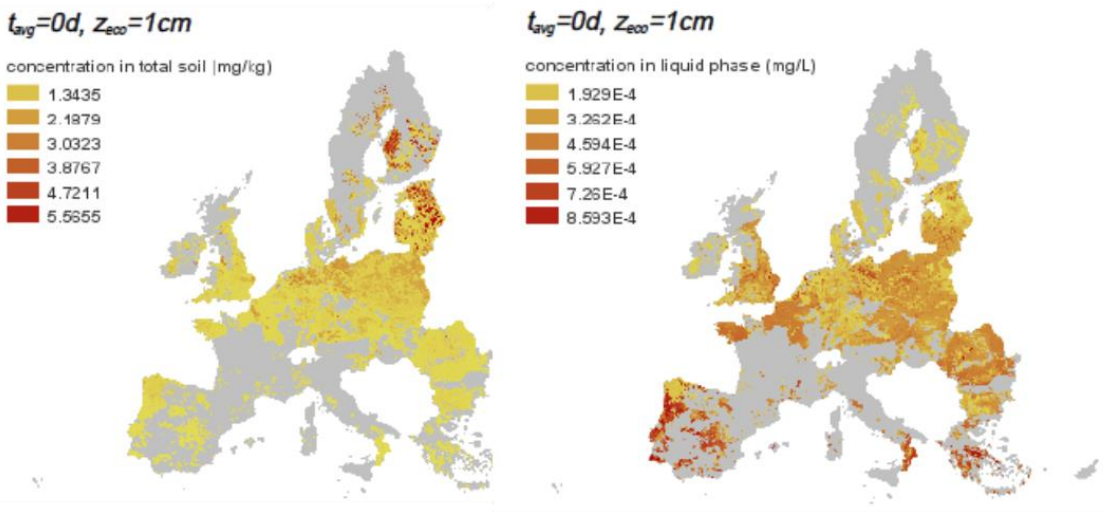

Figure 1SM. PEC-s of esfenvalerate in total soil (mg/kg) and pore water (mg/L), after a recommended worst case (T0 and 1 cm) application on potato crops in northern, central and south European agricultural soils.

Table 1SM: PEC-s of Esfenvalerate in total soil (mg/kg) and pore water (mg/L) for european soils at different times (0-56 days) and dephts (1-20 cm) after a recommended worst case esfenvalerate application on potatoes.

north

concentration in total soil C_T (mg/kg)

t_{avg} (d)	z_{eco} (cm) 1	2.5	5	20
0	1.3248	0.5442	0.284	0.0939
7	1.3082	0.5374	0.2804	0.0929
14	1.2919	0.5307	0.2769	0.092
21	1.2759	0.5241	0.2735	0.091
28	1.2601	0.5176	0.2701	0.0901
56	1.1995	0.4927	0.2572	0.0867

concentration in liquid phase C_A (mg/L)

t_{avg} (d)	z_{eco} (cm) 1	2.5	5	20
0	3.12E-4	1.283E-4	6.692E-5	2.154E-5
7	3.078E-4	1.267E-4	6.608E-5	2.131E-5
14	3.042E-4	1.251E-4	6.525E-5	2.108E-5
21	3.006E-4	1.235E-4	6.443E-5	2.086E-5
28	2.97E-4	1.22E-4	6.358E-5	2.063E-5
56	2.827E-4	1.161E-4	6.051E-5	1.977E-5

central

concentration in total soil C_T (mg/kg)

t_{avg} (d)	z_{eco} (cm) 1	2.5	5	20
0	1.2267	0.5002	0.258	0.0764
7	1.2083	0.4927	0.2541	0.0752
14	1.1904	0.4854	0.2504	0.0741
21	1.1728	0.4782	0.2467	0.073
28	1.1555	0.4711	0.243	0.072
56	1.0898	0.4444	0.2292	0.0679

concentration in liquid phase C_A (mg/L)

t_{avg} (d)	z_{eco} (cm) 1	2.5	5	20
0	4.027E-4	1.633E-4	8.381E-5	2.451E-5
7	3.956E-4	1.607E-4	8.24E-5	2.414E-5
14	3.888E-4	1.58E-4	8.101E-5	2.378E-5
21	3.825E-4	1.555E-4	7.969E-5	2.343E-5
28	3.763E-4	1.529E-4	7.845E-5	2.308E-5
56	3.524E-4	1.434E-4	7.382E-5	2.177E-5

south

concentration in total soil C_T (mg/kg)

t_{avg} (d)	z_{eco} (cm) 1	2.5	5	20
0	0.8129	0.3299	0.1684	0.0476
7	0.7982	0.3243	0.1653	0.0468
14	0.7849	0.3178	0.1626	0.046
21	0.7705	0.3117	0.1597	0.0452
28	0.7553	0.3063	0.1566	0.0444
56	0.7039	0.2852	0.1459	0.0415

concentration in liquid phase C_A (mg/L)

t_{avg} (d)	z_{eco} (cm) 1	2.5	5	20
0	6.558E-4	2.644E-4	1.33E-4	3.528E-5
7	6.378E-4	2.561E-4	1.297E-4	3.44E-5
14	6.203E-4	2.5E-4	1.263E-4	3.351E-5
21	6.064E-4	2.436E-4	1.223E-4	3.262E-5
28	5.878E-4	2.358E-4	1.193E-4	3.187E-5
56	5.357E-4	2.161E-4	1.094E-4	2.923E-5

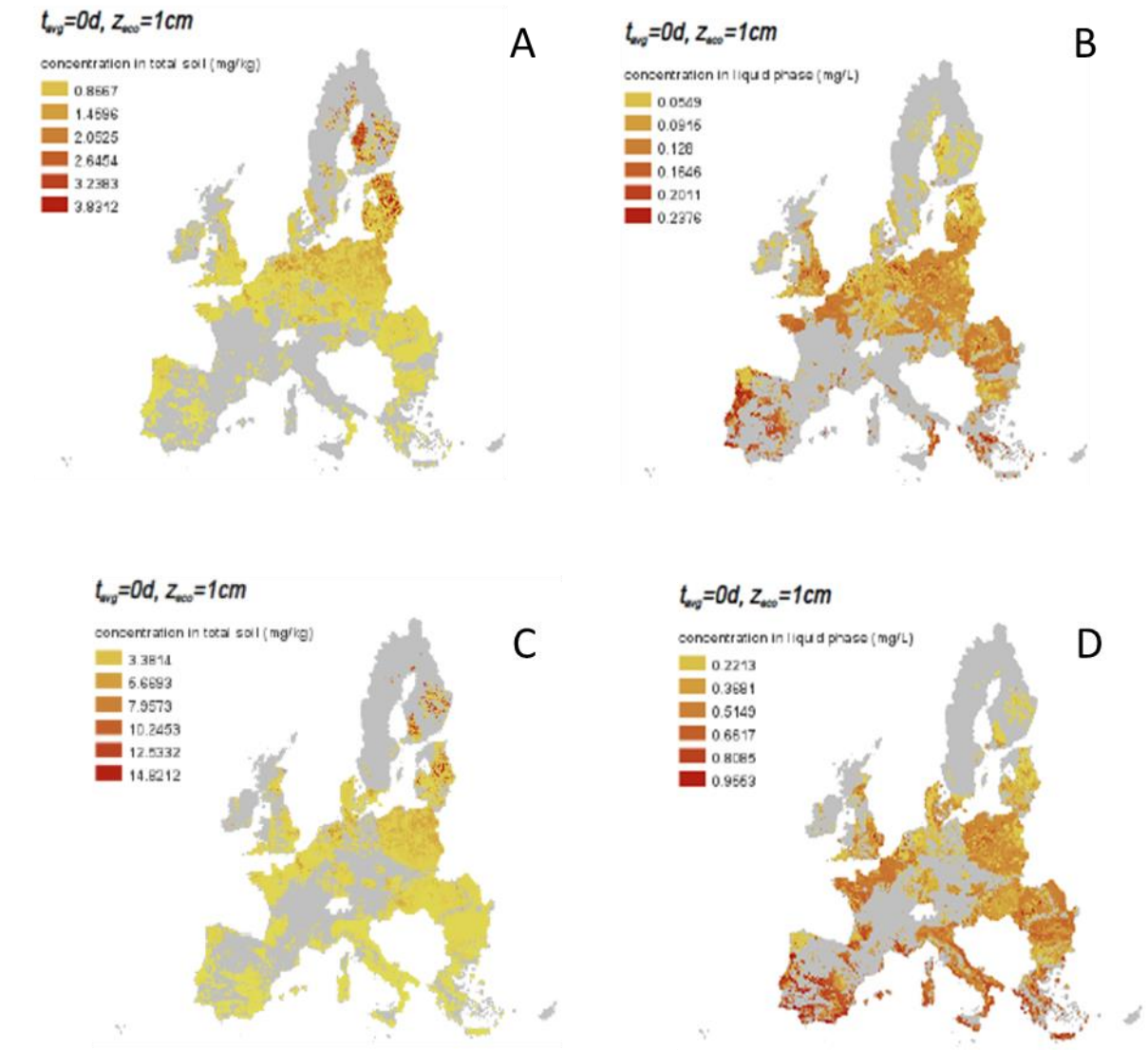

Figure 2SM. PEC-s of cyclaniliprole in soil (mg/kg) and pore water (mg/L), after a recommended worst case (T0 and 1 cm) application on potatoes(A, B) and tomatoes(C,D) in northern, central and south European agricultural soils.

Table 2SM. PEC-s of Cyclaniliprole in total soil (mg/kg) and pore water (mg/L) for european soils at different times (0-56 days) and dephts (1-20 cm) after a recommended worst case cyclaniliprole application on potatoes.

north

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.0996	0.0528	0.0378	0.0258
7	0.0994	0.0527	0.0378	0.0258
14	0.0992	0.0527	0.0377	0.0258
21	0.0991	0.0526	0.0377	0.0257
28	0.0989	0.0525	0.0376	0.0257
56	0.0982	0.0522	0.0374	0.0255

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.8339	0.4595	0.3311	0.2358
7	0.8325	0.4587	0.3307	0.2354
14	0.8313	0.458	0.3302	0.2351
21	0.8299	0.4573	0.3297	0.2348
28	0.8286	0.4566	0.3292	0.2345
56	0.8235	0.4538	0.3275	0.2331

central

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.1201	0.0606	0.041	0.0262
7	0.1198	0.0604	0.0409	0.0262
14	0.1196	0.0603	0.0408	0.0261
21	0.1193	0.0602	0.0408	0.0261
28	0.1191	0.0601	0.0407	0.026
56	0.1181	0.0596	0.0403	0.0258

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.7476	0.3785	0.2556	0.1633
7	0.7461	0.3778	0.2551	0.163
14	0.7446	0.377	0.2546	0.1627
21	0.7431	0.3763	0.2541	0.1623
28	0.7416	0.3755	0.2535	0.162
56	0.7357	0.3725	0.2515	0.1607

south

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.1795	0.0841	0.0516	0.0288
7	0.1787	0.0839	0.0515	0.0288
14	0.178	0.0835	0.0513	0.0287
21	0.1773	0.0833	0.0511	0.0286
28	0.1768	0.083	0.0509	0.0285
56	0.1743	0.082	0.0503	0.0282

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.4814	0.236	0.1556	0.095
7	0.48	0.2354	0.1552	0.0949
14	0.4786	0.2349	0.1549	0.0947
21	0.4777	0.2344	0.1545	0.0944
28	0.4766	0.2339	0.1542	0.0942
56	0.4718	0.2318	0.1528	0.0934

Table 3SM. PEC-s of Cyclaniliprole in total soil (mg/kg) and pore water (mg/L) for european soils at different times (0-56 days) and dephts (1-20 cm) after a recommended worst case cyclaniliprole application on tomatoes.

north

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.3985	0.2067	0.1422	0.094
7	0.3977	0.2064	0.1419	0.0939
14	0.397	0.206	0.1417	0.0937
21	0.3962	0.2056	0.1414	0.0935
28	0.3955	0.2052	0.1412	0.0934
56	0.3926	0.2037	0.1401	0.0927

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	3.1961	1.7728	1.2899	0.8971
7	3.192	1.7705	1.2883	0.8959
14	3.1879	1.7683	1.2868	0.8946
21	3.1838	1.766	1.2849	0.8932
28	3.1797	1.7634	1.2832	0.8918
56	3.1633	1.7544	1.2766	0.8862

central

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.5213	0.2563	0.1691	0.1045
7	0.5201	0.2558	0.1687	0.1042
14	0.5188	0.2552	0.1683	0.104
21	0.5175	0.2546	0.1679	0.1037
28	0.5162	0.2539	0.1676	0.1035
56	0.5111	0.2515	0.1661	0.1026

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	2.3576	1.1809	0.7901	0.4995
7	2.3526	1.1784	0.7885	0.4985
14	2.3476	1.1759	0.7868	0.4975
21	2.3427	1.1733	0.7852	0.4965
28	2.3377	1.1708	0.7835	0.4955
56	2.318	1.161	0.7769	0.4915

south

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.8472	0.3769	0.2213	0.1145
7	0.8433	0.3753	0.2203	0.1141
14	0.8394	0.3735	0.2193	0.1137
21	0.8355	0.3718	0.2182	0.1133
28	0.8317	0.3701	0.2172	0.1129
56	0.8165	0.3634	0.2131	0.1114

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	1.5923	0.779	0.5105	0.3101
7	1.5883	0.7771	0.5093	0.3094
14	1.5846	0.7752	0.5081	0.3087
21	1.5808	0.7734	0.5069	0.308
28	1.5771	0.7715	0.5057	0.3073
56	1.5622	0.7643	0.501	0.3045

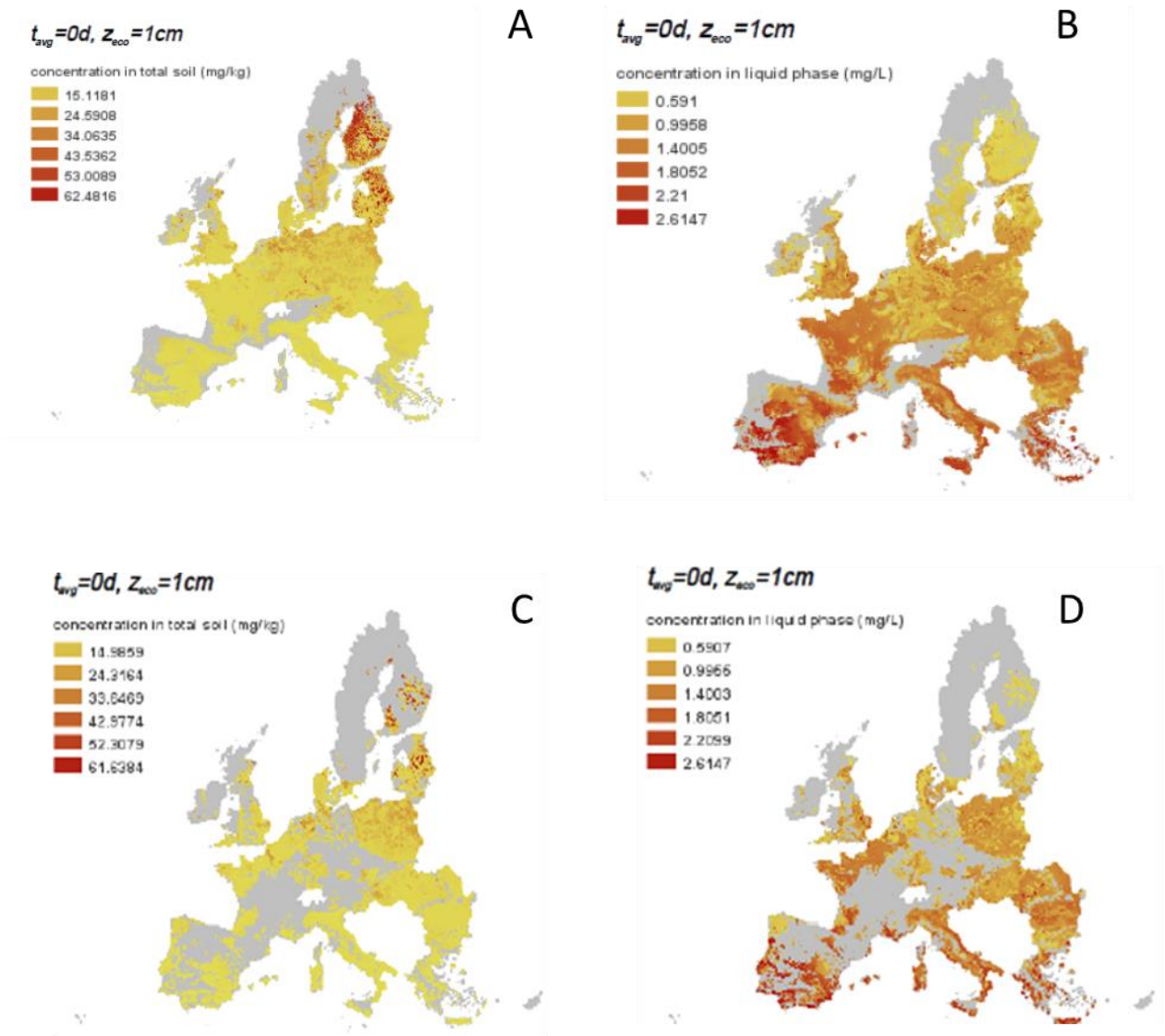

Figure 3SM. PEC-s of picoxystrobin in soil (mg/kg) and pore water (mg/L), after a recommended worst case (T0 and 1 cm) application on spring cereals (A, B) and tomatoes (C,D) in northern, central and south European agricultural soils.

Table 4SM. PEC-s of picoxystrobin in total soil (mg/kg) and pore water (mg/L) for european soils at different times (0-56 days) and dephts (1-20 cm) after a recommended worst case picoxystrobin application on spring cereals.

north

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	1.007	0.4051	0.2045	0.0541
7	0.9818	0.395	0.1995	0.0528
14	0.9577	0.3854	0.1946	0.0515
21	0.9346	0.376	0.1898	0.0503
28	0.9121	0.3669	0.1852	0.0491
56	0.8288	0.3335	0.1685	0.0449

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	16.7194	6.7735	3.4578	0.9703
7	16.4157	6.6505	3.3948	0.9526
14	16.1194	6.5299	3.333	0.9352
21	15.8303	6.4123	3.2729	0.9182
28	15.5463	6.297	3.2144	0.9016
56	14.4783	5.8651	2.9934	0.8396

central

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	1.2135	0.4879	0.2458	0.0644
7	1.1815	0.4748	0.2393	0.0627
14	1.1504	0.4624	0.233	0.0611
21	1.1205	0.4503	0.2269	0.0595
28	1.0914	0.4386	0.221	0.0579
56	0.9851	0.396	0.1996	0.0525

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	9.7663	3.9275	1.9851	0.5271
7	9.5205	3.8342	1.9359	0.5146
14	9.2961	3.7397	1.8863	0.5026
21	9.0669	3.649	1.843	0.4913
28	8.8517	3.5617	1.7994	0.4801
56	8.0557	3.2437	1.6394	0.4399

south

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	2.0196	0.8091	0.4048	0.1018
7	1.9264	0.7712	0.3862	0.0973
14	1.8446	0.7386	0.3699	0.0931
21	1.7666	0.7072	0.3541	0.0892
28	1.6919	0.6774	0.3393	0.0854
56	1.436	0.5749	0.2879	0.0725

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	7.9433	3.1895	1.6048	0.4158
7	7.7167	3.0983	1.5588	0.4037
14	7.4976	3.0102	1.5141	0.3921
21	7.2849	2.9246	1.4711	0.3809
28	7.08	2.8423	1.4297	0.3702
56	6.3344	2.5428	1.2789	0.331

Table 5SM. PEC-s of picoxystrobin in total soil (mg/kg) and pore water (mg/L) for european soils at different times (0-56 days) and dephts (1-20 cm) after a recommended worst case picoxystrobin application on tomatoes.

north

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	0.984	0.3962	0.2002	0.0533
7	0.9606	0.3867	0.1954	0.052
14	0.9378	0.3775	0.1908	0.0509
21	0.9158	0.3687	0.1863	0.0498
28	0.8944	0.3601	0.182	0.0487
56	0.8155	0.3284	0.166	0.0445

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	13.3356	5.4268	2.7793	0.7962
7	13.1252	5.3347	2.7311	0.7829
14	12.9191	5.2439	2.6851	0.7699
21	12.7006	5.155	2.6405	0.7572
28	12.4862	5.0672	2.595	0.7448
56	11.683	4.7413	2.4306	0.6978

central

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	1.3505	0.5421	0.2727	0.0704
7	1.3113	0.5264	0.2648	0.0683
14	1.2737	0.5113	0.2568	0.0663
21	1.2356	0.4959	0.2492	0.0642
28	1.1986	0.4809	0.2418	0.0623
56	1.0666	0.428	0.2151	0.0557

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	10.9317	4.3918	2.2119	0.5771
7	10.6339	4.2722	2.1517	0.5612
14	10.3471	4.157	2.0936	0.5461
21	10.0704	4.0459	2.0377	0.5315
28	9.8037	3.9385	1.9834	0.5174
56	8.8246	3.5452	1.7855	0.4659

south

concentration in liquid phase C_L (mg/L)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	2.4405	0.9763	0.4883	0.1222
7	2.2976	0.9191	0.4597	0.1151
14	2.1658	0.8664	0.4333	0.1085
21	2.0444	0.8179	0.409	0.1024
28	1.9327	0.7732	0.3867	0.0968
56	1.5678	0.6273	0.3138	0.0786

concentration in total soil C_T (mg/kg)

t_{av} (d)	z_{av} (cm) 1	2.5	5	20
0	7.5293	3.0223	1.5198	0.3911
7	7.3084	2.9334	1.4749	0.3791
14	7.0892	2.8451	1.4286	0.3677
21	6.8683	2.7542	1.3832	0.3564
28	6.6545	2.6699	1.3422	0.3453
56	5.9129	2.3718	1.1913	0.3071

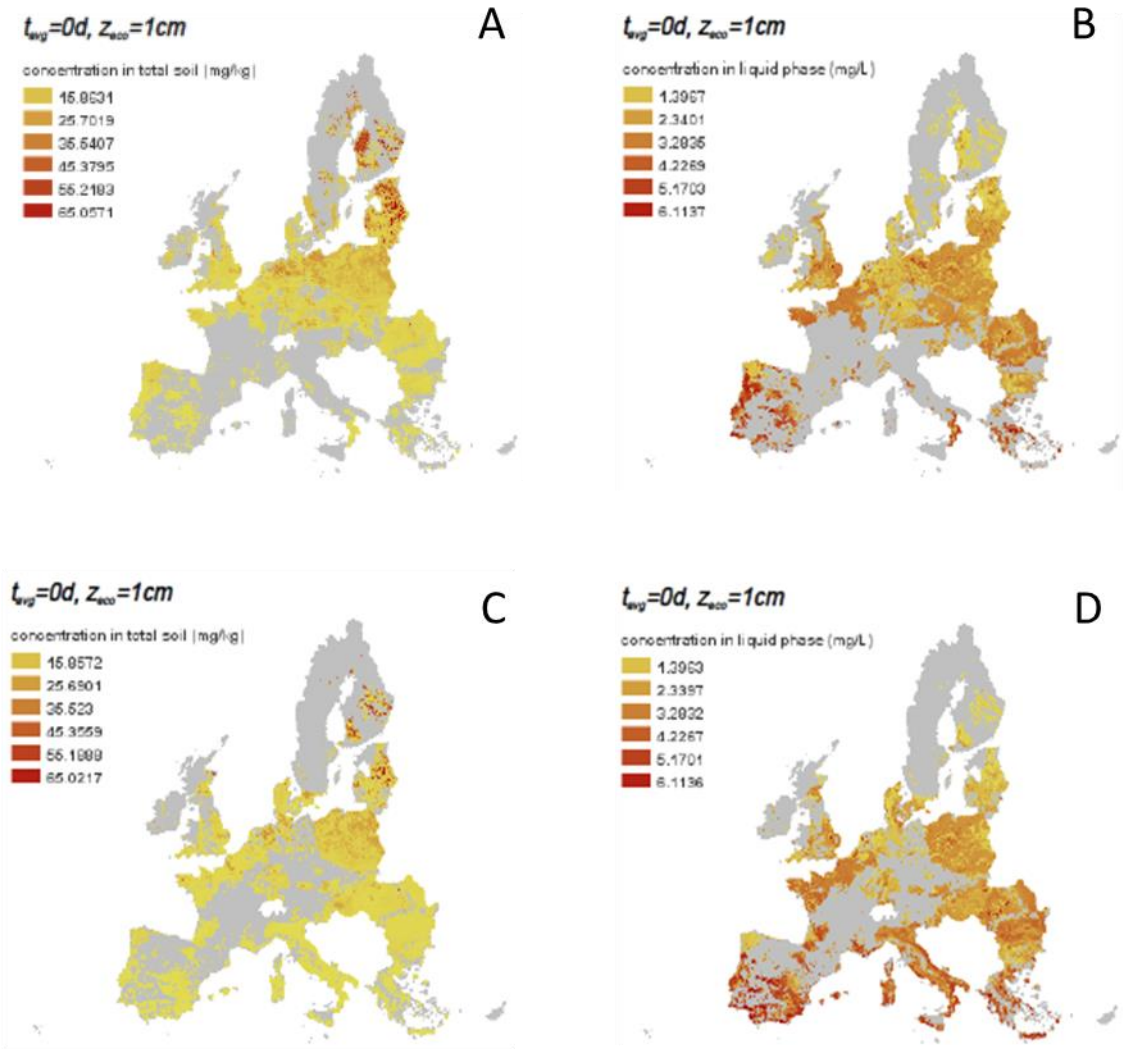

Figure 4SM. PEC-s of fenamidone in soil (mg/kg) and pore water (mg/L), after a recommended worst case (T0 and 1 cm) application on potatoes (A, B) and tomatoes (C,D) in northern, central and south European agricultural soils.

Table 6SM. PEC-s of fenamidone in total soil (mg/kg) and pore water (mg/L) for european soils at different times (0-56 days) and dephts (1-20 cm) after a recommended worst case fenamidone application on tomatoes.

north

concentration in liquid phase C_L (mg/L)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	2.3563	0.9427	0.4715	0.1181
7	2.2241	0.8898	0.445	0.1114
14	2.1018	0.8409	0.4205	0.1053
21	1.9889	0.7957	0.398	0.0997
28	1.884	0.7537	0.377	0.0944
56	1.5452	0.6183	0.3093	0.0775

concentration in total soil C_T (mg/kg)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	14.0555	5.63	2.8216	0.7152
7	13.5303	5.4197	2.7162	0.6859
14	13.0033	5.206	2.6075	0.6585
21	12.4891	5.0012	2.5049	0.6325
28	12.0098	4.8081	2.4077	0.6086
56	10.3401	4.1406	2.0741	0.5243

central

concentration in liquid phase C_L (mg/L)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	3.2099	1.284	0.6421	0.1606
7	2.9917	1.1967	0.5984	0.1496
14	2.7717	1.1087	0.5544	0.1387
21	2.5874	1.035	0.5175	0.1294
28	2.4156	0.9663	0.4832	0.1208
56	1.8777	0.7511	0.3756	0.094

concentration in total soil C_T (mg/kg)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	11.612	4.6451	2.3228	0.5811
7	10.8714	4.3489	2.1747	0.5441
14	10.192	4.0771	2.0388	0.5101
21	9.5704	3.8284	1.9144	0.479
28	8.9963	3.5988	1.7996	0.4502
56	7.1394	2.856	1.4281	0.3573

south

concentration in liquid phase C_L (mg/L)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	5.659	2.2636	1.1318	0.283
7	4.9064	1.9626	0.9813	0.2453
14	4.2886	1.7154	0.8577	0.2144
21	3.7836	1.5134	0.7567	0.1892
28	3.3513	1.3405	0.6703	0.1676
56	2.2631	0.9052	0.4526	0.1132

concentration in total soil C_T (mg/kg)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	8.0023	3.2011	1.6007	0.4003
7	7.4454	2.9783	1.4893	0.3725
14	6.9056	2.7624	1.3813	0.3455
21	6.4352	2.5742	1.2872	0.3219
28	6.0002	2.4002	1.2002	0.3001
56	4.6567	1.8628	0.9315	0.2329

Table 7SM. PEC-s of fenamidone in total soil (mg/kg) and pore water (mg/L) for european soils at different times (0-56 days) and dephts (1-20 cm) after a recommended worst case fenamidone application on potatoes.

north

concentration in total soil C_T (mg/kg)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	15.7298	6.2939	3.1486	0.7896
7	14.9296	5.9737	2.9884	0.7494
14	14.1832	5.675	2.839	0.7119
21	13.4866	5.3963	2.6995	0.677
28	12.8359	5.1359	2.5693	0.6443
56	10.7209	4.2922	2.1498	0.5448

concentration in liquid phase C_L (mg/L)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	2.2917	0.9171	0.4589	0.1152
7	2.1627	0.8735	0.4371	0.1098
14	2.0604	0.8325	0.4166	0.1045
21	1.9827	0.7934	0.397	0.0997
28	1.892	0.7571	0.3788	0.0949
56	1.5622	0.6251	0.3129	0.0787

central

concentration in total soil C_T (mg/kg)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	14.6375	5.8556	2.9283	0.7329
7	13.7538	5.5021	2.7515	0.6886
14	12.9407	5.1768	2.5889	0.6479
21	12.1916	4.8771	2.439	0.6104
28	11.5015	4.6011	2.301	0.5759
56	9.2274	3.6913	1.8459	0.4619

concentration in liquid phase C_L (mg/L)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	2.9469	1.1788	0.5894	0.1474
7	2.7478	1.0992	0.5497	0.1375
14	2.5678	1.0272	0.5136	0.1285
21	2.4084	0.9634	0.4818	0.1205
28	2.2677	0.9072	0.4537	0.1135
56	1.8108	0.7245	0.3623	0.0907

south

concentration in total soil C_T (mg/kg)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	9.7695	3.9078	1.9539	0.4885
7	9.0312	3.6125	1.8064	0.4518
14	8.3844	3.3539	1.677	0.4195
21	7.7907	3.1165	1.5585	0.3899
28	7.2765	2.9109	1.4557	0.3642
56	5.6693	2.2681	1.1343	0.2837

concentration in liquid phase C_L (mg/L)

t_{sc} (d)	z_{sc} (cm) 1	2.5	5	20
0	4.6745	1.8698	0.9349	0.2337
7	4.1972	1.6789	0.8394	0.2099
14	3.798	1.5192	0.7596	0.1899
21	3.4748	1.3899	0.695	0.1737
28	3.1399	1.256	0.628	0.157
56	2.288	0.9152	0.4576	0.1144

CHAPTER 5

Potential Ecosystem Service losses considering non-target organisms affection after PPP application in European soils

The present research has been prepared to be published as:

Urionabarrenetxea, E., Casás, C., Garcia-Velasco, N., Santos, M., Tarazona, J.V., Soto, M. *In preparation*. Potential Ecosystem Service losses considering non-target organisms affection after PPP application in European soils.

ABSTRACT

The increased usage of Plant Protection Products (PPPs) will lead to high exposure scenarios and thus, major accumulations in soil organisms, including non-target species. Assessment of the effects of PPPs on non-target organisms is one of the most important components of environmental risk assessment (ERA) since they play crucial functions in ecosystems, being main driving forces in different soil processes. In this framework, EFSA is proposing the use of the Ecosystem Services approach for setting specific protection goals. In fact, these services can be minimized, altered and degraded due to the misuse of PPPs in agroecosystems, principally by affecting soil function developing taxa. Thus, the aim of this work was to assess PPPs potential risk upon Ecosystem Services along European soils, considering impacts on earthworms and collembola. For that, four well-known and widely used pesticides (2 insecticide-esfenvalerate and cyclaniliprole- and 2 fungicide-picoxystrobin and fenamidone-) worst case application was studied; accounting for their MoA, predicted exposure, time-course effects in *Eisenia fetida* and *Folsomia sp.* and landscape variability. The selected fungicides exerted more effects than insecticides in *E. fetida*, whereas few reports were found for both pesticides regarding *Folsomia sp*. According to the reported effects, the most impacted Ecosystem Services after PPP application to crops appeared to be habitat provision, soil formation & retention, nutrient cycling, biodiversity, erosion regulation, soil remediation/waste treatment and pest & disease regulation. The spatial variability among European agricultural soil (pH, OM, temperature), PPPs physicochemical properties (MoA, Kom, solubility…), and non-target species behavior, habitat and role in ecosystem seemed to be the main factors to be taken into account for a correct PPP use management in crops.

Keywords: ERA, pesticides, Ecosystem Services, non-target sp., landscape variability

LABURPENA

Landare-osasunerako produktuen (PPP) emendioak, esposizio eszenarioen ugaritzea eta agente kimikoen metaketa eragiten du lurzoru organismoetan. Horrela, PPPek itu ez diren organismoetan eragiten dituzten efektuak ebaluatzea, ingurumen arrisku ebaluazioen (ERA-Environmental risk assessment ingelesez) giltzarrietako bat da; izan ere, organismo hauek ekosistemen funtzionamendurako ezinbestekoak diren zenbait prozesu burutzen dituzte. Harira, EFSAk (*European Food Safety Authority* ingelesez) zerbitzu ekosistemikoen erabilpena proposatzen du babes neurri zehatzen ezarpen egoki bat egite aldera. Izan ere, PPP-en isurketa ondorioz lurzoru funtzioak garatzen dituzten organismoak/taxak kaltetzen badira, hainbat zerbitzu ekosistemikok kalteak paira ditzakete. Horrela, PPPek zerbitzu ekosistemikoetan eragin dezaketen arriskua ebaluatzea bilatzen da lan honetan, horretarako, Europako nekazal lur ezberdinetan zizare eta kolenbolo organismoek paira ditzaketen efektuak aitzat hartuz. Ezagunak diren eta erabilera hedatua daukaten 4 pestiziden isurketa (2 insektizida -esfenvalerate eta cyclaniliprole- eta 2 fungizida -picoxystrobin eta fenamidone-) ikertzen da (onartutako isurketarik txarrena); berauen MoA (*Mode of Action* ingelesez), aurreikusitako kontzentrazioak, Europan zeharreko lur ezaugarrien aldakortasuna eta denboran zeharreko *Eisenia fetida* eta *Folsomia sp*-en gaineko efektuak kontuan izanik. Aukeratutako fungizidek, insektizidek baino inpaktu gehiago eragin zituzten *E. fetidan; Folsomia sp*. organismoekin burututako lanak urriak diren heinean. PPPen erabilera osteko efektu potentzialak ikertu ondoren, habitaten hornitzea, lurzoru eratzea eta heltzea, elikagaien ziklatzea, biodibertsitatea, higaduraren erregulazioa, lurzoru erremediazioa/hondakinen tratamendua eta izurrite/gaixotasunen erregulazioa izan ziren nekazal lurretan inpaktu gehien pairatutako zerbitzu ekosistemikoak. Europear nekazal lurren aldakortasun espazialak (pH, MO, tenperatura), PPPen ezaugarri fisikokimikoek (MoA, Kom, disolbagarritasuna…) eta itu espezieak ez diren organismoen habitat, rol eta jokaerek PPPen toxikotasuna zeharo baldintzatzen dute; hortara, PPPen arrisku maneiuan kontuan izan beharreko faktoreak dira.

Hitz-gakoak: Ingurumen arrisku ebaluazioa, pestizidak, zerbitzu ekosistemikoak, paisaia aldagarritasuna

1-INTRODUCTION

The use of Plant Protection Products (PPPs) has contributed to the high production of food during the last decades, making it possible to feed the increasing world population. Nevertheless, the worldwide rice, wheat and maize harvests doubled production yields as fertilizers and pesticides uses increased 20 and 7 times, respectively (Oerke, 2006; Silva et al., 2019). Around the world 3M pesticide tons are applied annually (Pimentel, 2006), while 374K tons are sold only in the EU (Eurostat, 2018; Silva et al., 2019). In conventional agriculture, crops can be sprayed with different insecticide and fungicides multiple times in a single year (10 and 25 applications, respectively), with more than 40 a.s (active substances) applied per year (Van Drooge et al., 2001; Garthwaite et al., 2015; Meyer et al., 2020). This usage and application rates of PPP, will definitely lead to high exposure scenarios and therefore, major accumulations in organisms inhabiting soils (Morris et al., 2016). In fact, apart from on croplands where PPPs are directly spiked, effects could be exerted in adjacent agricultural areas, such as field margins, hedges, non-cropped patches, groundwater, ditches, streams and lakes, or also in areas far away due to long range transport of pesticides (EFSA, 2010). Hence, the increased use of PPPs, in extension and intensity, has simultaneously enhanced social awareness about their risks (EFSA Journal 2010).

In order to regulate PPPs, EC 1107/2009 regulation was approved to ensure high protection level for PPPs in the market (Kluxen et al., 2021). This regulation specifies the requirement to carry out an Environmental Risk Assessment (ERA) in order to register a pesticide in OECD countries (Streloke, 2011); in the EU, this procedure is regulated by the European Food Safety Authority (EFSA) (Mayer et al., 2020). ERA is mandatory for all relevant organisms and populations exposed to pesticides and their residues (Kluxen et al., 2021). A quarter of world's biodiversity are soil living organisms and among them, invertebrate communities are especially sensitive to ecosystem changes (Velasquez and Lavalle, 2019). Hence, the absence of a proper evaluation of PPPs risk to soil invertebrates could lead to an imbalance in the ecosystems, with the consequent biodiversity loss, and the affection of the offered services (TEEB, 2008). Ecosystems Services are the benefits that society obtains from ecosystems as support (nutrient cycling, primary production), provisioning (food provision), regulatory (purification of

water, climate regulation) or cultural services (recreational, educational) (Millenium Assessment Ecosystems, MAE 2005). In this framework, soil plays a key role providing several functions and services (EC, 2006). Therefore, based on the Panel on Plant Protection Products and their Residues (PPR proposal (EFSA 2010) and the Scientific Committee (EFSA Scientific Committee, 2016), EFSA is proposing the use of the Ecosystem Services approach for setting specific protection goals. However, assessing the risk derived from an Ecosystem Service loss is the biggest challenge in ERA (Ostrom, 2009), and requires the identification and analysis of the mode of action (MoA) of PPPs, toxic effects upon non-target organisms and landscape variations.

Deciphering the MoA of the PPPs allows knowing which is the pathway followed by the a.s. to produce damages at different levels of biological complexity (molecular, cellular, tissue, reproductive, feeding and/or motility impairments among others). These alterations can affect soil health by affecting soil living organisms; and lastly Ecosystem Services. Among soil indicator organisms, *Eisenia fetida* earthworms and *Folsomia sp*. collembolans are widely used as non-target species to address the potential environmental risks of PPPs result of agricultural practicing (Ockleford et al., 2017). In this sense, EFSA panel has already developed concrete proposals covering different guidelines (EFSA PPR Panel, 2014; 2015 a, b), including standardized toxicity tests. Especially relevant tests are those promoted by OECD such as the Acute Toxicity (OECD-207) and Reproduction (OECD-222) tests with *E. fetida/andrei*; and Collembolan Reproduction test with *Folsomia sp.* (OECD-232) (Fountain & Hopking, 2005). This species are considered key organisms for the maintenance of soil functions and for the maintenance of supporting and provisioning services (Shroder et al., 2008; Pelosi et al., 2013; FAO and ITPS, 2015; Ockleford et al., 2017; Schon et al., 2017). Therefore, they could be used to address the potential Ecosystem Service losses derived from PPP application in soils.

Most of the soil functions and the Ecosystem Services derived from them depend on specific conditions such as climate, pH, organic matter (OM) content or the diversity of plants and organisms inhabiting soils (Pereira et al., 2018); and these may vary along European soils. Thus, environmental and ecological variabilities among Euroregions should be considered as they play a key role in assessing exposure and effects of PPPs.

In the present work the risk upon Ecosystem Services along European soils was assessed, considering impacts upon non-target organisms (*E. fetida* earthworms and *F. candida* collembolan) after 4 PPP (2 insecticide- esfenvalerate and cyclaniliprole-, and 2 fungicide- fenamidone and picoxystrobin-) worst case application into crops. For that, PPPs MoA, exposure, time-course effects and landscape variability were taken into account (Fig. 1).

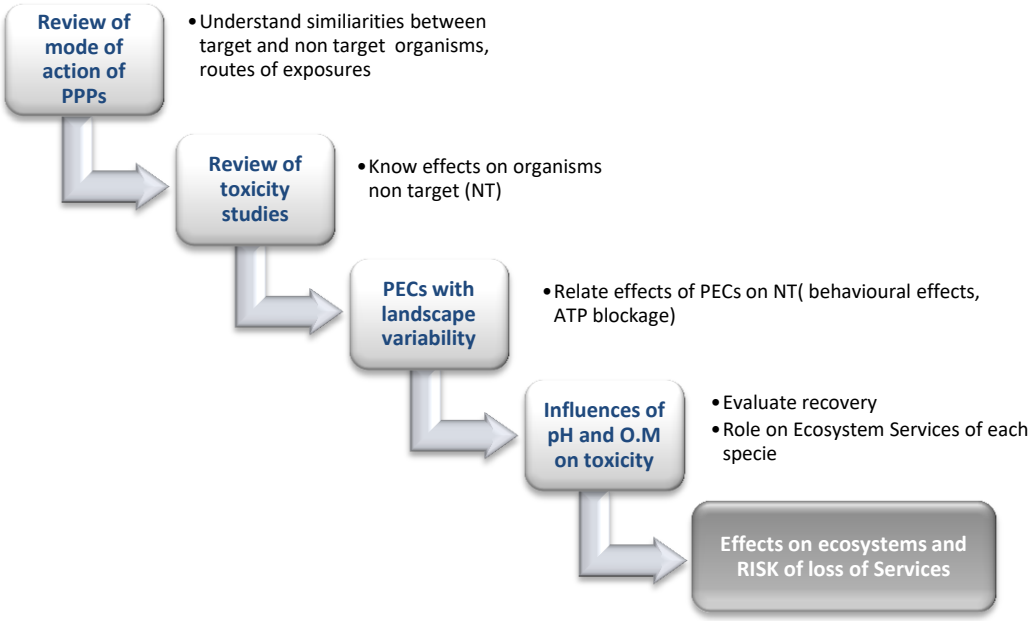

Figure 1: Process diagram. Activities to relate mode of action of PPP and the link with Ecosystem Services.

2.- MATERIALS AND METHODS

2.1. Review of PPP Mode of action and time course effects upon non-target organisms

Data regarding the MoA of esfenvalerate, cyclaniliprole, fenamidone and picoxystrobin was retrieved from available scientific literature and technical reports published in academic and environmental agency websites and databases (EFSA). In addition, exposure routes (of mentioned compounds) for target organisms were studied in order to extrapolate effects to non-target species. Information about geographical distribution of non-target organism according to characteristics of soil substratum, and modes of feeding and behavior were obtained from EFSA reports and scientific papers.

Ecotoxicological endpoints for *E. fetida* and *Folsomia sp*. at different PPP exposure times (7, 14, 28 and 56 days) were retrieved from toxicological studies and official guidelines made by and /or approved by EFSA, FAO and European Commission (Table 1. chapter 4). For each of the studies: test conditions (soil, pH, and temperature), compound purity, identified endpoints (NOEC, LOEC, LC_{50}) and observed effects were considered taking into account the most restrictive values. Chronic tests and acute toxicity tests were taken into account in the bibliographical survey.

2.2. Relating PPP exposure and potential effects to non-target organisms and Ecosystem Services along European soils

The toxicity data of pesticides on non-target organisms (2.1 section) was related with the predicted soil concentrations (PECs) obtained in previous works (Chapter 4). Additionally, the influence of each compound characteristics (e.g. pka, MoA) and European landscape variability (pH, organic matter, temperature) on toxicity was considered.

In order to assess the potential effects on Ecosystem Services, the Millennium Ecosystem Assessment (MEA; 2005) document was used, which listed Ecosystem Services in-crop and off-crop areas, and PPP potentially affected taxa for each service. From this list, services potentially affected (directly or indirectly-e.g. via trophic interactions-) were selected; choosing only the ones exhibiting a maximum importance (+++/+++) for the different spatial areas (in crop, off crop). Later, and having in mind the

MoA of the PPP and time-course effects (2.1 section), the potential impact of the selected pesticides upon ecosystem functions were determined. As PPP potentially affected taxa *E. fetida* and *Folsomia sp* were selected, representative for the impact upon earthworms and collembolans.

In a final step, a summarizing matrix was elaborated combining the pesticide, the non-target organism, the expected toxic effects for worst case PPP application in European soils and ecosystem functions and services affected.

3.- RESULTS AND DISCUSSION

3.1. Mode of action of PPPs

The MoAs of the different PPPs selected for this work have been illustrated in Table 1. The MoA can be defined as the functional change after the exposure of a living organism to a substance (Grant et al., 2010). Esfenvalerate belongs to the II group of pyrethroids chiral compounds, characterized by disruption of sodium channels in mammals, inhibiting their normal functioning (Casida et al., 2013; Bal-Price et al., 2017; Abreu-Villaça & Levin, 2017). Invertebrate sodium channels have similar biophysical properties to those of mammals, acquired before the evolutionary separation of the invertebrates from the vertebrates (Catterall, 2000); then, similar responses should be expected. Alike, the insecticide cyclaniliprole (diamine class) acts on the ryanodine receptors located in the endoplasmic reticulum of insects, inducing muscle paralysis by releasing the intracellular calcium necessary for muscle contraction (Qi et al.,2013; EFSA, 2015; Troczka et al., 2017). Indeed, ryanodine receptors are similar in insects, mammals and arthropods, leading to incapacity to increase pesticide efficiency without affecting non-target organisms (Casida & Durkin, 2013). Moreover, it is well known that calcium mobilization is a universally conserved (throughout the species) activation mechanism, essential in the signaling of immune cells (Opper et al., 2010). Thus, it is highly probable that cyclaniliprole behaves in a similar manner in invertebrates, causing a general paralysis of the organism.

Table 1. Summary of the Mode of Action of each PPP selected for the present work.

Type of PPP	**Mode of Action**	**Refs**
Esfenvalerate Insecticide (pyrethroid)	• Disruption Na channels by preventing the closure of the voltage-gated sodium channels in the axonal membranes. Hence, nerves cannot repolarize, leaving the axonal membrane permanently depolarized, thereby paralyzing the organism • Induces change in temperature and stomach disruption	Soderlund et al., 2002; Bal-Price et al. 2017 ; Casida et al. 2013; Abreu-Villaça, 2017.
Cyclaniliprole Insecticide (diamide)	• Enters in the organism through ingestion and by absorption through cuticle • Acts on the ryanodine receptors (*) located in the endoplasmic reticulum, inducing muscle paralysis by releasing the intracellular calcium necessary for muscle contraction	EFSA, 2015a ; Troczka et al. 2017 ; Opper et al. 2010
Picoxystrobin Fungicide (strobilurin)	• Inhibits mitochondrial respiration (block electron transfer between cytochrome b and cytochrome c1) # • disrupts the energy cycle within the fungus by halting production	Paramasivam & Chandrasekaran 2013; Tentu &

	of ATP. • Antisporulant	Tentu, 2016.
Fenamidone Fungicide (imidazolinone)	• Inhibits mitochondrial respiration by blocking electron transport at ubihydroquinone: cytochrome-c-oxidoreductase (Complex III).	

*Ryanodine receptors: responsible of Ca mobilization in the cell. Similar for some insects, mammals & arthropods.
Part of the cytochrome bc1 complex, located in the inner mitochondrial membrane of fungi and other eukaryotes that are present in a lot of organism from different species.

Regarding fungicides, picoxystrobin is a preventive and curative fungicide belonging to strobilurin group of chemicals, and fenamidone (imidazolinone class) is a systemic foliar fungicide. Both act inhibiting cytochrome bc1 complex in the inner mitochondrial membrane (also in fungi and other eukaryotes) responsible for cellular respiration of water molds and other fungal patogens (Paramasivam & Chandrasekaran, 2013; Tentu & Tentu, 2016). Once the inhibition occurs, the electron transference between cytochrome b and cytochrome c1 is blocked producing the disruption of the energy cycle and ATP production (Barlett et al., 2002). This alteration might affect individuals reproductive activity that itself implies enormous energy costs. So, may happen that ATP generation blockage could impact on the reproductive fitness of the species, decreasing the possibility to allocate this energy on individual growth or development.

3.2. Time course effects of PPP upon non-target organisms

At the individual level, survival, mortality (LC_{50}), fecundity, reproduction or growth are the main endpoints used to assess the toxicity of PPPs in soil macroinvertebrates (van Gestel, 1989; 2012). Behavior can be also taken into consideration in earthworms, with four main functions potentially affected by PPPs: avoidance behavior, burrowing behavior, bioturbation and burial of OM (Pelosi et al., 2014). However, in studies regarding pesticides effect on soil invertebrates summarized by Jänsch et al. (2006), 89% of studies used abundance and/or biomass as endpoint, followed by mortality (10%); while few studied behavior or development (< 1%). Among the most used tests, chronic test aiming sublethal effects must be highlighted, which are very sensitive and realistic for the prediction of environmental effects because exposure concentrations are usually quite low (Rombke et al., 2007). Both type of tests (acute and chronic) were available for *E. fetida* and *Folsomia sp*, although less information was

found for collembollans. Exposure concentration, time course effects and toxicity tests (both acute and chronic) applied to assess the toxicity of the selected PPP have been summarized in tables 2 and 3, for *E. fetida* and *Folsomia* sp., respectively.

Table 2. Exposure concentration, time course effects and toxicity tests applied to assess the toxicity of Esfenvalerate (A), Cyaniliprole (B), Picoxystrobin (C) and Fenamidone (D) on *Eisenia fetida*; with indication of the biological endpoints reported (NOEC, LOEC, EC, LCX) Legend: a.s., active substance.

(A) Esfenvalerate *Test conditions: 10% sphagnum peat, T: 19-20º·C (all references retrieved from EFSA, 2013).*						
Exposure	**Day 7**	**Day 14**	**Day 28**	**Day 56**	**Reference protocols**	**Endpoint summary**
0.07 -1.123 mg a.s./Kg soil	No effect	No effect	No effect	No effect	Teixeira (2003) Chronic. 56d OECD 222	NOEC: 1.123 mg a.s./Kg soil LOECmortality >5 mg a.s./Kg LC50 (7 days):13.6 mg a.s./Kg LC50 (14 days):10.625 mg a.s./Kg
3.125 - 50 mg a.s. /Kg of soil	Mortality rise up to 100% since 7 d	Weight loss above 125 mg/Kg			Whuthrich (1991) ATT, 14 d OECD 207	
5 - 50 mg a.s. /Kg of soil	Mortality.	Mortality Weight loss			Petto (1994). ATT. 14 d. OECD 207	
(B) Cyclaniliprole *Test conditions: sphagnum peat 5 -10%, temperature was 18-22º· C (all references retrieved from: EFSA, 2015a)*						
Exposure	**Day 7**	**Day 14**	**Day 28**	**Day 56**	**Reference protocols**	**Endpoint summary**
2.89 - 46.3 mg a.s. /Kg soil	No effects	No effects			Lührs (2012). ATT 14 d, OECD 207, ISO 11268-1	NOEC: 957.1 mg a.s./Kg soil
From 90.92 to 957.1 mg a.s./Kg soil	No effects	No effects	No effects	No effects	Lührs, Meinerling, 2012 OECD 222 ISO 11268-2	
957.1 mg a.s./Kg soil	No effects	No effects			Lührs, 2011 ATT 14 d. OECD 207. ISO 11268-1	
(C) Picoxystrobin *Tests conditions: sphagnum peat concentrations 10%; temperature, 22°C (retrieved from: EFSA, 2015b)*						
Exposure	**Day 7**	**Day 14**	**Day 28**	**Day 56**	**Reference protocols**	**Endpoint summary**
0.16 - 1.25 mg a.s./Kg soil	No effects	No effects	No effects	No effects	Friedrich (2003). ISO 11268-2. Jackson & Coulson (1998). ATT 14d. OECD 207	NOEC: 0.63 mg a.s./Kg soil ECreproduction: 1.25 mg a.s./Kg soil LOECmortality: 3.2 mg a.s./Kg soil LC50: 6.1 mg a.s./Kg soil
1.25 - 2.5 mg a.s./Kg soil	No effects	No effects	No effects	Reproduction	Friedrich, 2003. ISO 11268-2 (1998)	
5 mg a.s./Kg soil	No effects	No effects	Mortality (12.5%) but not sign. to control.	Reproduction		
3.2 - 10 mg a.s./Kg soil	Mortality. Behavioral alter.	Mortality. Behavioral alter.	Mortality. Behavioral alter.		Jackson & Coulson, 1998. ATT 14d. OECD 207	
(D) Fenamidone *Tests conditions: Sphagnum peat: 5-10%; temperature: 18-22·°C (all references retrieved from: EFSA, 2015c).*						
Exposure	**Day 7**	**Day 14**	**Day 28**	**Day 56**	**Reference protocols**	**NOEC**
0.628 mg a.s./Kg soil	No effects	No effects	No effects	No effects	Gossmann & Luehrs, 1998 ISO 11268-2 (Draft 1995), BBA VI 2-2 (1994)	NOEC: 0.628 mg a.s./Kg soil ECreproduction: 1.247 mg a.s./Kg soil ECmortality: 4.99 mg a.s./Kg soil
1.247 mg a.s./Kg soil	No effects	No effects	No effects	Reproduction		
2.495 mg a.s./Kg soil	No effect	No effects	Lose weight trend	Reproduction		
4.99 mg a.s./Kg soil	No effects	No effects	Mortality	Reproduction		
9.98 mg a.s./Kg soil	No effects	Weight loss		Reproduction		

Table 3: Exposure concentration, time course effects and toxicity tests applied to assess the toxicity of Esfenvalerate, Cyclaniliprole, Picoxystrobin and Fenamidone on *Folsomia sp*.; with indication of the biological endpoints reported (NOEC, LOEC, EC, LCX) Legend: a.s., active substance.

(A) Esfenvalerate Test conditions: %5 sphagnum peat, %20 Kaolin clay, % 74,7 fine quartz sand, %0,3 calcium carbonate. T=18-22·C (retrieved from: EFSA, 2013)						
Exposure	**Day 7**	**Day 14**	**Day 28 (adults)**	**Day 56 (youngers)**	**Reference, test protocols**	**Endpoint summary**
0.349 mg a.s./Kg	No effects	No effects	No effects	No effects	Luhrs (2010). OECD 232	NOEC: 0.349 mg a.s./Kgsoil LOECreproduction: 0.698 mg a.s./Kgsoil
0.698 mg a.s./Kg	No effects	No effects	No effects	Effects on reproduction		
1.405 mg a.s./Kg soil	No effects	No effects	No effects	Effects on reproduction		
2.811 mg a.s./Kg soil	No effects	No effects	No effects	Effects on reproduction		
5.62 mg a.s./Kg	No effects	No effects	No effects	Effects on reproduction		
(B) Cyclaniliprole Test conditions: %5 peat, 30g wet weight/100 ml glass beaker, Feed: after day 0 and 14: 2mg granulated dry yeast per test vessel (retrieved from: EFSA, 2015a)						
Exposure	**Day 7**	**Day 14**	**Day 28 (adults)**	**Day 56 (youngers)**	**Reference, test protocols**	**Endpoint summary**
From 0.625 to 2.5 mg a.s. /Kg soil	No effects	No effects	No effects	No effects	Ganßmann, 2012 OECD-232 ISO 11267-1999	NOEC: 2.5 mg a.s./Kgsoil ECreproduction: 5 mg a.s./Kg soil ECmortality: 10 mg a.s./Kg soil
5 mg a.s. /Kg soil	No effects	No effects	No effects	No mortality Reduction in number		
10 mg a.s. /Kg soil	No effects	No effects	Mortality	Mortality. Reduction in number		
(C) Picoxystrobin Test conditions: sphagnum peat= 5%; temperature 18-22°C (retrieved from: EFSA, 2015b)						
Exposure	**Day 7**	**Day 14**	**Day 28 (adults)**	**Day 56 (youngers)**	**Reference, test protocols**	**Enpoint summary**
From 2.5 to 20 mg a.s. /Kg soil			No effects	No effects	Lührs (2012) ISO 11267 (1999) OECD 232 (2009)	NOEC: 20 mg a.s./Kgsoil ECreproduction: 40 mg a.s./Kg soil
40 mg a.s. /Kg soil			No effects	Significative differences on reproductive capacity respect to control (29% reduction).		
(D) Fenamidone Test conditions: sphagnum peat= 5%; temperature= 20-22 °C (retrieved from: EFSA, 2015c)						
Exposure	**Day 7**	**Day 14**	**Day 28 (adults)**	**Day 56 (youngers)**	**Reference, test protocols**	**Endpoint summary**
99,8 mg a.s. /Kg soil			No effects	No effects	Frommholz (2011). OECD 232 (2009)	NOEC: 99.8 mg a.s./Kgsoil

3.2.1 Insecticides: esfenvalerate and cyclaniliprole

The studies in EFSA databases regarding esfenvalerate toxicity on *E. fetida* reported exposure concentrations ranging 0.07 and 50 mg a.s. /Kg soil. All toxicity tests (except Stabler 2009, in EFSA 2013) were carried at 10% of sphagnum peat and 19-20°C conditions, considering a product purity of around 5%. The NOEC reported was 1.132 mg a.s. /Kg of soil. Drastic effects (mortality and severe weight loss) were detected at medium exposure levels (3.125-50 mg/Kg, 7-14 d) while higher concentrations (>50 mg/Kg) exerted mortality at shorter exposure periods (7 d; Table 2A). Nevertheless, other sub-lethal effects such as difficulties to reach maturity have been detected in earthworms at low esfenvalerate exposure levels; even lower concentrations than those evaluated in reviewed toxicity assays (Schnug et al., 2014). This controversy deserves further research to establish accurate toxicity endpoints at different biological complexity levels.

Regarding collembolans, exposure concentrations in toxicity tests varied from 0.349 to 5.62 mg a.s./Kg, with a NOEC stablished at 0.349 mg a.s./Kg. Effects on reproduction were detected at high exposure levels (0.698-5.62 mg/Kg for 56 d, Table 3A).

The effects produced by cyclaniliprole in *E. fetida* (Lührs, 2011; 2012; Lührs, Meinerling 2012, EFSA, 2015a) were assessed at exposure concentrations ranging 2.89 - 957.1 mg a.s./Kg soil. NOEC was estimated at 957.1 mg a.s./Kg (Table 2B) so no evidence for lethal and sublethal effects was observed in all the toxicity studies reviewed for *E. fetida*. This might be due to the low solubility of the compound making it barely available for organisms. Although, oxidative stress glimpses have been observed in earthworms after diamide exposure with the same MoA (Liu et al., 2018), more information is needed to understand the toxicity mechanisms of the compound in these organisms. Meanwhile, the range of cyclaniliprole exposure concentrations for *Folsomia sp*. was between 0.625 and 10 mg a.s./Kg with the NOEC stablished at: 2.5 mg a.s./Kg soil. For reproduction and mortality, two ECs were determined as toxicity endpoints: 5 mg a.s./Kg for reproduction and 10 mg a.s./Kg for mortality. Exposures up to 10 mg/Kg did not produce mortality although a significant reduction in the number of juveniles was recorded after 56 d (Table 3B). At concentrations higher than 10 mg/Kg, enhanced

mortality and a significant reduction in the number of juveniles were observed in *Folsomia sp.* (Ganßmann, 2012; EFSA, 2015_a).

3.2.2. Fungicides: Picoxystrobin and Fenamidone

A concentration range between 0.16 and 10 mg/Kg was studied for picoxystrobin, estimating the NOEC for *E. fetida* at 0.63 mg/Kg. The reproductive capacity appeared to be affected after 56 d at 1.25-2.5 mg/Kg, while mortality was recorded at higher concentrations (LC50 6.1 mg/Kg) Table 2C). The toxicity of picoxystrobin for *F. candida* was tested in a range of exposure concentrations between 2.5 mg and 40 mg a.s/Kg (Lührs, 2012 retrieved from EFSA, 2015b). NOEC was established at 20 mg a.s/Kg, while significant differences on reproductive capacity were only observed after 56 d of exposure to the highest dose (40 mg/Kg, Table 3C).

Studies regarding fenamidone effects in *E. fetida* ranged concentrations between 0.628 and 9.98 mg a.s./Kg (Table 2D) with a NOEC at 0.628 mg a.s./Kg. Weight loss, impacts on reproduction and mortality effects were recorded, following time/concentration (exposure level) dependent trend: effects were observed in low-medium concentrations ($EC_{reproduction}$: 1.247 mg a.s./Kg) at longer periods (28-56 d); while, exposure to higher concentrations ($EC_{mortality}$: 4.99 mg a.s./Kg) exerted impacts at shorter periods (14 d). For *F.candida,* 99.8 mg a.s./Kg concentration used in the unique test available did not produce behavioral nor reproductive effects; so this concentration was established as NOEC (Table 3D). The lack of available data on *Folsomia sp*. should be susceptible of being improved with empirical data in the near future.

Among the studied compounds, effects produced by fungicides in *E. fetida* were more easily detected in comparison with insecticides caused impairments (Bünemann et al.,2006; Jänsch et al., 2006; Pelosi et al.,2014). In contrast, *Folsomia sp*. seems to be less affected by fungicides, while insecticides affected reproductive capacity after chronic exposures.

3.3- Potential Ecosystem Services loss derived from the PPP application in European soils

Agricultural landscapes provide several important Ecosystem Services, which could be significantly affected by the massive use of PPPs. The main affections would be

mostly associated with biodiversity losses, affecting organisms playing main roles (e.g. bioturbators, shredders) and root biota (Coleman et al.,2004). In this context, the Water Framework Directive (2000/60/EC) established that ecosystems protection level will be defined by the protection of the most sensitive species; among them earthworms and collembolans must be highlighted due to their important role on several ecosystem functions in crop and off crop areas (EFSA, 2010). Earthworms contribute to gene pool and biodiversity, play a key role in soil formation and retention, nutrient cycling, erosion regulation, soil remediation (or waste treatment) and habitat provision (EFSA, 2010; Wang et al, 2012; Ockleford et al., 2017). Meanwhile, collembolans are important for the maintenance of pest and disease regulation (food support), the nutrient cycling, biodiversity (Filser, 2002; Ockleford et al., 2017), habitat provision, soil formation and retention (EFSA, 2010).

For esfenvalerate PECs in Europe (ranging 1.123-3.125 mg a.s./Kg) no ecotoxicological data was reported in EFSAs databases for *Eisenia sp*. Even if at lower concentration no effects were detected, no impacts on Ecosystem Services could be discussed due to lack of data in the mentioned range (Table 4). In some northern spots (Finland, Sweden, Estonia, Latvia and Lithuania), concentrations from 5 mg a.s./Kg onwards could be expected, and thus mortality and weight loss could occur (LOEC>5 mg a.s./Kg). From this concentration on, supporting, regulating and provisioning services could be affected; principally by affecting habitat provision, soil formation & retention, nutrient cycling, erosion regulation, soil remediation/waste treatment and biodiversity (Table 4). Thus, in those spots with concentrations >5 mg a.s./Kg soil, a specific soil management could be required in order to recover a proper soil functioning. Meanwhile, esfenvalerate could induce reproduction impairment in collembolans for PECs in Europe ranging 1.343-5.565 mg a.s./Kg soil. Thus, pest & disease regulation, biodiversity, habitat provision, soil formation & retention, or nutrient cycling carried out by *Folsomia sp.* could be affected; impacting soil supporting, regulating and provisioning services.

Cyclaniliprole would not exert deleterious impacts upon earthworms after a worst case PPP application in European soils; however, multiple impacts could be expected for collembolan. For the lower PECs (close to 3.381 mg a.s./Kg soil) no effects

would be estimated in collembolans; while, concentrations ranging 5.66-7.957 mg a.s./Kg (spots in Germany, Poland, Estonia, Latvia and Lithuania) would exert reproductive impairment (Table 4). Meanwhile concentrations ranging 10.24-14.82 mg a.s./Kg soil (hot spots in Finland, Estonia and Latvia) could suppose significant collembolans mortality. In these specific spots within northern Europe, reproductive and lethal effects upon collembolans could impact on supporting, regulating and provisioning services (by affecting pest & disease regulation, habitat provision, soil formation & retention, nutrient cycling and biodiversity; Table 4).

Matching ecotoxicological data and picoxystrobin PECs, potential mortalities for earthworms are expected in European concentrations (14.58-61.63 mg a.s./Kg). Thus, affecting soil Ecosystem Services; principally supporting, regulating and provisioning ones (Table 4). For collembolans no effects were expected for main picoxystrobin concentrations along Europe (14.58-20 mg a.s./Kg soil), whereas reproductive impairment could be expected in agricultural areas with 42.97-61.63 mg a.s./Kg soil; affecting pest & disease regulation, habitat provision, soil formation & retention, nutrient cycling and biodiversity. It must be highlighted that the lack of ecotoxicological endpoints for European concentrations ranging 24.91- 33.64 mg a.s./Kg (mainly in certain areas of Germany, Poland, Estonia, Latvia and Lithuania) made impossible the estimation of the potential Ecosystem Service losses.

Fenamidone worst case application showed concentrations ranging 15.95- 65.02 mg a.s./Kg soil in Europe. For this concentration range, lethal and chronic effects could be expected; by affecting earthworms role on habitat provision, soil formation & retention, nutrient cycling, erosion regulation, soil remediation /waste treatment and biodiversity (Table 4). Meanwhile, no impacts on collembolans communities are expected along European soils after worst case fenamidone application.

In conclusion, mainly impacted Ecosystem Services by earthworm and collembolan affection (in off-crop areas and in-crop areas) would be habitat provision, soil formation & retention, nutrient cycling and biodiversity (Table 5). Additionally, impacts on earthworms would affect erosion regulation and soil remediation/waste treatment; while, impacts on collembolans will affect pest & disease regulation. Overall, a higher ecosystems risk was observed in northern soil, especially in hot spots with

significantly higher PECs (Finland, Sweden, Estonia, Latvia and Lithuania). Soils in northern Europe are characterized by cold climates, low pH and high OM contents leading to strong bindings between soil and pesticides, being accessible for soil living organisms by oral route (Zou et al., 2018; Xuy et al., 2019; Ogungbemi and van Gestel, 2018).

Moreover, the impact of PECs upon Ecosystem Service loses could be conditioned by the species habitat, role and behavior in the media. In fact, the multiple habitats covered by different earthworm species enhance Ecosystem Services resilience. Epigeic earthworm species (e.g. *Lumbricus rubellus, Eiseniella tetraedra, E. fetida*) are more susceptible to environmental changes and pollutant exposure (including pesticides) than the anecic species; principally due to their close to surface habitat (Ockleford et al., 2017; Paoletti, 1999). In addition, epigeic species feed on humus, allowing incorporating PPPs attached to OM through dietary route. Opposite, anecic species feed principally on soil litter being exposed only in cases with soluble PPPs. Therefore, anecic organisms could fill the absent role of epigeic organisms affected by the use of pesticides. This scenario could occur in soils with esfenvalerate application, where high mortalities could be expected at surface due to the low solubility and the high affinity to OM, making impossible PPP leachate to deeper layers of soil (Ogungbemi and van Gestel, 2018). This is a key factor for risk managers when protecting soil Ecosystem Services; especially when managing highly lipophilic compounds.

Table 4: Potential Ecosystem Services loss derived from the PPP worst case application in European soils based on ecotoxicological endpoints (illustrated in tables 2 and 3) and PECs.

Pesticide – organism	Effects upon NT-organisms along Europe after worst case PPP application	Ecosystem function	Ecosystem Services
Esfenvalerate ***Eiseina***	For PECs 1.123-3.125 mg a.s./Kg (inside main European concentrations) effects are uncertain due to the lack of data	Uncertaint	*
	For 3.876-4.721 mg a.s./Kg soil concentrations in some C and N European soils (Germany, Lithuania, Latvia and Estonia) no effects are expected	-	-
	At >5 mg a.s./Kg concentrations in N soil spots (Finland, Sweden, Estonia, Latvia and Lithuania), mortality and weight loss could happen	Habitat provision, Soil formation & retention, Nutrient cycling, Erosion regulation Soil remediation /waste treatment Biodiversity	Supporting Regulating Provisioning
Esfenvalerate ***Folsomia***	For all concentrations estimated along Europe (1.343-5.565 mg a.s./Kg soil) effects on reproduction are expected	Pest & disease regulation, Habitat provision Soil formation & retention, Nutrient cycling Biodiversity	Supporting Regulating Provisioning
Cyclaniliprole ***Eisenia***	No effects were expected for all European soils	-	-
Cyclaniliprole ***Folsomia***	Predominant PECs along Europe were ≈ 3.381 mg a.s./Kg so no effects could be expected	-	-

	For PECs ranging 5.669-7.957 mg a.s./Kg soil in some C and N European spots (Germany, Poland, Estonia, Latvia and Lithuania), effects upon reproduction could be expected For PECs ranging 10.24-14.821 mg a.s./Kg in some N European spots (Finland, Estonia and Latvia) effects upon mortality could be expected	-Pest & disease regulation, Habitat provision Soil formation & retention, Nutrient cycling Biodiversity	Supporting Regulating Provisioning
Picoxystrobin ***Eisenia***	Mortalities expected in European concentration ranges (14.58-61.63 mg a.s./Kg)	Habitat provision, Soil formation & retention, Nutrient cycling, Erosion regulation Soil remediation /waste treatment Biodiversity	Supporting Regulating Provisioning
Picoxystrobin ***Folsomia***	For main concentrations in Europe (14.58 mg a.s./Kg soil) no effects are expected No data is available in EFSA databases for 24.91-33.64 mg a.s./Kg soil range; so effects for this agricultural areas (spots in Germany, Poland, Estonia, Latvia and Lithuania) is uncertain	- Uncertainty	- *

	For concentrations ranging 42.97-61.63 mg a.s./Kg soil (spots in Finland, Estonia and Latvia) reproductive effects are expected.	Pest & disease regulation, Habitat provision Soil formation & retention, Nutrient cycling Biodiversity	Supporting Regulating Provisioning
Fenamidone ***Eisenia***	For all European concentrations (15.95- 65.02 mg a.s./Kg) mortalities are expected	Habitat provision, Soil formation & retention, Nutrient cycling, Erosion regulation Soil remediation /waste treatment Biodiversity	Supporting Regulating Provisioning
Fenamidone ***Folsomia***	No effects were expected for all European soils	-	-

* The lack of information in the official sources makes impossible an accurate estimation. More empiric information is needed in order to relate effects and Ecosystem Service loss.

Folsomia sp. features high reproductive rates, as well as a well-developed exoskeleton that minimizes the possible effects exerted by pesticides. But, their ventral tube enables water and oxygen exchange with the environment (Lock and Janssen 2003; Fountain and Hopkin, 2005); making them particularly vulnerable to contamination via pore water (Filser et al., 2014; Ogungbemi and van Gestel, 2018). Moreover, these animals live on soil surface and could be exposed to pesticides for long periods; especially when sprayed PPPs are poorly lipophilic compounds, enabling high PPP concentrations in pore water. Only their ability to move quickly to buffer (clean) zones (Verhoef & Van Selm, 1983; Detsis, 2000; Tsiafouli et al., 2005; Holmstrup, 2019) could enhance resilience, favoring preservation or recovery. This factor (pollution avoidance and migration to clean zones) should be taken into account regarding the management of large agricultural areas with pesticide disposal. Besides, collembolans are considered one of the most abundant species in soil (Fountain and Hopkin, 2005), but their limited habitat in depth makes them a weak group in terms of resilience comparing to earthworms. Thus, designing wild field margins in crops could be helpful to allow collembolans migrate to these non-spiked zones. This crop management allows potential off-crop to in-crops area recolonization after PPP degradation (recovering lost Ecosystem Services). Besides, the scarce ecotoxicological information available on collembolans for the selected PPPs, makes impossible to assess properly a good managing strategy; being required further ecotoxicological studies in order to evaluate more accurate thresholds and effect ranges.

Degradation time of PPPs could also contribute to Ecosystem Service losses. For instance, low degradation times could pose significant effects on soil fauna for long periods. This could be crucial when managing PPPs causing chronic effects as reproductive impairment. Esfenvalerate, cyclaniliprole and picoxystrobin showed the slowest degradation times while fenamidone showed to be the fastest degrading compound (Deg50: 15.4 days). Although the later degradation period is the lowest among the selected PPPs, it should be desirable to minimize at maximum the degradation time in order to avoid effects on non-target species that could impact Ecosystem Services.

Table 5. The most important Ecosystem Services in agricultural landscapes (according to EFSA 2010) due to PPP disposal.

Ecosystem Service category	In crop areas	Off crop areas
Provisioning	Food	Food
	Fibre and fuel	Biodiversity
		Fresh water
Regulatory	Pollination	Pollination
	Pest & disease regulation	Pest & disease regulation
	Erosion regulation	Erosion regulation
		Water regulation
		Soil remediation/waste treatment
Cultural	Education & inspiration	Education & Inspiration
		Recreation & ecotourism
		Cultural heritage
		Aesthetic value
Supporting	Primary production	Primary production
	Photosynthesis	Photosynthesis
	Habitat provision	Habitat provision
	Soil formation & retention	Soil formation & retention
	Nutrient cycling	Nutrient cycling
	Water cycling	Water cycling

Note: Only the services exhibiting a maximum importance (+++/+++) after PPP application according to EFSA (2010). Ecosystem Services affected by impacts upon soil macroinvertebrates (earthworms and collembolan) marked in red.

4.- CONCLUSIONS

Effects upon functions developed by non-target species have been useful to identify the most impacted Ecosystem Services: habitat provision, soil formation & retention, nutrient cycling, biodiversity, erosion regulation, soil remediation/waste treatment and pest & disease regulation would be affected in off-crop areas and in-crop areas. Moreover spatial variability among European agricultural soil (pH, OM, temperature), PPPs physicochemical properties (MoA, Kom, solubility…), and non-target species behavior, habitat and role in ecosystem seemed to be the main factors to be taken into account for a correct PPP use management in crops.

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CHAPTER 6

Araztegi lokatzak jasotako lurzoruaren analisi toxikologikoa zizare eta landareak erabiliz

(Toxicological analysis of soils poured with sewage sludges using worms and plants)

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ABSTRACT

Increasing global population, along with a rising industrial activity and soil scarceness led to landfill, spill and filling point extension. Among this landfills, “Landfill 17” can be found with 48046-00181 code; located in Gernika-Lumo (43°19'28,9"N 2°40'30,9"W, Basque Country). There, sewage sludges coming from Gernika waste water treatment plant were poured with a hose with agricultural purposes; thus, many pollutants with industrial origin (heavy metals, PAHs and pesticides among others) ended up in the mentioned soils. Among the species mostly affected by the contamination, plants and earthworms must be highlighted due to their close relation with soil matrix; and therefore, soil pollution. In this context, the aim of this work is to evaluate the effects exerted by pollutants in the spilling in order to assess potential Landfill future uses; using for that widely known biomarkers and standardized tests in different organisms (lettuce and earthworms), exposure times (3, 7, 28 and 56 days) and complexity levels (from cellular level to populational level). Indeed, OECD-204 (acute toxicity test), OECD-222 (reproduction test) and calcein AM tests were applied in *Eisenia fetida* earthworms; while, germination and elongation tests were applied on *Lactuca sativa* plants. After carrying out ecotoxicological assays, significant differences respect to the control were observed in: extruded coelomocyte quantity, cell viability, tissue metal accumulations, growth parameters (weight loss) or reproductive parameters; proving pollutants affection upon soil biota. However, no significant differences were observed between experimental groups in those tests carried out with *L. sativa*.

Keywords: sewage sludge, heavy metals, ecotoxicology, *Eisenia fetida*, *Lactuca sativa*, biomarkers, standard toxicity tests

LABURPENA

Urtetik urtera areagotzen diren giza-populazio eta aktibitate industrialak, zabortegi, isuri puntu eta betetze guneen emendioa eragin du. Zabortegi hauen artean, 48046-00181 kodearekin, “17 zabortegia” aurki daiteke; Gernika-Lumon kokatua (43°19'28,9"N 2°40'30,9"W.). Zabortegi honetan, Gernikako araztegiko depurazio lokatzak isuri izan ziren ongarri gisa urteetan zehar; horrela, Gernikako industrian sortutako hainbat kutsatzaile (metal astunak, PAH-ak, pestizidak, e.a.) kokapen honetan lurperatuz. Isurketaren eraginez gehien kaltetutako espezien artean, landareak eta zizareak bezalako lurzoruko ornogabeak azpimarratzekoak dira; batez ere, berauen eta lurzoru matrizearen arteko kontaktu estuagatik. Testuinguru honetan, lan honen helburua lokatz horien isurketak eragindako efektuen ebaluazio bat burutzen da lurzoruari beste erabilera bat emate aldera; horretarako, zabalki ezagunak diren test estandarizatu eta biomarkatzaileak erabiliz: organismo (uraza eta zizare), denbora (3, 7, 28 eta 56 egunetara) eta konplexutasun biologiko desberdinetan (zelula mailatik-populazio mailararte). Besteak beste, OECD-204 (toxikotasun akutuaren testa), OECD-222 (ugalketa testa) eta kaltzeina AM bideragarritasun testak aplikatu ziren *Eisenia fetida* zizarean; paraleloki, *Lactuca sativa* landareetan ernetze eta elongazio testak burutu ziren heinean. Azterketa toxikologikoen ostean, kontrolarekiko desberdintasun esanguratsuak ikusi ziren: erauzitako zelomozito kantitatean, bideragarritasun zelularrean, ehunetan metatutako metal kontzentrazioetan, hazkuntza parametroetan (pisu galeran) eta ugalketa parametroetan; kutsatzaileek lurzoru biotarengan eragindako afekzioa berretsiz. *L. sativa* espeziarekin egindako frogetan aldiz, ez zen desberdintasun nabarmenik preziatu talde esperimentalen artean.

Hitz gakoak: araztegi lokatzak, metal astunak, ekotoxikologia, *Eisenia fetida*, *Lactuca sativa*, biomarkatzaileak, toxizitate proba estandarrak

1-SARRERA

Azken mendean nekazaritza eta industrian eman diren aurrerakuntza zientifiko eta teknologikoek munduko biztanleria laukoiztea ahalbidetu dute (Gomez-Sagasti et al., 2019). Eztanda demografiko horrek, lurzoru erabilgarriaren eskasiarekin eta kultura kontsumista batekin batera, zabortegien, isuri puntuen edota betetze puntuen emendatzea eragin du. Euskal Autonomia Erkidegoko irailaren 30eko 165/2008 dekretuan, lurzorua kutsa dezaketen jarduera zein instalazioak izan dituzten lurzoruak inbentariatzen dituen hartan, 1277 zabortegi daude. Inbentario horretan, 17-Zabortegia aurki daiteke Gernika-Lumon kokatua. Egun zabortegia den lurzoruetan, hainbat hamarkadetan zehar Gernika-Lumo araztegiko lokatzak bota izan ziren ongarri gisa, nekazaritza aktibitatea bultzatuz. Baina uztak elikatuko zituen materia organikoarekin batera, araztegiak garbitu ezin zitzakeen kutsatzaileak heldu ziren lurzoruetara. Izan ere, Gernika-Lumoko saneamendu sistema unitarioa da; hots, ez ditu ur zuri eta beltzak bereizten. Modu horretan, Gernikako industrian euriak garbitutako edo ilegalki kolektiboetara jaurtitako kutsatzaileek araztegian bukatu zuten; eta handik, lokatzen bitartez, lurzoruetan. Lokatzak 250 m-tara mahuka bitartez isurtzen zirela jakinda, Eusko Jaurlaritzak (GV/EJ) 3.38 hektarea–tako zabortegia aitortu zuen araztegiaren iparraldeko lur esparrua; 17-zabortegia alegia. Gaur egun lur esparru osoa (zabortegiari dagokiona) itxita mantentzen da; edozein aisialdi, edo ekintza sozioekonomiko debekatuak egonik. Hala ere, Gernika-Lumoko udalak, zabortegiaren hegoaldean aurkitzen den Urbietako kirolekua handitzeko aurreikuspena dauka; posible bada, zabortegiaren zati bat hartuz. Ondorengo lana testuinguru horretan aurkitzen da; 17-zabortegiko lurzoruen toxikotasunaren jarraipen eta kontrolean. Horretarako, *Eisenia fetida* zizarea bezalako organismo lurtarrekin eta *Lactuca sativa* landareekin bioentseguak burutu dira.

Zizareak metalen metatzaile eraginkorrak dira eta kutsaduraren aurrean erantzun sentikor eta neurgarria dute; horregatik, organismo behale hauen erabilera oso zabaldua dago lurzoruen kutsadura ikertzeko. Zizareen artean, *E. fetida/E. andrei* espezieak dira erabilienak, mundu mailan onarturiko lurzoruen toxikotasun test estandarretarako erabiltzen direlarik (OCDE, 1984; ISO, 2008). Kutsatzaileek zizareetan eragin dezaketen arrisku potentzialak ebaluatzeko toxikotasun test akutuak eta kronikoak aurkitu daitezke. Toxikotasun test akutuek, konposatu batek denbora konkretu batean

organismoen %50eko heriotza eragiteko dosia (LC50) ezartzea ahalbidetzen dute, arriskuen ebaluazioan edo konposatu kimikoen erregulazioan lagunduz (Spurgeon et al., 1994). Entsegu mota horien artean, zizareak kutsatutako lurzoruarekin kontaktuan jartzean datzan Artificial soil testa aurkitzen da (OECD, 1984). Honako testa, animalien eta probatu beharreko substantzien arteko kontaktu dermikoan eta lurzoruaren ahoratzean oinarritzen da; toxikotasuna, animalien heriotza-tasa eta pisu galeraren bitartez ebaluatuz. Test hauek burutzeko, OECD edo LUFA bezalako lur estandarizatuak erabiltzen dira; honela, herrialde ezberdinetako laborategietan emaitza berdintsuak lortzea ahalbidetzen da, konparaketak erraztuz eta ezarritako toxikotasun neurketen fidagarritasuna areagotuz (Lopes Alves eta Nogueira Cardoso, 2016).

Toxikotasun kronikoa aztertzerakoan, ugalketa garrantzi handiko faktorea da (Joosse eta Verhoef., 1983; Kooijman eta Metz., 1984; Spurgeon et al., 1994) populazioen dinamiketan daukan garrantziagatik. Horrela, hazkuntza edo ugalketan eman daitezkeen aldaketak elementu erabilgarriak izan daitezke populazio mailako efektuak aurreikusteko (Moriarty., 1983). Hauen artean, OECD-222 (2004) testa aurki dezakegu; zeina, ugalketaren gaineko agente kimiko ezberdinen eragina aztertzeko diseinatu zen. Entsegu hauetan, ugalketa efektuak 8 astetako esposizioaren ondoren neurtzen dira, lurzoruan hazi izan den zizareen oinordekotza zenbatuz. Entsegu horien bitartez gainera, hainbat indikatzaile lortzea posible da: ECx, LC0, LC50, LC100, LOEC, NOEC eta ugalketa tasak besteak beste.

Aipatutako entsegu estandarizatuez gain, azken urteotan biomarkatzaile sorta zabala garatu izan da zizareetan efektu subletalak behatzeko; eta hortara, lurzoruaren osasun egoera ebaluatzeko. Biomarkatzaileek, konplexutasun biologiko maila ezberdinetan (organismo behaletan) ematen diren aldaketen gaineko informazio baliagarria ematen dute. Azterketa kimiko orokorrek edo ehunetan metatutako kutsatzaile kantitateak ez bezala, biomarkatzaileek, kutsatzaileek organismoetan eragiten duten inpaktu biologikoaren gaineko informazioa ematen dute (Marigomez et al., 1996; Cajaraville et al.,2000). Zizareen kasuan, euren zelula immuneetan (zelomozitoak) neurtu ohi dira biomarkatzaileak. Izan ere, zelomozitoek kutsatzaile kontzentrazio baxuen aurrean azaldu duten erantzun goiztiarrek, konplexutasun maila altuagoetan (ehun, organismo) pairatutako kalteak aurreikustea ahalbidetzen dute

(Curieses et al., 2017; Garcia-Velasco et al., 2020). Zelomozitoekin egindako in vitro testen barruan, ondo hedatutako teknika da kaltzeina AM bideragarritasun testa; zeina, substantzia kimikoek eragin ditzaketen efektu subletalak hautemateko teknika sentikor eta arina den. Honen bitartez, substantzia kimikoek zelulen euste (kaltzeinaren eustea) ahalmenean daukaten eragina aztertzen da; potentzialki toxikoak diren kimikoek mintz zelular eta lisosomikoetan sortzen dituzten kalteak kuantifikatuz. Azken hamarkadetan, banakako elementuen (Cr, Ni, Pb, Cd, Cu, kerosenoa, etab.), nahaste konplexuen (errefusak, pestizidak, metalak eta kutsatzaile organikoak) zein lurzoru naturalen (utzitako meategiak, zabortegi ilegalak, e.a.) toxikotasuna aztertu izan da *E. fetida*-n; horretarako, analisi kimikoak, test estandarizatuak eta biomarkatzaile ezberdinen konbinazioak erabiliz (Asensio et al., 2013;Irizar et al., 2014a; 2014b; 2015).

Gaur egun badira kutsatzaile ezberdinen toxikotasuna zehazteko landare prozesuetan oinarritzen diren ikerketak ere. OECD-ak (2003) eta EPA-k (1996) urazekin burututako bioentseguak gomendatzen dituzte; batez ere, kutsatzaileek sor dezaketen ingurumen arriskua ebaluatzeko metodo sinple, bizkor eta sentikorrak direlako (Adamo et al., 2014). Urazak, metalak sustrai bitartez xurgatzeko eta sustrai zein kimuetan metatzeko duten ahalmen altuagatik, lurzoruaren osasuna ikertzeko erabiltzen dira (Adamo et al., 2014). Letxugekin burututako entsegu fitotoxiko estandar erabilienen artean, hazien ernetze eta sustraien elongazio testak aurkitzen dira. Lehenengoa epe laburreko testa kontsideratzen da; 5 egunetan zehar (120 h) plantulen garapenean ematen diren efektu toxiko akutuak zehazten lagunduz. Aldiz, biomasa begetala eta sustraien elongazioa balioztatzen diren epe luzeko entseguek (2 eta 8 aste bitartekoak), efektu toxiko akutu zein kronikoen zehaztapenean lagun dezakete (In, 2013).

Lan honen helburua, 17 Zabortegian isuritako araztegi lokatzek sortutako toxikotasunaren ebaluazioa egitea da; irizpidetzat, espezie ornogabe eta landareen gaineko efektu biologikoak izanik. Aipatutako testen bitartez, lokatzen isuriak espezie ornogabeengan eragiten duen pisu galera, heriotza, ugalketa kalteak edo landare espezietan eragindako ernagarritasun kalte zein sustrai kalteak ebaluatzea bilatzen da. Honek, eragindako ingurumen kalteen gaineko jakintzan sakontzea ahalbidetuko du, kutsatutako lurzoruaren kudeaketan lagunduz.

2. MATERIAL ETA METODOAK

2.1- Esperimentaziorako organismoak

2.1.1- *Zizareak: Eisenia fetida*

Eisenia fetida espezieko zizareak Lombricor SCA (Kordoba) enpresan erosi ziren. Zizareak tenperatura (19°C) eta hezetasun konstantean (% 60) mantendu ziren *stock* gisa, astero zaldi gorotzekin elikatuz. Esperimenturako zizare osasuntsu, klitelodun (heldu) eta tamaina antzekodunak (300-600 mg pisu hezea) erabili ziren.

2.1.2- *Uraza: Lactuca sativa*

Hazien ernetze eta sustraien elongazio testetarako, *L. sativa* haziak erabili ziren. Haziak tenperatura konstantean (5°C) eta iluntasun baldintzetan mantendu ziren, % 10eko hezetasuna gainditzen ez zuten hermetikoki itxitako paketeetan. Esperimenturako, irregulartasunik gabeko haziak aukeratu ziren.

Ernetze testean haziak erabili baino lehen, ernagarritasun azterketak burutu ziren uraza stock-aren % 75eko ernagarritasun minimo bat bermatzeko.

2.2- Lurzoruen egokitzapena

17-zabortegiko lurzoruetan (43°322434 N, -2°675425 W), 20 puntu ezberdinetako lur laginak hartu ziren berauen analisi kimiko eta fisikokimikoa egiteko. Proposatutako testak burutzeko, hartutako 20 laginetatik, MAN-7, MAN-14 eta MAN-17 puntuetako lurrak aukeratu ziren beraien Pb, Ni, Cd eta Cr kontzentrazioak irizpidetzat hartuz; kutsatzaile hauetarako GV/EJ-k markatutako mugak gainditu zirelako. Lurzoru horiek behin lehortuta, bakoitza bere zelai kapazitatearen %60-era hezetu zen 48h-z; egonkortzea, tenperatura (19°C) eta hezetasun baldintza konstanteetan burutuz. Erreferentzia gisa, LUFA 2.3 (Speyer, GER) lurzoru natural eta estandarrak erabili ziren.

1. Taula. Aztertutako lurzoruen (MAN-7, MAN-14 eta MAN-17) materia organiko, pH kadmio, kromo, nikel, berun kontzentrazioak. Pisu lehorra P.L bezala adierazita

	LUFA 2.3	MAN-17	MAN-14	MAN-7
Materia organikoa (LOI) % P.L.	±1	6.2	11	19
pHa	6.81	7.42	6.28	7.30
Kadmioa (Cd) mg kg^{-1} P.L.	<LOD	0.33	8.7	26
Kromoa (Cr) mg kg^{-1} P.L.	<LOD	41	190	400
Nikela (Ni) mg kg^{-1} P.L.	<LOD	32	56	120
Beruna (Pb) mg kg^{-1} P.L.	<LOD	27	96	170

2.3- Lixibiatuen prestaketa

Lurzoru kutsatuen lixibiatuak prestatzeko legislazio Espainiarrak ezarritako (BOE, 2005 516/id) DIN 38414-S4 (Deutsches Institut fur Normung, 1984 480/id) estandar Alemana jarraitu zen. Horretarako, 10 g lurzoru (pisu lehorra) 100 mL ur distilaturekin nahastu eta 24h-z irabiatu ziren giro tenperaturan. Prozesu hori kontrol (LUFA 2.3), MAN-7, MAN-14 eta MAN-17 taldeetan egin zen. Prestatutako lixibiatuak kimikoki analizatu eta *L. sativa* hazien ernetze zein sustraien elongazio testetan erabili ziren.

2.4.- *Lactuca sativa* hazien esposizioa kutsatutako lurzoruetan

2.4.1 –*Lactuca sativa* hazien ernetze testa

Petri kutxetan, 15 g hare eta 15 *L. sativa* hazi jarri ziren, jarraian, MAN-7, MAN-14 eta MAN-17 lurretarik eratorritako lixibiatuekin hezetzeko (3 ml, n=3). Era berean, testa bermatzeko intentzioarekin, kontrol positibo talde bat gehitu zen, zeinetan, haziak, parke publikoetarako muga kontzentrazioen pean (25 mg kg^{-1} Cd, 400 mg kg^{-1}Cr, 500 mg kg^{-1}Ni eta 450 mg kg^{-1}Pb) hasi ziren (n=3). Hazkuntza, iluntasun, tenperatura (20°C) eta hezetasun konstantepean garatu zen, 48 eta 168 ordutara ernaltzea aztertzeko.

2.4.2 Sustraien elongazio testa

25 hazi 48 orduz iluntasun baldintzetan eta kutsatzailerik gabe aurre-ernetu ondoren, sustraien luzera neurtu eta hoziak lixibiatuen pean jarri ziren. Kontrolen kasuan ordea, ura erabili zen. 8 orduz hezetasun, tenperatura eta iluntasun baldintza konstantepean haziak mantenduta gero, sustraien hazkuntza neurtu zen. Analisi kimikoak burutze aldera landareak labean 36°C-tan 72 orduz lehortu ziren, ondoren ehotuak eta digerituak izateko (HNO_3). Analisi kimiko guztiak masen espektrometria akoplamendu induktiboko plasmarekin (ICP-MS) burutu ziren.

2.5.- *Eisenia fetida* zizareen esposizioa kutsatutako lurzoruetan

2.5.1-Toxikotasun akutuaren testa

Esperimentaziorako aukeratutako MAN-7, MAN-14 eta MAN-17 talde bakoitzetik 750 g lurzoru kristalezko ontzietan jarri ziren; zeintzuetan, pisatutako 10 zizare sartu ziren (3 erreplika). Ontziak tenperatura baldintza optimopean (19°C) eta argipean mantendu ziren zizare-lurzoru kontaktu etengabea bermatuz. 3 egunetara, zizareen pisu galera eta hilkortasuna neurtzeaz gain, Kaltzeina AM bideragarritasun testa (ikusi 2.6.2) egin zen zelomozitoetan. Kontrol taldeak LUFA 2.3 lurzoru estandarrean mantendu ziren esperimentu-baldintza berdinetan.

2.5.2- *Eisenia fetida* zizareen ugalketa testa (OECD-222, 2004)

500 g kontrol, MAN-7, MAN-14 eta MAN-17 lur kristalezko ontzietan jarri ziren, hauetara, 0,3-0,6 g bitarteko 10 zizare sartzeko (4 erreplika). Esperimentazio ontziak, 19°C-tako tenperatura konstantepean eta argitasun-iluntasun (16:8h) ziklopean mantendu ziren 56 egunez. Lehenbiziko 28 egunetan 5 g zaldi gorotz gehitu zitzaizkien astero materia organiko ekarpen gisa; hezetasun mailaren kontrola aldiz, astero burutu zen esperimentu osoan zehar. Behin 28 egun igarota, zizare helduak lurzoruetatik atera, balantza analitikoan pisatu eta analisi kimikoak egiteko erabili ziren; lurrean, arrautza eta jubenilak utziz lehenago aipatutako baldintza berdinetan. Esperimentuaren 56. egunean, jubenilak kontatu eta jubenilen biomasa pisatu zen.

Esperimentazio erreplika bakoitzeko, bost zizare erabili ziren analisi kimikoak egiteko. Behin zizareen liseri-traktua garbituta, labean lehortu ziren 120°C-tara 48 orduz. Behin lehortuta, zizareen pisu lehorra neurtu eta HNO_3-arekin (Tracepur®, % 69, 1M) estali ziren digestioa burutu zedin; ondoren, disoluzio azidoak plaka bero batean lurrundu arte jarri ziren. Azkenik, 6 mL HNO_3 0,01 M gehitu zitzaizkien neurketak burutu bitartean laginak 4 °C-tan mantenduz.

2.6-Zelulen bideragarritasun testak kutsatutako lurzoruetan izandako zizareen zelomozitoekin

2.6.1-Zelulen erauzketa eta kontaketa

3 egunez MAN-7, MAN-14 eta MAN-17 lurpean izandako zizareak erauzketa soluzioan (1 mL PBS % 0,02 EDTA/zizare) sartu eta 9V-ko korronte elektrikoa aplikatu zitzaien poro dortsaletatik zelomozitoak eraucteko (Irizar et al., 2014a). Lortutako zelulen soluzioa zentrifugazio-hodietan jaso ziren; jarraian, 530 g- tan eta tenperatura konstantean (10°C) 10 minutuz zentrifugatzeko. Behin prezipitatua lortuta, garbiketak burutu ziren PBS eta antibiotikodun (*Saline phosphate buffer*, pH 7.0-7.2) soluzio batekin suspendituz. Jarraian, zelulak hemozitometroan zenbatu ziren talde bakoitzeko zelula dentsitate balioa lortze aldera; ondoren talde esperimental guztiak $1 \cdot 10^6$ zelula/mL kontzentraziora eramateko. Soluzio zelularrak 96 putzuko mikroplaketan gehitu (200.000 zelula/putzuko), eta testak egin orduko, 30 minutuz egonkortzen utzi ziren 18 °C-tan, zelulen atxikitzea bermatzeko.

2.6.2-Kaltzeina AM bideragarritasun testa

Plakak zentrifugatu (1500 rpm, 5 min, 10°C) ostean, gainjariakina kendu eta zelulak 40 minutuz 2.5 M Calcein-AM-rekin inkubatu ziren (n=3, 100 µL putzuko). Tratamendu guztietan 100 µL PBS (n=3) gehitu ziren kontrol tekniko gisa; garbiketa prozesuetan zehar taldeen arteko zelula galera berdintsu bat bermatu, eta zelulen autofloreszentzia estimatu ahal izateko. Zelomozitoak birritan garbitu ostean (zentrifugatu, gainjariakina kendu eta 100 µL PBS gehitu) fluoreszentzia plakentzako FLx fluorimetroan neurtu ziren: 490 nm-tako uhin luzeran kitzikapenerako eta 520 nm-tan emisiorako.

2.7-Analisi estatistikoa

Lortutako emaitzak SPSS softwarearen 22. bertsioan aztertu ziren. Datu normalitatea Kolmogorov–Smirnov eta Shapiro-Wilk testen bitartez frogatu zen. Distribuzio normala erakutsi zuten parametroak norabide bakarreko bariantza-analisi bidez ikertu ziren. Kontrolaren eta kutsatutako laginen arteko desberdintasun esanguratsu posibleak ordea Dunnet ($p<0,05$) post-hoc testaren bitartez analizatu ziren. Gainera, laginen arteko desberdintasunak Tukey testaren bitartez aztertu ziren. Aldiz, emaitza ez parametrikoak Kruskal-Wallis testaren bitartez prozesatu ziren.

3.- EMAITZAK

3.1-Analisi fisikokimikoak

3.1.1- Lixibiatuen metal determinazioa

Lurzoru ezberdinetatik eskuratutako lixibiatuen artean metal maila ezberdinak kuantifikatu ziren. MAN-14 lixibiatuak, metal astunen kontzentrazio altuenak erakutsi zituen; aitzitik, metal astunen kontzentrazio baxuenak MAN-17 taldean neurtu ziren.

2. taula: Aztertutako lurretako lixibiatuetan determinatutako Cr, Ni, Cd eta Pb kontzentrazioak (µg/L). *Kontrol taldean lixibiatutako metalen kontzentrazioak detekzio mugen azpitik neurtu ziren (<LOD).

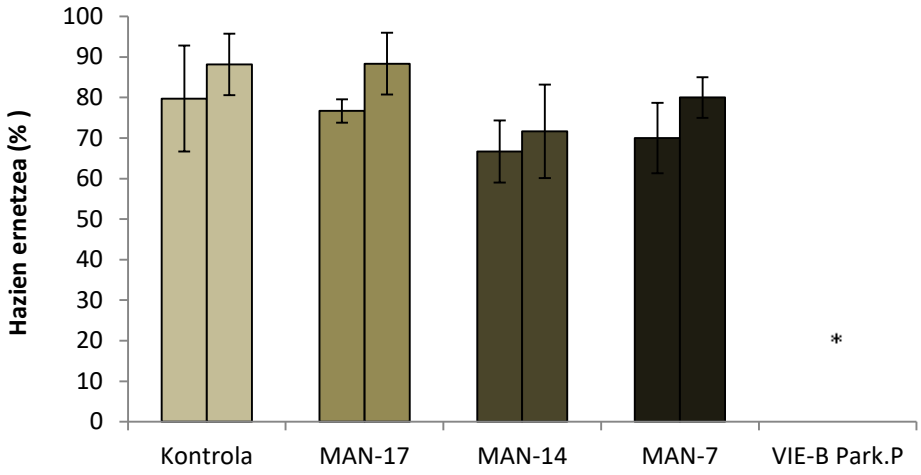
	Kontrola	MAN-17	MAN-14	MAN-7
Cr	<LOD	4.7	31.5	12.6
Ni	<LOD	4	17.7	2.9
Cd	<LOD	0.5	2	1
Pb	<LOD	2.7	12.4	5.8

3.2-Analisi toxikologikoak

3.2.1- *Lactuca sativa*-rekin egindako probak

3.2.1.1- Lactuca sativa hazien ernetze testa

Hiru egunetara neurtutako ernetzeek balio berdintsuak erakutsi zituzten bai aztertutako lurretan eta baita kontroletan ere. Ernetze balio baxuenak MAN-14 and MAN-7 taldeetan kuantifikatu ziren; %66 -ko eta %70 -eko ernetze portzentajeekin.



2. irudia: *Lactuca sativa* hazien ernetze portzentaiak, 20°C-tara, 3 (1.go zutabea) eta 7 (2. zutabea) egunez lurzoruetatik erauzitako lixibiatuen pean izan ondoren. * Ez zen ernalkuntzarik eman (% 0).

7.egunean, 3.egunean antzemandako joera beretsuak ikusi ziren (2. Irudia); hots, ez zen desberdintasun esanguratsurik aurkitu talde ezberdinen artean. MAN-14 eta MAN-7 lurretara esposatutako hazietan ernetze baxuagoak ikusi ziren, MAN-14a izanik ernetze portzentaje baxuena erakutsi zuen taldea. Ernalkuntza altuenak kontrol taldean (% 88) eta MAN-17 lurretara esposaturiko hazietan aurkitu ziren.

Bestalde, parke publikoetarako limiteetan oinarritutako kontrol positiboan ez zen hazirik ernaldu.

3.2.1.2- Sustraien elongazio testa

Bi egunetako esposizioaren ondoren ez zen sustraien elongazioan ezberdintasun esanguratsurik aurkitu taldeen artean. Balio altuenak MAN-14 taldean (0.39 mm batez beste) ikusi ziren; eta baxuenak, MAN-7 taldean (0.15mm batez beste).

3.2.1.3- Urazen analisi kimikoa

Sustraiak bi egunez lixibiatuen pean izan ondoren, landareen analisi kimikoak metal metatze joera ezberdinak erakutsi zituen talde esperimental desberdinetan.

3. taula: Cr, Ni, Cd eta Pb kontzentrazioak *Lactuca sativa* ehunetan (µg Cd/g uraza), sustraiak 2 egunez kutsatutako lurzoruetatik erauzitako lixibiatuetara esposatu ondoren. <LOD bezala adierazita detekzio limiteen azpitik (ICP-MS) zeuden kasuak.

	Kontrola	MAN-17	MAN-14	MAN-7
Cr	<LOD	<LOD	<LOD	0.26
Ni	<LOD	4.64	3.29	8.79
Cd	<LOD	0.08	0.12	0.16
Pb	<LOD	3.56	3.9	13.89

Orokorrean ez zen Cd eta Cr metaketa handirik ikusi aztertutako hiru talde esperimentaletan. Pb eta Ni-a aldiz kantitate altuetan metatu izan ziren. MAN-7 taldea izan zen metal balio altuenak erakutsi zituen taldea; hala ere, ez zen desberdintasun esanguratsurik aurkitu talde esperimentalen artean.

3.3 Esperimentuak zizareekin

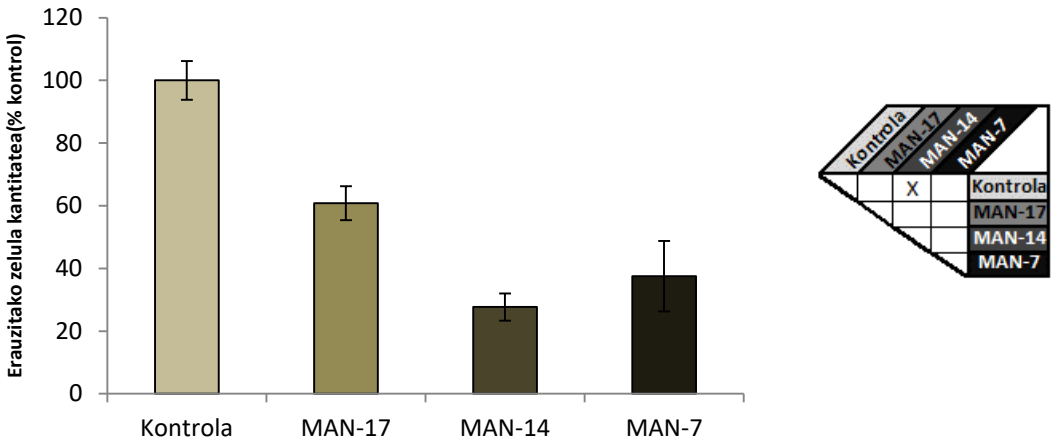
3.3.1- Toxikotasun akutuaren testa

3.3.1.1- Pisu galera

Hiru egunez esposatutako zizareen pisu galerak ez zuen inolako patroirik erakutsi esposizio taldeen artean. Pisu galera altuenak MAN-17 eta MAN-7 taldeetan antzeman ziren, pisuaren %8.01 eta %9.6a galduz hurrenez hurren. Aldiz pisu galera txikiena, MAN-14 taldeko organismoek pairatu zuten (%7.39)

3.3.1.2- Erauzitako zelomozito kantitatea

Hiru egunetako esposizioa pasata, zelula gutxiago erauzi ziren kutsatutako lurretan mantendutako zizareetatik.

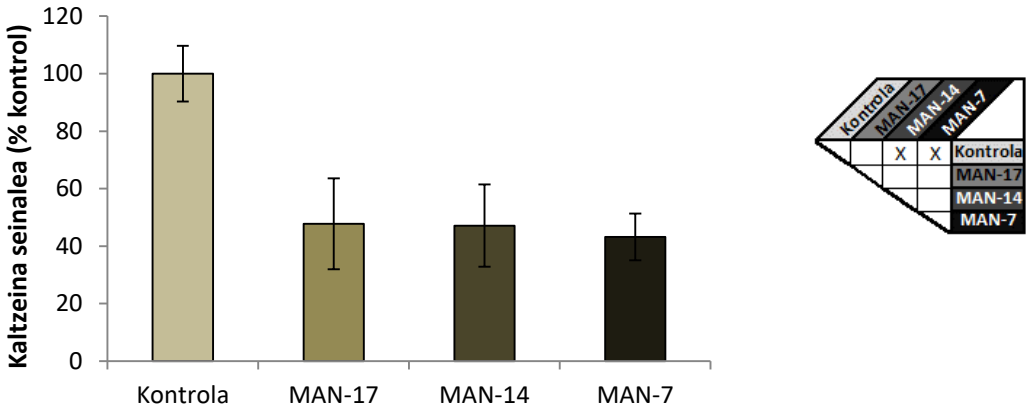


5. irudia: *Eisenia fetida* zizareetatik erauzitako zelula portzentajeak (kontrolarekiko normalizatua; %) 3 egunez kutsatutako lurzoru kutsatuen pean izan ostean. Desberdintasun esanguratsuak X batekin adierazi dira eskuineko matrizean.

Erauzitako zelula kontzentrazio baxuena MAN-14 lurzorura esposatutako organismoetan antzeman zen; non, kontrolean baino % 73 zelula gutxiago kuantifikatu ziren (5. irudia). MAN-17 eta MAN-7 lurzoruetan, kontrolean zenbatutako zelulen % 60 eta % 37a kuantifikatu ziren besteak beste. Kontrol taldea izan zen erauzitako zelula kantitate altuena erakutsi zuen taldea.

3.3.1.3- Kaltzeina-AM bideragarritasun testa

3 egunez kutsatutako lurzoruetan izandako zizareen zelomozitoek, kaltzeina gutxiago metatzeko joera erakutsi zuten kontrolekin alderatuz; hala nola, kontrol taldean zeudenak baino % 53-54 gutxiago (6. irudia).

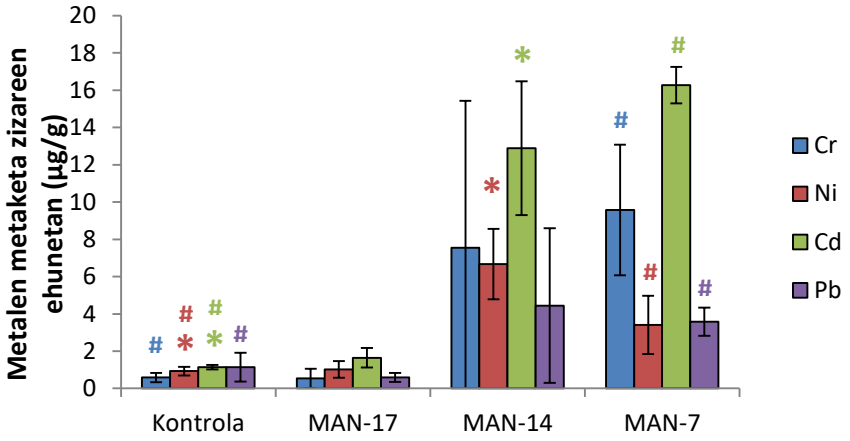


6. irudia: Kaltzeina AM bideragarritasun testean lortutako seinalea (kontrolarekiko normalizatua; %) zizareak 3 egunez kutsatutako lurzoru kutsatuen pean izan ostean. Desberdintasun esanguratsuak X batekin adierazi dira eskuineko matrizean.

3.3.2- *Eisenia fetida* zizareen ugalketa testa (OECD-222, 2004)

3.3.2.1- Zizareen analisi kimikoa

Ugalketa testaren lehen 28 egunak pasa ostean, analisi kimikoek, metalen metaketa joera ezberdinak erakutsi zituzten talde ezberdinen artean.



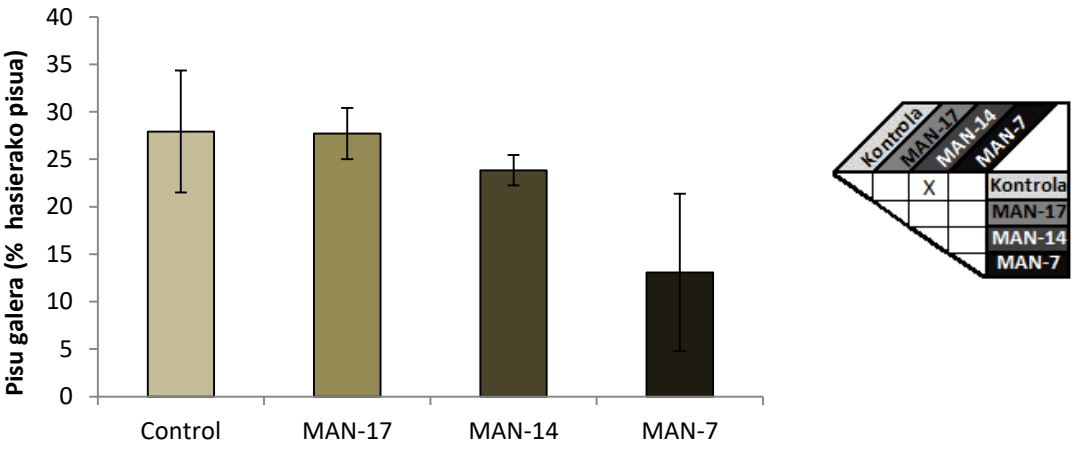
7. irudia: Cr, Ni, Cd eta Pb (µg Cd/g zizare) kontzentrazioak *Eisenia fetida* ehunetan, hauek 28 egunez kutsatutako lurzoru kutsatuen pean izan ondoren.

MAN-7 eta MAN-14 taldeek metal metaketetan gorakada erakutsi zuten, MAN-17 taldeko zizareek inolako metaketa esanguratsurik erakutsi ez zuten bitartean. Talde

esperimentalak estatistikoki konparatuz, MAN-17ak ez zuen kontrolarekiko desberdintasunik erakutsi; MAN-14ak, MAN-17ak eta kontrol taldeak baino Cd eta Ni nabarmen gehiago erakutsi zuen. MAN-7 taldeak ordea, neurtutako metal guztietan, MAN-17ak eta kontrolak baino balio esanguratsuki altuagoak erakutsi zituen.

3.3.2.2- Pisu galera

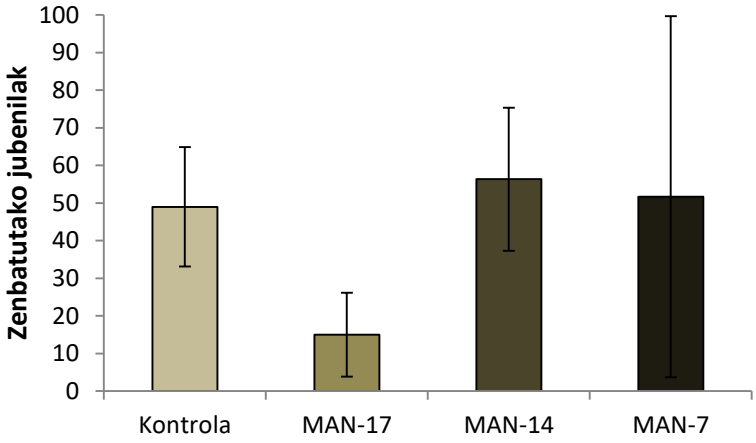
Ugalketa testaren lehen 28 egunak igaro ondoren, galera altuenak kontrol taldeetan aurkitu ziren; hurbiletik MAN-17 esposizio taldeak jarraituta (kontrolak baino % 0.75 gutxiago). Aitzitik, pisu galera baxuenak MAN-7 taldean antzeman ziren, non, hazitako organismoek, kontrolek (LUFA 2.3 lurzorua) galdutako pisuaren erdia baino gutxiago galdu zuten (kontrolek galdutakoaren % 47).



8. irudia: *Eisenia fetida* zizareen pisu galera (Hasierako pisua; %) 28 egunez kutsatutako lurzoru kutsatuen pean izan ostean Desberdintasun esanguratsuak X batekin adierazi dira eskuineko matrizean.

3.3.2.3- Jubenil kantitatea

56 egun eta gero kontatutako jubenil kantitateak ez zuen desberdintasun esanguratsurik erakutsi talde esperimental ezberdinen artean (9. irudia).

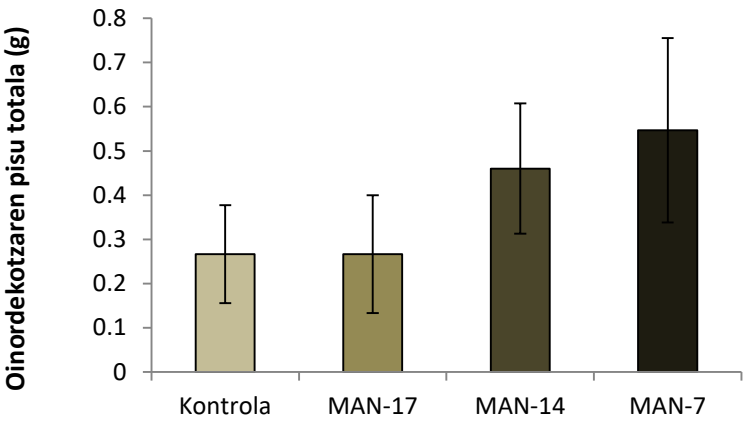


9. irudia: Esperimentuaren 56. egunean zenbatutako jubenil kantitatea *Eisenia fetida* zizare helduak lurzoru kutsatuen pean 28 egunez izan ondoren.

MAN-14 taldea izan zen jubenil kantitate altuena erakutsi zuen tratamendua, bataz besteko 56 jubenilekin; MAN-7 eta kontrol taldeak jarraituta 51 eta 49 banakorekin besteak beste. Aitzitik, jubenil kantitate baxuena MAN-17 lurzoruan kontabilizatu zen, non 15 jubenil zenbatu ziren bataz bestez.

3.3.2.4- Oinordekotzaren pisu totala

Desberdintasun estatistikorik aurkitu ez arren, oinordekotzaren pisu totalaren balio altuenak MAN-7 eta MAN-14 taldeetan jaso ziren, MAN-17 taldean eta kontrol taldeetan neurtutako batez besteko pisuak bikoiztuz (MAN-7 lurzoruarekiko, 10. irudia).



10. Irudia: Esperimentuaren 56. egunean kuantifikatutako oinordekotzaren pisu totala *Eisenia fetida* zizare helduak lurzoru kutsatuen pean 28 egunez izan ondoren.

4. EZTABAIDA

4.1- Karakterizazio fisikokimikoak

Lurzoruen propietateek metalen eskuragarritasuna, eta hartara toxikotasuna, zeharo baldintzatzen dituzte. Giltzarri diren ezaugarri hauen artean, buztin kantitatea, elkartruke kationikorako ahalmena, pH-a edota materia organiko (MO) kantitatea aurki daitezke (Spurgeon eta Hopkin, 1995; Smit et al., 1998; Gondek eta Kopek, 2006; Leduc et al., 2007). Epe laburreko toxikotasun testetarako zein zizareen ugalketa testetarako erabilitako lurren artean pH diferentziak ikusi izan ziren talde esperimentalen artean. Datu hauek, MO kantitateekin batera, talde ezberdinen arteko bioeskuragarritasun desberdintasunak azalduko lituzke (Visioli et al., 2013); izan ere, pH azidoetan, lixibiatzeko ahalmena eta metalen bioeskuragarritasuna pH neutro edo basikoetan baino handiagoa da. Lurreko materialetara atxikitako metalak, lurzoruetako materialetara (Katioak trukatzeko gaitasun altuak izaten dituzten buztin eta materia organikoak) afinagoak diren hidrogeno protoiengatik elkartrukatzen dira; era honetan, askatuz. Era berean, MO eta kutsatzaileen arteko elkarketa indartsuak, ligando organometalikoen bitartez ematen denak, kutsatzaileak lurrean fixatzen ditu; hauek, fase urtarrera eta beste medio batzuetara migratzea ekidituz. Honela, toxikotasuna aztertzerakoan, metalak lurzoruan igarotako denbora kontuan izan beharreko beste faktore bat da; izan ere, metalaren eskuragarritasuna ko-prezipitatu hauen eraketarekin batera jaisten da (Martinez eta McBride, 2001). Era honetan, kutsatuta dauden lurzoru zahartuetan metalen bioeskuragarritasuna zein toxikotasuna ahulagoak izatea espero da; berriki kutsatutako lurzoruekin alderatuz (Smolders et al., 2009).

Fenomeno hauek ondo islatzen dira lixibiatuen analisi kimikoetan, non, MAN-14 taldea izan zen metalen kutsadura maila altuenak erakutsi zituen taldea, printzipioz lurzoruan metal maila altuenak zituen MAN-7 taldea gaindituz. Honen arrazoi, MAN-14 taldeak erakutsitako materia organiko kantitate baxuagoa (%11 ML MAN-14an vs %19 ML MAN-7an) eta azidotasuna (6.28 MAN-14an vs 7.42 MAN-7an) izango liratekeelarik.

4.2- *Lactuca sativa* hazien ernetze eta elongazio testak

3. eta 7. egunetan kuantifikatutako ernetzeak ez zuen desberdintasun esanguratsurik erakutsi esposizio talde ezberdinen artean. Hala ere, MAN-7 eta MAN-14

taldeetan ikusitako ernetze ahalmenaren beherakada arina lixibiatuetan kuantifikatutako metal kontzentrazioekiko alderantziz proportzionala zela ikusi zen. Test hau balioztatzeko aipuaz, beronen sentikortasuna frogatu eta balioztatu zen kontrol positibo batekin; horretarako, haziak GV/EJ-k erabilera industrialerako ezarritako VIE-B balioetara esposatuz.

Haziaren elongazio testari dagokionez, emaitza berdintsuak lortu ziren esposizio taldeen artean, lixibiatuetan dauden kutsatzaileak uraza kaltetzeko adinako toxikoak ez direla frogatuz. Germinazio testean ez bezala, 2 egunetako sustraien esposizioa ez zen nahikoa izan elongazioaren eta metalen erlaziorik ikusteko. Ez zen talde kutsatuenetan inongo erantzunik sumatu, nahiz eta metal metaketa altuenak eta lurzoru kontzentrazio altuenak MAN-7 taldean ikusi izan. Erantzun gabezia honen arrazoia, esposizio kontzentrazio baxuetan legoke. Izan ere, lixibiatuetan neurtutako metal kontzentrazioak, beste lan batzuetan letxugekin argitaratutako LOEC kontzentrazioetatik urrun leudeke: 2 ppb *versus* 14.4 ppm Cd, 12.4 ppb *versus* 53.0 ppm Pb, 17.7 ppb *versus* 6.9 ppm Ni (Di Salvatore et al., 2008) eta 31.5 ppb *versus* 50.0 ppm Cr (Hou et al., 2013). Berun kontzentrazioek uraza sustrai guztietan, Europar (EU) 2015/1005 arautegiak begetalentzako ezarritako 0.1 ppmko atalasea gainditu zuten. Kadmio kontzentrazioek ere komisioaren 488/2014 araudiak (elikagaietan gehienezko kadmio kontzentrazioak ezartzen ditu) ezarritako legezko muga (0.05 ppm) gainditu zuten. MAN-7 taldean, Cr eta Ni kontzentrazioek legislazio Brasildarrak (Cr eta Ni-arentzako ez dago legezko atalaserik legislazio Espainiarrean edo Europarrean) gomendatutako atalaseak gainditzen zituzten (0.1 ppm Cr eta 5 ppm Ni).

Sustraietan metatutako metal kontzentrazioetan, MAN-7-a izan zen metal gehien metatu zituen talde esperimentala; orokorrean Ni eta Pb kontzentrazioak izanik gehien metatutako metal taldeak. Hala ere, esposizio lixibiatuetara erreparatuz, MAN-7 taldea ez da metal kontzentrazio altuenak dituena, ezta Ni-a eta Pb-a kutsatzaile kontzentratuenak. Fenomeno hau, etorkizuneko lanetan argitu beharreko absortzio mekanismo konplexuen bidez bakarrik justifika daiteke.

4.3- *Eisenia fetida* zizareen esposizioa kutsatutako lurzoruetan

4.3.1- Efektuak 3 egunen buruan

Hiru eguneko esposizioaren ostean, kuantifikatutako pisu galerak ez zuen patroi edo tendentzia aipagarririk erakutsi talde ezberdinen artean. Baliteke esperimentazio denbora laburregia erabili izana (testak berez 14 egunetako espozizioak gomendatzen ditu) kalte fisikorik edo pisu galerarik antzeman ahal izateko. Izan ere, esperimentu hauen bitartez, heriotza eragiteko kontzentrazioetatik beratago ematen diren erantzunak ebaluatzea bilatzen da; maila zelularrean gertatutako erantzunak ebaluatuz adibidez. Modu honetan, esposatutako organismoen estres maila aztertzea ahalbidetzen da, pisu galerak, heriotzak edo emendiorik gabeko efektuak gertatu baino lehen.

Shock elektriko bitartez eginiko erauzketan, lurzoru kutsatuen pean izandako organismoetatik erauzitako zelula kantitatean beherakada nabarmena ikusi zen; batez ere, MAN-14 eta MAN-7 taldeetan. Gainera, desberdintasun nabarmenak ikusi izan ziren kontrol taldearen eta MAN-14 taldearen artean, non, kontrolean baino % 73 zelula gutxiago zenbatu ziren. Aipatutako beherakada hauek, zelomozito erauzketa eta lixibiatuen analisi kimikoen arteko joera negatiboarekin batera, zelulen beherakada zabortegiko kutsadurak eragindako erantzun zitotoxiko bat dela iradokitzen dute. Hipotesi hau zeharo indartu zen zelomozito erauzketa emaitzen, kaltzeina test emaitzen eta ernetze test emaitzen artean aurkitutako joera antzekoekin.

Kaltzeinari dagokionez, kontrol taldetik lurzoru kutsatuetara zihoan beherakada nabarmena ikusi zen; batez ere, MAN-7 eta MAN-14 taldeetan, non, kontrolarekiko desberdintasun nabarmenak ikusi izan ziren. Talde hauetako zizareen zelulek, kaltzeina erretenitzeko ahalmen esanguratsuki baxuagoa erakutsi zuten kontrol taldearekin alderatuz; 3 egunen ostean kalte zelularra zegoela konfirmatuz (Plytycz et al., 2007). Zelulen tindagai atxikitze edo hartze ahalmenaren jaitsiera horrek eleozito/amebozito (zelomozito mota nagusiak) ratioaren alterazio batean izan zezakeen jatorria (Plytycz et al., 2009). Izan ere, kaltzeina seinalean ikusitako jaitsiera hori, metalen esposizioak induzitutako eleozito kantitatearen beherakada (amebozitoekin alderatuz) batengatik gerta zitekeen; amebozitoak izanik hildako eleozitoak fagozitatzen dituztenak (Irizar, 2013). Era berean, kaltzeina AM bideragarritasun entseguak, hemozitometroarekin ikusitako joera baieztatu zuen.

Era honetan, eta pisu galeran dosi-efektu erantzunik antzematen ez zen arren, maila zelularrean aplikaturiko entsegu ezberdinen bitartez, MAN-7 eta MAN-14 taldeetan 3 egunez esposatutako zizareek estres subletala pairatzen dutela frogatu zen.

4.3.3- Efektuak 28 egunen buruan

28 egun igarota, biomasa galera txikiagoa ikusi izan zen kontrol taldetik esposatutako taldeetara mugitzean. Pisu gehien kontrol taldeak eta MAN-17 taldeak galdu zuten; pisu gutxien aldiz, MAN-7 taldeak. Hala ere, talde guztiek galdu zuten hasierako pisuaren % 20-a baino gehiago; hots, talde guztiek pisu galera kritikoa pairatu zuten (Garcia-Velasco et al., 2017)

3 egunen buruan ikusi izan zen bezala, ez zen ikusi esposizioarekin erlazionaturiko joerarik; pisu galera txikienak kutsatuen zeuden taldeetan aurkitu ziren. Aldiz, 3 egunean lortutako emaitzetan ez bezala, MAN-7 eta MAN-14 taldeetan ikusitako pisu galera, lurzoruetan aurki daitekeen materia organikoaren bitartez azal daiteke. Izan ere, 1.go taulan ikus daitekeen bezala, MAN-7 eta MAN-14 taldeak dira materia organiko kantitate altuena aurkezten dutenak (% 19 eta % 11). Hau indartuz, kontrako joera argi bat ikusi izan zen 28 egunean neurtutako pisu galeren eta lurzoruetako MO datuen artean. Modu honetan, kuantifikatutako pisu galera, lurzoruek daukaten MO kantitatearen araberakoa dela iradoki daiteke; eta ez aldiz, kutsaduraren ondorio zuzena denik.

28 egunez esposatu ziren zizareen analisi kimikoetan, MAN-7 eta MAN-14 taldeek, kontrol eta MAN-17 taldeak baino metal metaketa altuagoak erakutsi zituzten. Talde hauetan, kontzentrazio azpimarragarriak neurtu ziren lurzoru kutsatuetara esposatuak izan zirela kontuan hartzen bada. Aipagarriak dira ehunetan metatutako Cd kontzentrazioak; zeintzuak, 18 eta 16 ppm-tara heltzen diren MAN-7 eta MAN-14 taldeetan. Kontzentrazio hauek, beste ornogabe askotan frogatu izan den bezala, nabarmenki igotzen dira esposizio denborarekin batera (Callahan et al., 1979). Nabarmentzekoa da baita Cr eta Cd kontzentrazioen eta MO kantitateen arteko joera berdintsua, metal astun ioien sortzio arrazoi den metal-MO ligandoen eraketa iradokituz (Gondek et al., 2014). Gondek et al.-ek (2006) argitaratutako lanak, besteak beste, araztegi lokatzetako MOaren eta metalen arteko interakzioak aztertzerakoan Cd-aren

% 25-59-a eta Cr-aren % 34-81-a lurrean MO-arekin estekaturik atxikita gelditzen dela dio. Hipotesi honek indarra hartzen du 28 egunen buruan jasandako pisu galeren eta metatutako Cd zein Cr kontzentrazioen alderantzizko proportzionaltasunarekin. Era honetan, MAN-7 eta MAN-14 taldeetako pisu galera baxua, metalekin ligandoak eratzen legokeen MOaren kontsumo altuago batekin azalduko litzateke. Hau da, MAN-7 eta MAN-14 taldeetan metaturiko metalen kontzentrazioak, metal astunekin kutsatutako MOaren barneratze batengatik emango lirateke.

Bestalde, nabarmentzekoak dira zelomozitoen kontaketa eta Cd, Cr edo Ni kontzentrazioen arteko erlazio negatiboak; baita germinazioaren eta Pb edo Ni aren artekoak ere. Joera hauek, metatutako kontzentrazioen eta kuantifikatutako parametro/markatzaileen artean erlazio zuzen bat existitzen dela ematen dute aditzera. Beste behin ere behatutako erantzunak, aipatutako kutsatzaileen esposizio eta metaketaren ondorio denaren hipotesia indartzen du.

4.3.4-Ugalketa testa

56 egun eta gero, ez zen populazio dinamiketan eragin zezakeen ugalketa kalterik ikusi esposatutako talde ezberdinen artean; hau da, ez zen desberdintasun nabarmenik ikusi zizare jubenilen produkzioan.

Oinordekotzaren pisuetan kontrolarekiko eta MAN-17 taldearekiko gorakada bat ikusi zen MAN-7 eta MAN-14 taldeetan; MAN-7 taldean bereiziki, kontrolean eta MAN-17 taldean neurtutakoa bikoizteraino. Fenomeno hau, MAN-7 (% 19 MS) eta MAN-14 (% 11 MS) taldeetako materia organiko kantitate altuen bitartez azal daiteke; non, arrautzek eklosionatu zuten momentutik, elikagai gehiago eskura zezaketen.

5. ONDORIOAK

Lan honetan burututako lurzoruen osasun ebaluazioan, emaitza kimikoak beste test toxikologiko batzuekin osatzeko beharra indartzen da; emaitzak osagarriak direla frogatuz. Bestalde, MO kantitatea eta pH-a determinatzea kritikoa da; batetik, analisi ekotoxikologikoen interpretazio egokia egiteko; eta bestetik, kutsatzaileen bioeskuragarritasunean aurki daitezkeen desberdintasunak azaltzeko.

Metal toxikoen presentziak, maila altuko metaketak eragin zituen 28 egunen buruan 17 Zabortegiko lurzoruetan jarritako zizareetan. Are gehiago, metaketa hauek, denboran zehar emendatu egiten dira. Parametro zelularretan oinarritutako analisiek, zabortegiko kutsatzaile anitzek eragindako efektu zitotoxikoa frogatu zuten. Erantzun zelular hau oso sentikor eta goiztiarra da; organismo mailako efektuak (biomasa galera, heriotza…) gertatu baino lehen arriskua kuantifikatzeko tresna eraginkorrak direla bermatuz. Era berean, organismo ezberdinak erabiltzen dituzten entseguen konbinazioa efektu toxikoak antzemateko metodo baliagarria dela balioetsi da.

Ondorioz, lan honetan MAN-7 eta MAN-14 lurzoruetan dauden ornogabeak arriskuan egon daitezkeela frogatu da; konponbide gisa, lurzoruen erremediazioa gomendatzen delarik.

6. ESKER ONAK

Egileon esker onak: Gernikako Udalari, elkarrekin xedatutako hitzarmenak (2018/12/11) lan hau posible egin baitu; Rafa Ensunza Gernika-Lumoko teknikari ohiari bere jakintza eta gomendio ordainezinengaitik; Biorem proiektuari (IHOBE, Ekoberrikuntza 2017ko deialdia) lan hau finantziatzeagatik eta ZBIT ikerkuntza taldeari emandako sostengu eta azpiegiturengatik.

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CHAPTER 7

Application of *in situ* biorremediation strategies in soils amended with sewage sludges

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ABSTRACT

Increasing soil loss and the scarcity of useful land requires new reusing strategies. Thus, recovery of polluted soils offers a chance for economic and social regeneration. With this objective, different soil cleaning technologies have been developed during the last few decades. On one hand, classical physical and/or chemical technologies can be found; which are efficient, but have high costs and impacts upon ecosystems. On the other hand, biological methods (such as phytoremediation, bioremediation and vermiremediation) are relatively cost effective and eco-friendly, but also more time-consuming. These biological methods and their yields have been widely studied but little is known about the interaction between different soil cleaning methods. The combination of different biological strategies could lead to an improvement in remediation performance. Hence, in the present work, different micro-, vermi- and phyto-remediation combinations are applied in a sewage sludge polluted landfill in Gernika-Lumo (Basque Country) which was used as a disposal point for decades, in search of the treatment (single) or combination (dual or triple) of treatments with best remediation yields. Eight experimental groups were applied (n=3) placing earthworms (E), bacteria (B), plants (P), bacteria+earthworms (B+E), bacteria+plants (B+P), plants+earthworms (P+E) plants+bacteria+earthworms (P+B+E) and a non-treated (N.T) group in the experimental plot (Landfill 17), for 12 months. In order to assess the efficiency of each treatment, a complete characterization (chemical and ecotoxicological) was carried out before and after remediation. Results showed high removal rates for dieldrin (between 50% and 78%) in all the experimental groups. In contrast, removal rates around 20-25% were achieved for heavy metals (Cd 15%-35%; Ni 24%-37%; Pb 15%-33%; Cr 7%-39%) and benzo(a)pyrene (19.5%-28%). The highest reductions were observed in dual (P+E, B+E) and triple (P+B+E) treatments. The best elimination yields were obtained after P+B+E treatment, as highlighted by the battery of ecotoxicological tests and bioassays performed with earthworms, plants and bacteria.

Keywords: Bioremediation, bacteria, plants, earthworms, sewage sludges, *in situ*, metals, PAHs, pesticides

LABURPENA

Gaur egungo lurzoru galerak, nekazal lurzoru eta lurzoru naturalen eskasiarekin batera, berrerabilpenerako estrategi berriak garatzearen beharrizana galdatu du; zentzu honetan, kutsatutako lurzoruen berreskuratzea, berpizte ekonomiko eta sozialerako aukera bikaina izanik. Intentzio honekin, lurzoruen deskontaminaziorako hainbat teknologia garatu izan dira azken urteotan. Gehienek, kostu handia duten eta ekosisteman inpaktu handiak dituzten teknologia fisiko edo/eta kimikoak erabiltzen dituzte. Aldiz, teknologia biologikoak, errentagarriagoak eta ingurumenarekiko jasangarriagoak dira; hauen artean, fitoerremediazioa, bioerremediazioa eta bermierremediazioa nabarmenduz. Teknologia hauek eta berauen errendimenduak, sakonki ikertua izan dira azken hamarkadetan; hala ere, teknologia batek gainontzekoengan sor ditzakeen eraginen inguruko informazioa eskasa da. Izan ere, estrategia biologiko ezberdinen konbinazioak erremediazio etekinen emendioa eragin dezake.

Lan honetan, mikro-, bermi- eta fito-erremediazio konbinazioak araztegi lokatzekin kutsatutako lurzoru batean (17 Zabortegia; Gernika-Lumo; Euskal Herria) aplikatzen dira; errendimendu altuenak dituen bakarkako, binakako edo hirukako teknologia bilatzea xedez. Horretarako, zizareak (E), bakterioak (B), landareak (P), bakterioak+zizareak (B+E), bakterioak+landareak (B+P), landareak+zizareak (P+E) eta landareak+bakterioak+zizareak (P+B+E) konbinatzen zituen 7 esperimentazio talde (3 erreplika bakoitzeko) jarri ziren esperimentazio esparruan (17 Zabortegian). Paraleloki, tratamendurik gabeko kontrol talde bat ezarri zen. Tratamendu bakoitzaren efizientzia kuantifikatze aldera, biak, fokatze ekotoxikologiko eta kimikoak barneratzen zituen karakterizazio orokorrak burutu ziren erremediazioa hasi eta bukatzerakoan (urte bete). Egindako probek dieldrin eliminazio ratio altuak erakutsi zituzten; %50 eta %78 arteko eliminazioak aurkeztuz. Bestalde, metal eta benzo(a)pireno kontzentrazioak %20-%25 bitartean jaitsi ziren (Cd 15%-35%; Ni 24%-37%; Pb 15%-33%; Cr 7%-39%; Benzo(a)pirenoa 19.5%-28%). Tratamenduen arteko desberdintasunei erreparatuz, jaitsiera handienak P+E, B+E, eta P+B+E taldeetan antzeman ziren; hala ere, zizare, landare ta bakteriekin burutako entsegu ekotoxikologiko ezberdinek, P+B+E

tratamendua azpimarratu zuten lurzoru osasuna gehien emendatzen zuen tratamendu gisa.

Hitz gakoak: Bioerremediazioa, bakteriak, landareak , zizareak, araztegi lokatzak, *in situ*, metalak, PAHak, pestizidak.

1.- INTRODUCTION

Industrial activity, together with a high human population density and scarcity of useful land, have contributed to the proliferation of dumps, landfills and waste disposal points at different urban, peri-urban and natural areas around the world. This soil loss and the scarcity of natural or agricultural lands require new strategies for reuse. In this sense, the recovery of polluted soils (for other uses) offers a chance for social and economic regeneration. With this purpose, several decontamination technologies/treatments have been developed. Most of them employ physical and/or chemical technologies with high cost and big impact for the ecosystems (Jan et al., 2015). By contrast, biological technologies offer good remediation results in a cost effective way, although long times are needed to produce significant improvements. Among these techniques microbial bioremediation, phytoremediation and vermiremediation are widely known.

Microbial remediation employs microorganism metabolism and its synergies to degrade pollutants in presence of optimal environmental conditions and nutrient availability. Moreover, the introduction of individual strains or consortia with desired catalytic skills (Agnello et al., 2016) can enhance biodegrading capabilities at polluted sites (bioaugmentation). Among the most used strains *Pseudomonas aeruginosa* improves metal and hydrocarbon availability for further decontamination procedures (Visca et al., 2007; Zhang et al.,2012; Agnello et al., 2016). Besides, its role as growth-promoting rhizobacteria improves plant growing; therefore boosting phytoremediation yields (Agnello et al., 2016).

Phytoremediation is based on plant (and associated microorganisms) capacity to tolerate, absorb, accumulate and degrade pollutants. Recently is widely used in several countries to remediate organically and inorganically polluted landfields (Meagher, 2000; Nwoko, 2010; Ali et al., 2013). This eco-friendly and *in situ* applicable technology, is one of the best options for extensive areas in which the application of other techniques would be unprofitable or impracticable (Garbisu & Alkorta, 2003). The modern phytoremediation techniques are based on different mechanisms, including: (1) phytoextraction (extraction of pollutants out of soil to harvestable parts), (2) phytostabilization (reduction of pollutants mobility and availability), (3)

phytoevaporation (volatilization of pollutants into atmosphere), (4) rhizofiltration (roots filtration of pollutants from polluted flows) and (5) rhizodegradation (Garbisu & Alkorta, 2003; Halim et al., 2003; McIntyre, 2003; Pulford & Watson, 2003; Ghosh & Singh, 2005; Yang et al., 2005; Kotrba et al., 2009).

Epigeic earthworms are organic feeder bioagents capable of decompose and remediate quick and efficiently soils receiving organic and industrial wastes (Hickman & Reid, 2008). Therefore, vermiremediation/vermicomposting processes have been described as one of the best options for the stabilization of solid or waste water treatment plant (WWTP) residues (Neuhauser et al., 1988; Sinha et al., 2008; Suthar et al., 2012; Negi and Suthar, 2013; Rodriguez-Campos et al., 2014), but also to degrade non-recyclable compounds (Gupta and Garg, 2009; Rodriguez-Campos et al., 2014). The positive effect of earthworms on the removal of different contaminants (e.g. oils, PAHs, PCBs, pesticides or metals) has been widely described by a variety of authors along the last decades (Contreras-Ramos et al., 2008; Geissen et al., 2008; Hickman et al., 2008; Tejada & Masciandaro, 2011; Rodriguez-Campos et al., 2014). Earthworms crush the residual materials into finer particles, allowing their intestinal microflora to transform non-bioavailable elements into bioavailable forms for expelling of the mineralized materials. In a similar way, earthworms fix different metals by accumulating them in target tissues (e.g. digestive tract) associated to cellular ligands as metallothioneins (Goswami et al., 2014; Sahariah et al., 2015). These proteins bind metal ions inside organometallic compounds and, when earthworms die, these fixed metals are exposed to edaphic conditions and retained in soil as immobile forms by the humic substances (Nannoni et al., 2011).

As mentioned before, bioremediation technologies and their yields are widely known and applied world-wide. However, little is known about the impact of each technology upon the rest. That is, whether single remediation performances could be enhanced when different biological strategies are combined. The study of these remediation yields can be carried out from two different approaches; chemical and ecotoxicological. The former provides data on changes in the concentration of pollutants, while the latter aggregates data on the improvement of the "health" of the soil. An analysis that encompasses both evaluation techniques is essential to assess the

performance of this type of soft decontamination techniques (bioremediation) especially if the techniques are based on biostabilization or contaminants unavailability. International organizations (i.e. OECD, USEPA) have developed and promoted the use of standard tests (Kong, 2013), where the toxicity of chemical agents is examined by using different species, including bacteria, algae, plants, worms or fish (US EPA, 1993). Hence, standard toxicity tests, OECD-207 (Earthworm Acute Toxicity Test, 1984), OECD-222 (Earthworm Reproduction Test, 2016), seedling emergence and root elongation plant bioassay, and other non-standard bioassays (soil respiration, enzymatic activities and community level physiological profiles) have been widely used to assess the effects of soil contamination and could be used to address bioremediation success.

In the present work combinations between microbial remediation, phytoremediation and vermiremediation strategies were applied in a public landfill located in Gernika-Lumo (Basque Country) in which active sludges from a WWTP were deposited for at least two decades. The aim was to search the treatment (single) or combination of treatments rendering the best remediation yields. In order to assess the efficiency of each treatment, the Landfill soil was fully characterized before and after applying the remediation techniques, integrating both chemical and ecotoxicological approaches.

2.- MATERIALS AND METHODS

Landfill 17 from Basque Country Landfill Inventory (2005) located in Gernika-Lumo (Basque Country,43°19'28.9"N 2°40'30.9"W) was selected due to its usage as disposal point receiving sewage sludge from a WWTP for decades starting in 1980s. The study was divided in two phases. First, Landfill 17 was chemically and ecotoxicologically characterised. The second phase of the study was aimed to establish the best soft bioremediation technology or combination to reduce contaminant levels *in situ*.

2.1- Phase I: Chemical and ecotoxicological characterization

2.1.1. Soil sampling and chemical characterization of soils

Field sampling was carried out in the Landfill 17 at two different periods, May 2015 and May 2018. Taking into account the diffuse character of the pollution, sampling points were homogeneously distributed in order to cover the largest area of the landfill. In May 2015, 20 points were sampled with piezometers manually and mechanically (Fig.1SI, Supplementary information). In May 2018, additional 8 soil sites were added in order to complete previous characterization campaigns (Fig. 1SI). Soil samples were manually collected from the soil surface. The aim of this second sampling (May 2018) was (1) to observe natural changes on pollutants distribution/concentration in 3 years and (2) to determine the points with the highest pollution levels in order to apply remediation techniques in Phase II. Samples were always transported under controlled temperature (4-7 °C), humidity and darkness conditions to the laboratory.

All chemical analysis of soils were carried out in SYNLAB, homologated laboratory (NEN-EN-ISO/IEC 17025:2005 nº L028) with certification NEN-EN-ISO 9001:2015. The following compounds were quantified according to quality standards: Metals (As, Cd, Cr, Cr VI, Cu, Hg, Pb, Mo, Ni, Zn), cyanides, volatile compounds (benzene, toluene, ethylbenzene, o-, p- and m-xylene, total BTEX, styrene), phenols, PAHs (16 EPA); organo-halogenated volatile compounds (14), chlorobenzenes (5), chlorophenols (6), PCBs (7), chlorinated pesticides (22), hydrocarbons (C5-10, C10-40, Total) and amino compounds. In parallel soil pH and organic matter (OM) content were measured. A second set of soil samples was sieved (4 mm) and stored at 19°C and constant humidity for ecotoxicological characterization.

The sampling point with the highest concentrations of critical pollutants (worst case scenario) was selected in order to carry out ecotoxicological bioassays. The study was focused on the critical pollutants: Cd, Cr, Pb, Ni, benzo(a)pyrene and dieldrin, estimated on a previous Quantitative Environmental Risk Analysis ordered by the Basque Government. Data for each chemical compound were normalized according to the legal thresholds established by the Basque norm and Pollution index (PI) was calculated as described in Håkanson (1980):

$$PI = C/Cn$$

where C is soil concentration and Cn is the Basque reference level for the substance.

Then, the sum of all individual pollution indexes was obtained:

$$PI_{sum} = \sum_{i=1}^{m} Pi$$

2.1.2. Toxicity bioassays

Earthworms, plants and microorganisms, were used as model organisms for ecotoxicological assays in order to assess initial soil health in the worst case scenario.

Acute toxicity tests, both filter paper test and artificial soil test, and earthworm reproduction test were carried out with *Eisenia fetida* earthworms strictly following OECD-207 (1984) and OECD-222 (2016) standard guidelines. OECD artificial soil was used as control. *E. fetida* earthworms were purchased from the commercial supplier Lombricor SCA (Cordoba, Spain) and were all healthy, sexually mature with a weight range between 300-500 mg (f.w.). Specimens were maintained under stock conditions: constant humidity (60%) and temperature (19°C) until experimentation. After earthworm exposure chemical analysis to determine pollutant tissue accumulations were carried out. Briefly, five earthworms per experimental group were used to perform chemical analysis. After 24 h depuration, earthworms were dried, weighted and subjected to chemical digestion according to García-Velasco et al. (2016, 2017). All measurements were performed in SGiKER General Service of Analysis, University of the Basque Country, Leioa.

A series of standard toxicity tests with plants (*Lactuca sativa*, *Allium cepa*, *Cucumis sativus*) were performed to assess soil phytotoxicity. The seed germination was conducted with three plant species: two dicotyledonous *Cucumis sativus* and *Lactuca sativa* and one monocotyledonous *Allium cepa* by following EPA 850.4100 standard guideline. The root elongation bioassay was developed with the same species following Lacalle et al. (2018,2020) (a modified test from EPA 850.4230). Root Elongation (RE) (RE = $RET_{final} - RET_{initial}$) was calculated for each seedling (Lacalle et al., 2018).

Community-level physiological profiles (CLPPs) of cultivable heterotrophic bacteria were determined with Biolog EcoPlates™- (Insam, 1997) following Epelde et al. (2008). Data were calculated from Gompertz regressions (three parameters) of the obtained curves as proposed by Preston-Mafham et al. (2002), (i) average well colour development (AWCD) was determined by calculating the mean of every wells absorbance; (ii) number of utilized substrates (NUS), i.e. number of substrates with an absorbance value>0.25; and (iii) Shannon's diversity index ($H' = -\Sigma p_i \log_2 p_i$) was calculated considering absorbance values at each well as equivalent to species abundance.

2.2- Phase II: *In situ* application of bioremediation techniques

2.2.1. Plot design, subdivision and homogenization

One area with dimensions of 18 m x 12 m surrounding the worst case point (according to results obtained in Phase I) was selected for the application of remediation techniques. The whole area was stirred and homogenized several times and 24 subplots were defined (Fig. 2SI): 2m (width) + 1m (width margin) x 2 m + 1 m (height) (Fig. 2SI). Soil samples were obtained in each of the 24 subplots in order to characterise chemical and toxicological profiles at time 0 (May 2018).

2.2.2. Application of biological remediation technologies

Bioremediation technologies and their dual and triple combinations were randomly applied on each subplot for 12 months. Hence, 8 experimental groups (with 3 replicates each) were randomly prepared: earthworms (E); bacteria (B); plants (P); bacteria + earthworms (B+E); plants + bacteria (P+B); plants + earthworms (P+E); plants

+ bacteria + earthworms (P+B+E), and a non-treated (N.T) group without remediation techniques (Fig. 2SI-B).

The plant species selected for the phytoremediation process was alfalfa (*Medicago sativa,* variety "Tierra de campos"). The selection of alfalfa cultivation was made based on its physiological characteristics as well as the phytoremediation capacity of polluted soils. Alfalfa is a multi-annual crop, with rapid growth, that supports the harvest of the aerial part and develops an important root biomass. The sowing was carried out in June 2018 to ensure a good implantation of alfalfa. The sowing dose was 20 kg/Ha (as recommended by the distributor) and was applied by hand. No selective treatment with herbicides was applied to prevent the growth of the plant species present in the soil seed bank.

Bioremediation through the application of bacteria was performed with: *Burkholderia xenovorans* LB400 and *Paenibacillus sp. Burkholderia xenovorans* LB400 strain has broad catabolic versatility for aromatic compound degradation (Chain et al., 2006). The *bphK* gene located in the *bph* operon of *Burkholderia xenovorans* LB400 encodes a protein, BphKLB400, with significant sequence similarity to glutathione-S-transferases (GSTs). GSTs are a superfamily of enzymes involved in the detoxification of many endobiotic and xenobiotic substances. Regarding *Paenibacillus sp*. this strain was isolated in previous studies from real contaminated soils and was selected for having genes related to pollutants degradation. Bacterial strains were grown individually in 2 L bottles at 1.5 L of sterile Luria-Bertani medium, at 28 °C and orbital agitation, following the method described by Burges et al. (2016). After 72 h, the culture medium was centrifuged in 250 mL bottles for 5 min at 4500 rpm, discarding the supernatant. The cells were resuspended in deionized water in a final volume of 2 L. Subsequently the two strains were combined in the same container before applying them in the field. The optical density was determined at 660 nm using a spectrophotometer (Shimadzu UV-1800) in order to estimate cell density. The determined optical densities were in a range of 1.9-2.1 (an optical density of 1.25 is equivalent to 109 CFU/mL). Once in the field, the bacterial broth was diluted 10 times in distilled water and 2 L of the mentioned dilution were sprayed on each plot. Three applications were made in autumn (October 2018) and

three more in spring (April-May 2019) taking advantage of the periods of greatest microbial activity.

For vermiremediation, and in order to achieve a density of 170 worms/m^2, earthworms were manually counted and transported from laboratory stock to Landfill 17 in moistened containers. Then, earthworms were spread evenly in the subplots and their entry into the soil was carefully observed and verified. For treatments in which worms and bacteria were combined (B+E; P+B+E), worms were sprayed together with bacterial culture medium. This action sought the incorporation and impregnation of the remediating bacteria to worms, thus helping in the transport and segregation of the colonies through the soil column. This process was repeated twice (October 2018 and April 2019) during the treatment of 12 months that finished in June 2019.

2.2.3. Final chemical and ecotoxicological characterization

Once the remediation period was finished (June 2019) soil samples were collected again in each of the 24 subplots for chemical and ecotoxicological analyses. The measurement of the same critical pollutants and the most responsive and accurate assays carried out during phase I were repeated. Thus, Cd, Cr, Pb, Ni, benzo(a)pyrene and dieldrin were quantified in the 24 subplots in order to calculate chemical yielding (C_i-C_f/C_i; in %) and reproduction test for earthworms, seed germination and root elongation bioassays for plant species and AWCD, NUS and Shannon diversity index for microbial communities were applied. Additionally, other soil parameters with indicator potential as soil respiration or enzymatic activities were also measured: β-glucosidase (EC 3.2.1.21), β-glucosaminidase (EC 3.2.1.30), xylosidase (EC 3.2.1.37), acid phosphatase (EC 3.1.3.2), L-Ala-aminopeptidase (EC 3.4.11.12) and L-Leu-aminopeptidase (EC 3.4.11.1). Soil respiration was determined by measuring CO_2 evolution according to ISO-16072 (2002). Enzimatic activities were determined according to ISO/TS-22939 (2010), using fluorogenic substrates [4-methylumbelliferyl (MUF) and 7-amino-4-methylcoumarin (AMC)] in 96-microwell plates.

2.3. Statistical analyses

Data obtained was analysed by SPSS software vers. 22. Normality and equality of variances of the datasets were checked through Shapiro-Wilk and Levene's tests respectively. Statistical significant differences ($p<0.05$) between control group and Landfill 17 soils were studied by one way ANOVA followed by Tukey´s comparison and Dunnet´s test for parametric data. On the other hand, non-parametric datasets were analysed with Kruskal- Wallis followed by Dunn´s post-hoc test.

3.-RESULTS

3.1- Phase I: Chemical and ecotoxicological characterization

3.1.1. Chemical characterization of soils

Soil samples collected in May 2015 did not show any pattern or distribution gradient of pollutants. In fact, MAN-2 point, the closest one to the WWTP, showed the highest concentrations for Cd, Cr, Pb, Ni and dieldrin (Table 1SI) while the highest contents for benzo(a)pyrene were obtained at northern MAN-8 and MAN-10 points. After 3 years (May 2018) values were unaltered (Table 2SI). Indeed, MN3 point (equal to MAN-2 in 2015) exhibited the highest concentrations of Cd, Cr, Pb and dieldrin and quite high levels of benzo(a)pyrene, while the highest concentrations of nickel and benzo(a)pyrene were measured at MN8 (Table 2SI; equal to MAN-8 in 2015). The highest Pollution index was obtained for MN3 and therefore was selected as the worst case scenario for further analyses.

3.1.2. Toxicity bioassays

Regarding Filter paper test (OECD-207, 1984) with earthworms, after 48 h of exposure no differences on weight loss were observed among control, water control and MN3 groups (Fig. 3SI). Weight loss was lower than 20% for all the groups and no mortality was observed in the experimental groups. Nevertheless, 100% mortality was observed in the positive control group (OCED soil spiked with then cocktail of pollutants at the Basque legal threshold for industrial use).

Earthworms exposed to MN3 polluted soils showed significantly higher contents of Cr, Cd and Pb, than the OECD control group (Fig. 4SI-A) while Ni concentration was not significantly different to the control group. Although cadmium was largely accumulated after 14 d (8.19 µg Cd/g), the highest increase occurred with Cr (>8 fold). After performing Reproduction test (OECD-222; 2016), Cr, Ni, Cd and Pb concentrations in earthworms maintained in MN3 soils were significantly higher than in controls after 28 d. Cd increase was the highest >10 times reaching 18.91 µg Cd/g (Fig. 4SI-B). The accumulation of the rest of metals (Cr, Ni and Pb) was always below 5 mg/kg soil. Benzo(a)pyrene and dieldrin concentrations were under detection limit for all the experiments.

After 7 and 14 d of exposure, no mortality neither significant differences in weight loss were recorded in all the treatments (Fig. 5SI-A and 5SI-B). Earthworms at MN3 showed similar weight losses (<20% in all) to the control groups after both exposure times (7 and 14 d). Earthworms maintained in MN3 soil showed a trend to lose more weight than controls at long exposure periods. Adult earthworms removed from control and polluted soils at day 28 showed similar weight losses. Nevertheless, as a mean value, earthworms at soils MN3 showed a higher weight loss than those at control group, losing more than the 20% of their initial weight.

Cocoon production in MN3 soils was significantly lower than in the control group (37.6 vs 10.3, Fig. 1A). Juvenile number (Fig. 1B) and biomass of the offsprings (Fig. 1C) showed also significantly lower values in earthworms at MN3 in comparison to OECD control.

Plant bioassays performed with three plant species (*C. sativus, L. sativa and A. cepa*) to evaluate initial phytotoxicity of soil MN3 shown that root elongation was a parameter very sensitive as it was affected in all species (Fig. 2). However, the percentage of germination was not significantly affected (Fig. 6SI). Although the 3 species exposed to polluted soil exhibited different root elongation rates, all of them were statistically reduced ($p<0.05$) in MN3 soil compared with control soil. *C. sativus* was the most sensitive species with a 50% of root elongation inhibition (Fig. 2A), followed by and *A. cepa* and *L. sativa* (35 and 28%, respectively).

The results of toxicity bioassays with microbes showed that the values of the three parameters determined (AWCD, NUS and Shannon diversity index) were significantly lower in MN3 samples with respect to the control (Fig. 3). After 42 h, AWCD in MN3 soil was about 20% with respect to the control soil, while the NUS used and the Shannon diversity index were close to zero.

3.2- Phase II: Chemical and ecotoxicological evaluation of bioremediation treatments

3.2.1. Chemical yielding

Dieldrin was the contaminant with the highest removal rates with degradations between 50% and 78% in all experimental groups. Meanwhile, elimination rates around

20-25% were achieved for heavy metals and benzo(a)pyrene (Table 1): Cd 15-35%; Cr 7-39%; Pb 15-33%; Ni 24-37%; benzo(a)pyrene 19.5-28%. The best yields and the lowest intragroup variability were achieved in groups with double and triple combination of remediation techniques. The variability in groups with individual treatments (B, L, P) exceeded 100% of the average value.

Table 1. Concentrations (mg/kg soil) of cadmium, chromium, lead, nickel, dieldrin and benzo(a)pyrene –B(a)P- in soils of each subplot, after individual, dual and triple application of vermi-, phyto- and microremediation technologies. Mean values and standard deviations for each treatment are shown. Values in bold correspond to values exceeding Basque legislation threshold for polluted sites. E earthworms; B bacteria; P plants; P+B plants + bacteria; P+E plants + earthworms; B+E bacteria + earthworms; P+B+E plants + bacteria + earthworms.

Compound		Basque Thresholds	B		P		E	
			Pre	Post	Pre	Post	Pre	Post
Cadmium	mg/kgdw	**5**	**11.73 ± 3.4**	**9.7 ± 4.2**	**15.33 ± 3.8**	**12.1 ± 2.6**	**19.03 ± 10.5**	**11.9 ± 4**
Chromium	mg/kgdw	**200**	140 ± 10	131.9 ± 49.5	186.66 ± 35.1	160 ± 26.2	**236.6 ± 135.8**	164.3 ± 61.3
Lead	mg/kgdw	**120**	86.33 ± 3.2	73.6 ± 19.7	102 ± 17.1	80 ± 0.0	113 ± 50.9	80 ± 24
Nickel	mg/kgdw	**110**	60.66 ± 4.7	46 ± 12.4	69.33 ± 7.6	51.3 ± 5.7	78.33 ± 30.6	52.8 ± 14.7
BaP	mg/kgdw	**0.02**	**0.08 ± 0.01**	**0.06 ± 0.03**	**0.09 ± 0.02**	**0.06 ± 0.01**	**0.11 ± 0.09**	**0.06 ± 0.02**
Dieldrin	µg/kgdw	**10**	**13.26 ± 13.2**	5 ± 5.1	**33.33 ± 21.5**	8.1 ± 3.7	**49.5 ± 16.3**	8.2 ± 5.8

Compound		Basque Thresholds	P+E		B+E		P+B	
			Pre	Post	Pre	Post	Pre	Post
Cadmium	mg/kgdw	**5**	**19 ± 7**	**11.7 ± 4.1**	**18.2 ± 11.2**	**11.3 ± 5**	**12.5 ± 2.8**	**12.8 ± 0**
Chromium	mg/kgdw	**200**	**220 ± 79.4**	144.9 ± 44.2	**234 ± 134.7**	151.4 ± 68.7	156.66 ± 40.4	155.7 ± 0
Lead	mg/kgdw	**120**	115 ± 35	75.1 ± 21.9	115.66 ± 56.6	75.8 ± 26.6	89.33 ± 11	86.7 ± 0
Nickel	mg/kgdw	**110**	81 ± 20.8	49.8 ± 11.6	82.33 ± 37.5	52.1 ± 17.7	58.33 ± 8.5	51.7 ± 0
BaP	mg/kgdw	**0.02**	**0.09 ± 0.03**	**0.06 ± 0.03**	**0.07 ± 0.03**	**0.06 ± 0.02**	**0.06 ± 0.01**	**0.06 ± 0**
Dieldrin	µg/kgdw	**10**	**27.03 ± 22.6**	3.6 ± 3.5	**34.96 ± 31.1**	8.1 ± 7.6	**24.96 ± 22.3**	6.1 ± 0

Compound		Basque Thresholds	P+B+E	
			Pre	Post
Cadmium	mg/kgdw	**5**	**15.1 ± 6.5**	**10.5 ± 2.5**
Chromium	mg/kgdw	**200**	180 ± 70	142.7 ± 29.7
Lead	mg/kgdw	**120**	98 ± 23.1	77.3 ± 10.9
Nickel	mg/kgdw	**110**	66.3 ± 17.6	47.3 ± 6.9
BaP	mg/kgdw	**0.02**	**0.08 ± 0.02**	**0.06 ± 0.01**
Dieldrin	µg/kgdw	**10**	**19.6 ± 16.9**	4.9 ± 2.8

3.2.2. Toxicity bioassays

Chromium accumulations along the reproduction test in earthworm tissues ranged from 7.19 (non-treated group) to 25.45 µg Cr/g tissue (B+E). Low accumulation rates were observed in earthworms maintained in soils with a single treatment, while

higher accumulation rates were registered in dual and triple treatments (Fig. 7SI-A). Nickel concentrations ranged from 4.48 µg Ni/g tissue (non-treated group) to 9.71 µg Ni/g tissue in the B+E dual treatment. Low accumulation rates were observed in non-treated and individually remediated soils (6.58 µg Ni/g tissue, B treatment). Dual and triple treatments showed higher accumulation rates in earthworms; being observed significant differences between non-treated soil earthworms and earthworms in B+E treated soil (Fig. 7SI-B). Cadmium was the metal with the highest accumulation in earthworm tissues, with values ranging from 28.12 (non-treated) to 41.62 µg Cd/g tissue (P treatment). No statistical differences on accumulation were recorded among earthworms maintained in different bioremediated soils. However, significant differences were observed among non-treated group and individual (P, B), dual (P+B and P+E) and triple (P+B+E) treatments (Fig. 7SI-C). Lead concentrations showed a similar trend to Cr and Ni. Non-treated group exhibited the lowest lead accumulation values (3.25 µg Pb/g tissue); while, B+E dual treatment achieved 10.89 µg Pb/g tissue (Fig. 7SI-D). The rest of the treatments ranged between 3.57 and 8.66 µg Pb/g tissue. However, a slight trend to accumulate more Pb was observed in dual and triple combined treatments comparing to individual treatments and non-treated group. In fact, statistical differences were reported between non-treated group and B+E dual treatment.

Weight loss in adult earthworms after 28 d showed similar values in all groups with losses smaller than 5% of their initial weight for all cases except for the E group, where the worms increased their weight very slightly (Fig. 8SI). No earthworm mortality was observed.

Cocoon production (Fig. 1A) showed the lowest values in the non-treated group, with high values in remediated subplots. The highest cocoon number was accounted in the triple (P+B+E) treatment, showing significant differences with the non-treated group (23.3 vs 7.5). The dually combined P+E was the second treatment with highest cocoon number accounted (17.25).

The highest juvenile production was recorded in the triple P+B+E treatment (70.3), and was significantly different to non-treated group (37) and all the individual (B: 37, P: 37 and E: 22.75) and dually combined treatment (P+B: 35.5)(Fig. 1B). Double and

triple treatments showed higher juvenile numbers comparing to individual treatments. Meanwhile, lowest juvenile number was accounted in the E single treatment.

The biomass of the offspring showed highest values also in the triple P+B+E treatment, while the lowest weight were measured in the E individual treatment (Fig. 1C). However, P+B+E treatment showed statistical differences respect to the non-treated group, B and E individual treatments and P+B double treatment. As already seen in juvenile counting, double and triple treatments showed higher offspring biomass comparing to individual treatments and non-treated group.

Comparing pre- and post- remediation reproductive parameters, an increase on cocoon (10.3 vs 23.3) and juvenile production (66.33 vs 70.3) could be accounted in the triple treatment. Nevertheless, all post remediation subplots showed lower offspring biomass values tan those measured pre-remediation.

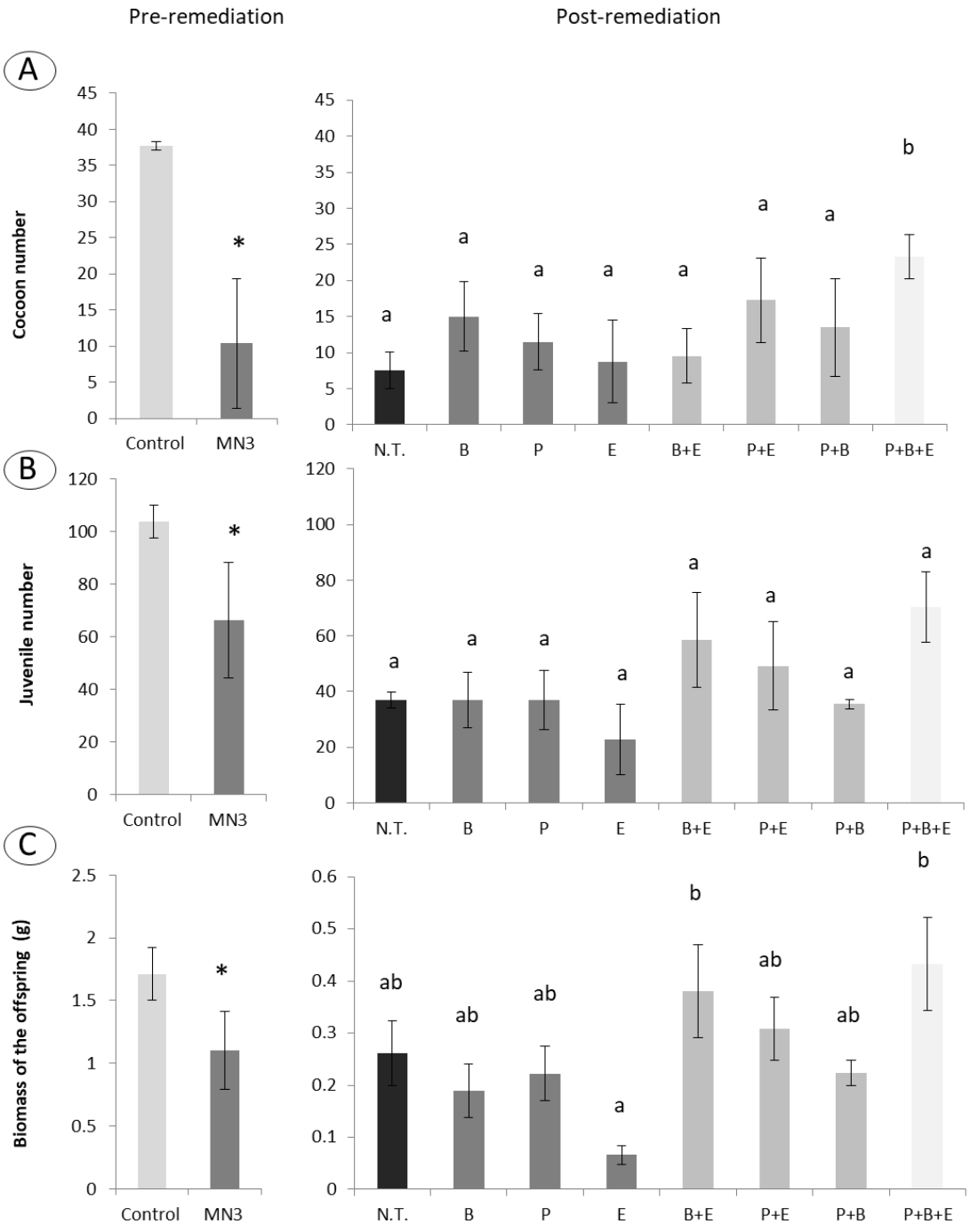

Figure 1. Cocoon number (A), juvenile number (B) and biomass of the offspring (C) after exposure of *E. fetida* adult earthworms to OECD and MN3 soils (Pre-remediation); and remediated soils from Landfill 17 (Post-remediation). Remediated soil are classified depending on used cleaning technology (N.T means non-treated Landfill 17 soil). Mean values and standard deviations for each treatment are shown. Significant differences marked with asterisks and letters (ANOVA test, $p<0.05$).

Similar to the results of initial characterization, germination rate of *C. sativus, L. sativa* and *A. cepa* was very high (80-100%) in the non-treated group, and was not affected by the presence of contaminants after bioremediation treatments application (Fig. 6SI). Root elongation after bioremediation treatments showed a partial recovery depending on the differential sensibility of species to contaminants (Fig. 2). Thus, the most sensitive species in the initial characterization (*C. sativus;* Fig. 2B) was the species most benefited by the assayed treatments, particularly in treatments where earthworms were present (Fig. 2B). According with our results the increase of root elongation after biological treatments was low for *L. sativa* (Fig. 2A) and insignificant for *A. cepa* (Fig. 2C), precisely the species less affected in the initial characterization. In overall terms, all biological treatments decrease soil phytotoxicity, and treatments that include earthworms could be the most effective ones.

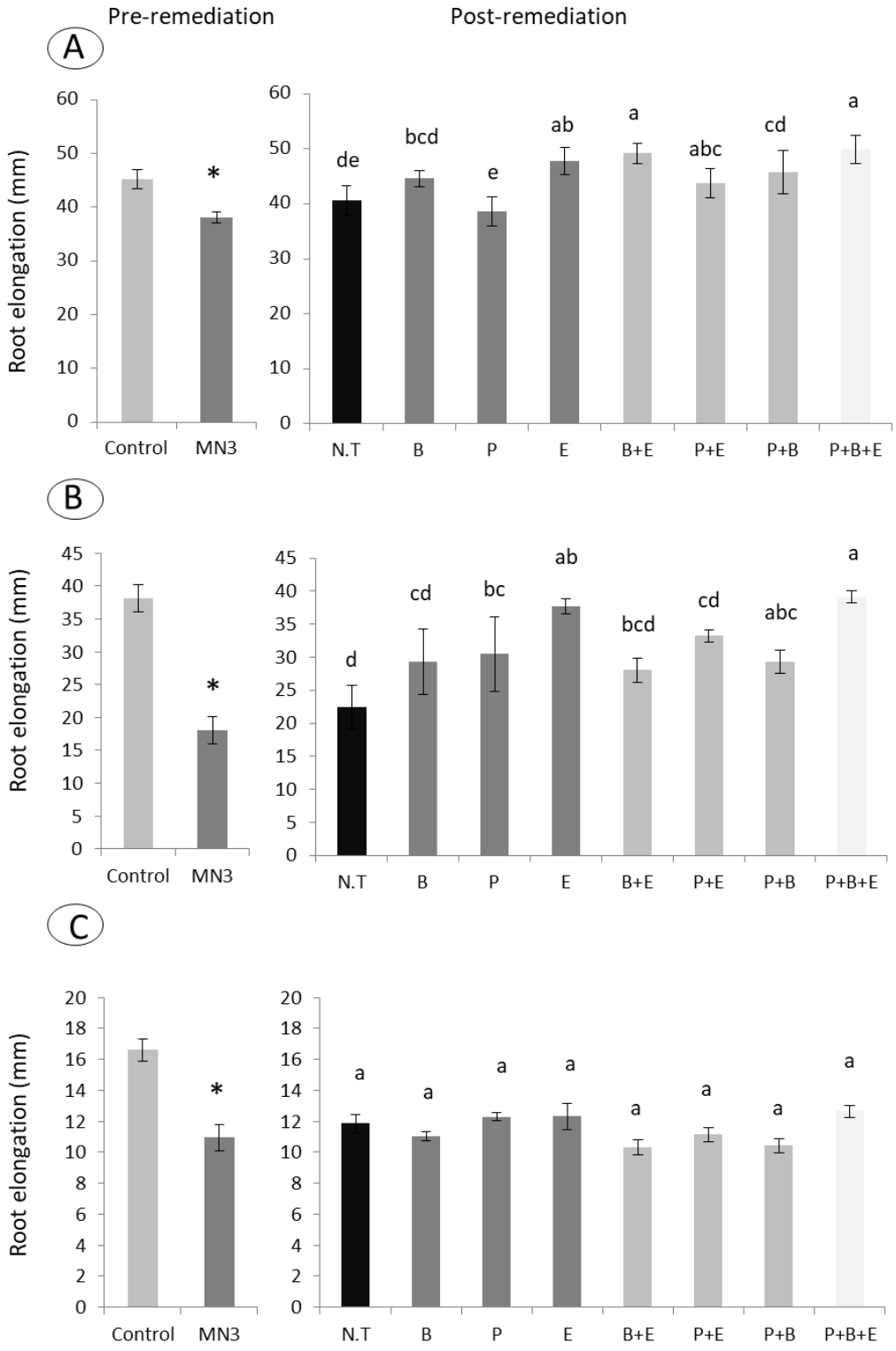

Figure 2. Root elongation (mm) bioassays of three plant species: *Lactuca sativa* (A), *Cucumis sativus* (B) and *Allium cepa* (C), exposed to OECD and MN3 soils (Pre-remediation); and remediated soils from Landfill 17 (Post-remediation ; N.T means non-treated). Treatments with different letters show significant differences according to Duncan's Test ($p<0,05$).

The microbial soil respiration values obtained showed no significant differences with respect to the non-treated group, except in the P treatment, where the highest values were observed (Fig. 9SI). The values of the enzyme activities β-glucosidase, arylsulfatase, L-Alanine-aminopeptidase and L-Leucine-aminopeptidase were lower in the B+E treatment (Fig. 10SI). In contrast, B+E showed the highest values of acid phosphatase activity.

The values of the community level physiological profiles obtained from the EcoPlates BiologTM plates did not reveal significant differences between the microbial communities studied (Fig. 3). Thus, the AWCD was similar in all treatments, while the NUS and the Shannon diversity index showed no significant differences (Fig. 3).

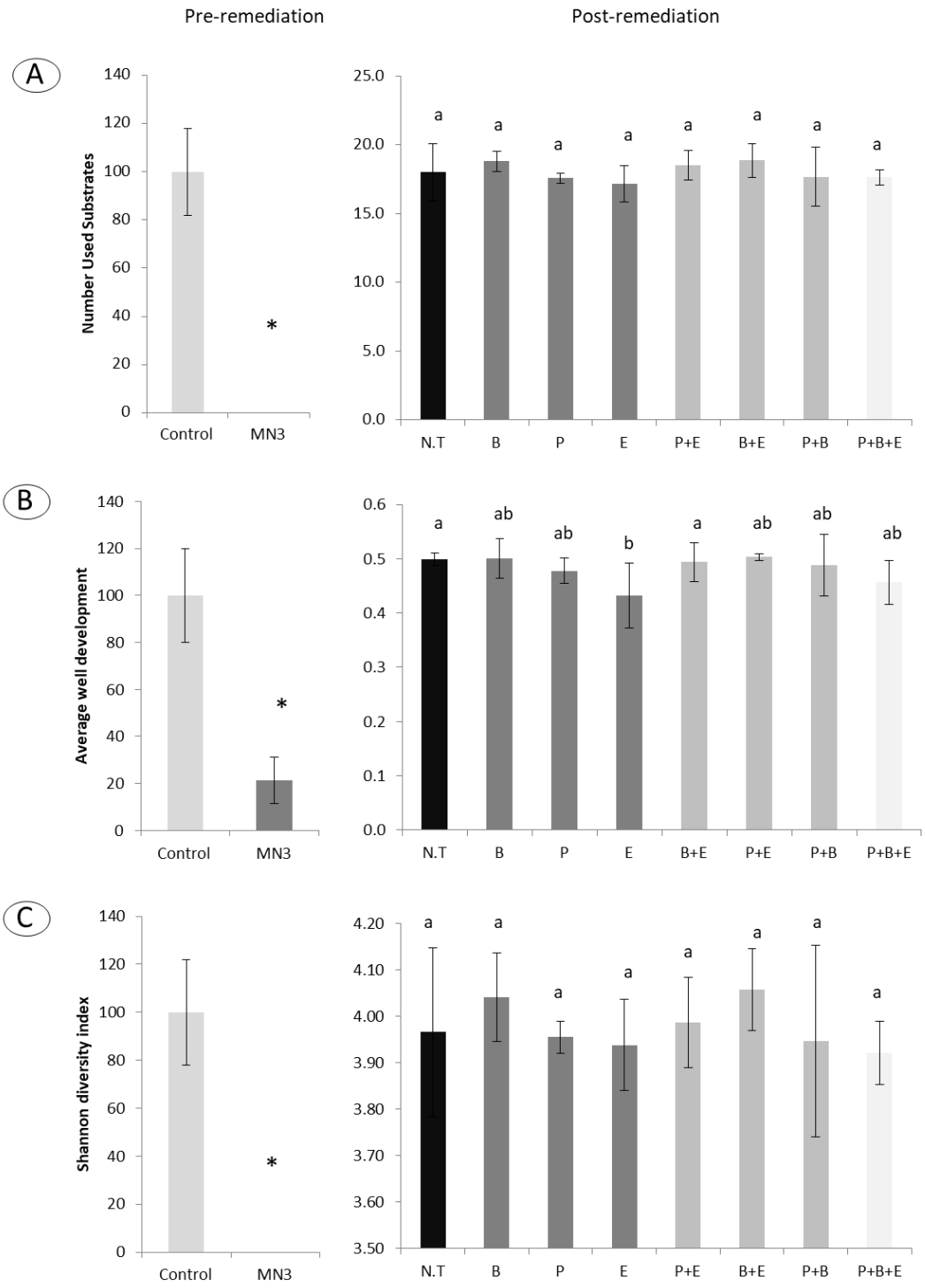

Figure 3. Number of substrates used (NUS) (A), Average well colour development (AWCD)(B) and Shannon diversity index (C) in OECD and MN3 soils (Pre-remediation); and remediated soils from Landfield 17 (Post-remediation; N.T means non-treated). Treatments with different letters show significant differences according to Duncan's Test ($p<0.05$).

4.- DISCUSSION

Chemical and ecotoxicological characterisation prior remediation

Soils amended with sewage sludges in Landfill 17 were chemically characterized for a further ecotoxicological characterization of the worst case scenario soil. The initial chemical characterization (May 2015) as well as the second one, repeated 3 years later (May 2018), showed moderate concentrations for those compounds marked by the administration (Basque Government) as dangerous for public health (Cd, Cr, Ni, Pb, benzo(a)pyrene and dieldrin). This stagnation of metal (Cd, Cr, Pb, Ni), benzo(a)pyrene and dieldrin in time, together with the high amount of OM measured at points with highest pollutant load (Pearson coefficient> 0.7) suggest an adhesion of the pollutants to the organic fraction of the soil; already high considering the origin of the contamination. According to Gondek and Kopec (2006,2014) the proportion of heavy metals associated to the organic fraction of sewage sludge can be very different and even reach 80% of the total content. This was reinforced by the distribution of pollutants in Landfill 17 where contaminants are diffusely scattered, suggesting that pollutants have persisted in points where sewage sludge was spilled. Thus, contaminant could be removed or degraded in points with less amount of OM to which adhere. Moreover, the problematic of the Landfill 17 corresponds to a clear casuistic of aged soil: the 30 years from the disposal and also the origin of the pollution (WWTP), make expectable consolidated and persistent contaminant-OM junctions.

The use of bioassays, apart from assessing the acuteness or chronicity of the toxicity of the Landfill 17, intended to understand the uptake route of the contaminants in soils. In filter paper test with earthworms the exposure occurs mainly via dermis, while in the soil tests the main contact occurs via the digestive tract (Rodriguez-Campos et al., 2014). Leachates can be an important source of toxic and persistent heavy metals when coming from landfills (Karak et al., 2012; Saharia et al., 2015) but presently no significant differences in worms mortality or weight loss were observed between controls and MN3 groups after 48 h of filter paper contact. However, the high mortality in the positive control (VIE-B or Basque Government regulation intervention value) with 100% of organisms dead, endorsed the test as a sensitive and adequate technique for the study of the toxicity exerted by contaminants in this landfill. The absence of weight

loss and mortality in the MN3 group suggests that the toxicity of Landfill 17 soils it does not occur through pore water. This result matches the relationship already observed during chemical characterization between OM and pollution. The strong link between OM and pollutants, forming organometallic ligands, make pollutants stay fixed into soil and does not allow them pass into the aqueous fraction. Thus, since the soil is rich in OM, it is feasible that organic ligands are strong enough to sequester contaminants and minimize their effect. Something expectable considering the age of the soils (discharged three decades ago); so if there were something in the aqueous fraction of the soil it would have already been removed or leached. In order to study the effects generated by soils beyond those generated by elements in pore water the acute toxicity of Landfill 17 soils were evaluated according to OECD standard soil test (1984); more representative of the earthworm environment than the filter paper test (Udovic and Lestan, 2010; Rodriguez-Campos et al., 2014). After 7 and 14 d of exposure, no mortality nor significant differences in weight loss were observed between both treatments. In addition, weight loss was not severe (<20%) in any of the cases. This absence of acute toxicity is justified by: (1) the high amount of OM in the control soil (10% dw; dry weight) and in the soil from the Landfill (19% dw), which, apart from being a food source, is capable of buffering moderate levels of pollution (Irizar et al., 2015), and (2) the dermal uptake from pore water occurring during short exposures. Saxe et al. (2001) estimated that during the first 3 weeks of exposure more than 96% of the total Cd and Cu intake occurs dermally (*E. andrei*). However, the accumulation of different lipophilic contaminants does not only occur by passive absorption of the fraction dissolved through the body wall; it also occurs through intestinal absorption (Belfroid et al., 1994; Contreras-Ramos et al., 2006).

It is known that mortality/survival is less sensitive and less relevant, from an ecological point of view, than growth or reproduction (Moriarty, 1983; Van Gestel et al., 1992; Spurgeon et al., 1994). After 28 d of adult earthworm exposure no significant differences in weight loss were observed between the control group and the Landfill 17 soil group. Nevertheless, weight loss in the group exposed to MN3 was slightly higher, exceeding the threshold of 20% loss and therefore, indicating a severe effect on the growth of adult earthworms. All this despite the contribution of horse manure as food;

which, as several studies showed, acts positively on different vital parameters of worms (Rodriguez-Campos et al., 2014). Reproductive capacity was also decreased in the MN3 group since the production of cocoons, production of juveniles and juvenile biomass were significantly lower than in the control group. It is well known that the exposure of earthworms to high concentrations of metals in soils, can affect the production of cocoons, growth, sexual development, vital behaviour, viability and density of worms (Andre et al., 2010; Uwizeyimana et al., 2017). In fact, the effect of metals upon the cocoon production has been widely reported in earthworms accumulating cadmium, copper, lead, zinc or chromium in their tissues (Van Gestel et al., 1993; Spurgeon et al., 1994). In this case, the cocoon production in the studied soils was significantly lower than the control group, and lower than 1.2 cocoons per earthworm/week reported by Van Gestel et al. (1989). Presently, metal accumulations in earthworms maintained in Landfill 17 soils were significantly higher comparing control group.

The accumulations of metals in tissues are a reflection of the detritivorous lifestyle of worms, their highly permeable body walls and the extensive tissue composed of chloragocytes with organelles capable of sequestering high concentrations of certain metals in relatively insoluble states (Morgan et al.,2002; 2004; Mohee et al., 2014). Mainly, metal accumulation occurs through two routes, dermal absorption and absorption through digestive tissues (Saxe et al., 2001; Hobbelen et al., 2006; Nannoni et al., 2011; Rodriguez-Campos et al., 2014; Wang et al., 2018). Among the analysed compounds, Cd showed the highest accumulation in earthworm tissues (20 µg Cd/g tissue) after exposure to MN3 soils for 28 d and was the only compound showing significant increases between 14 and 28 d of exposure. All this despite the fact that worms were fed along the Reproduction test with clean horse manure allowing them evade contamination (Edwars and Lofty, 1977; Van Gestel et al., 1991; Van Gestel et al., 1993; Spurgeon et al., 1994). Even at sublethal concentrations Cd can affect the immunity, growth and reproduction of worms (Spurgeon et al., 1994; Spurgeon and Hopkin, 1996; Rorat et al., 2017). In general, the accumulation of metals in tissues is a species-specific phenomenon and each metal has its physiological mechanism of assimilation or excretion during its metabolism in the digestive tract of worms (Suthar et al., 2014). In fact, the low values of lead accumulation in earthworm tissues may be due

to the union of the metal to the oxidizable fraction in OM (Nannoni et al., 2011), which makes the metal less mobile and less bioavailable (Wang et al., 2018). Nickel did not show high accumulation values suggesting the lack of accumulation not causing significant effects on growth, as reported by Nakashima et al. (2008) even after long exposure times. Presently, the high levels of Cd accumulated after 28 d in earthworm tissues probably affected their reproductive capacity in comparison to other metals (i.e. Cr, Ni). It is well known the strong affinity of Cd for cellular ligands such as metallothioneins due to their high cysteine content being an important detoxification mechanism for worms (Van Gestel et al. 1993; Stürzenbaum et al., 2004; Mirmonsef et al., 2017; Rorat et al., 2017; Wang et al., 2018) leading to accumulation in target cell compartments (Brulle et al., 2011; Uwizeyimana et al., 2017). In conclusion, increased cadmium accumulation in earthworm tissues may be responsible for their reproductive impairment.

In a similar way to earthworm bioassays, plant bioassays have been widely used to assess the ecotoxicity of soils polluted by both metals and organic compounds (Baderna et al., 2014; Kaur et al., 2017). Although there was no clear effect on seed germination, the three species tested (*C. sativus, L. sativa and A. cepa*) exhibited significant reduction in root elongation. The fact that the reduction percentage was different for each species is a phenomenon described not only for different species but also for different varieties within the same species (Wang et al., 2001). Thus, the mixture of available pollutants produces a toxic effect on the roots of the species tested, but *C. sativus* species was the most sensitive to this cocktail of contaminants. These results were consistent with the worm reproduction test that showed weight loss and reproductive capacity in the MN3 soil compared to the control soil.

In parallel results obtained through microbial parameters reinforced the adverse response observed in MN3 with significantly lower values measured in the assessed ecotoxicological parameters.

Chemical and ecotoxicological evaluation of bioremediation techniques

After the application of the different remediation techniques and their combinations in MN3 soils for 12 months, removal efficiencies were analyzed in order to settle which one was best fitting Landfill 17 edaphic and weather conditions. Dieldrin

elimination rates stood out with removals between 50% and 78% in most of experimental groups. For heavy metals and benzo(a)pyrene, removal rates around 20-25% were achieved. Cadmium was reduced between 15% and 35%; chromium between 7% and 39%; lead between 15% and 33%; nickel between 24% and 37%; and benzo(a)pyrene between 19.5% and 28%. Accordingly, other authors obtained similar yields for some of these metallic elements in a 5-week vermiremediation process in composted soils: Cr, 23%; Pb, 6%; Ni, 18% (Rorat et al., 2017). Regarding benzo(a)pyrene, much higher reduction values (96.6%) have been reported (Rorat et al., 2017), probably because the starting concentration was much higher (1649.57 ± 476.25 µg/kg), and also because the compost was rich in OM. Nevertheless, Contreras-Ramos et al. (2008) have also previously reported a similar removal of benzo(a)pyrene (24%). It is feasible that worms can accelerate the removal of PAHs from the soil due to their capacity to mix and aerate the soil, improving the edaphic structure and increasing microbial activity (Singleton et al. 2003; Drake and Horn 2007; Lapied et al. 2009; Dendooven et al., 2011; Lacalle et al., 2020). However, several authors reported a decrease in the extractability and an increase in the sequestration of PAHs as an effect of soil ageing (Kottler and Alexander, 2001; Nam and Alexander, 2001; Bogan and Sullivan, 2003; Contreras-Ramos et al., 2006). Hydrocarbons present in soils for longer periods are less available than those recently added (Morrison et al., 2000; Contreras-Ramos et al., 2006). Moreover, Northcott and Jones (1999) and Contreras-Ramos et al. (2006) demonstrated that benzo(a)pyrene extraction decreases around 17% after 525 days of ageing. In this hydrocarbon sequestration by the soil OM is a key retention factor although other factors such as cation exchange capacity, soil texture, or the cycles of moisture can act together (Chung and Alexander 1998, 2002). All this is reinforced by the intrinsic conditions of benzo(a)pyrene, which has a low water solubility (3.8 µg/L), a high octanol water coefficient (Kow = 6.04) and a high molecular weight; making benzo(a)pyrene a compound easily sorbable by OM and difficult to degrade (Juhasz and Naidu., 2000; Contreras-Ramos., 2006). All these factors conditioned the degradability and therefore the remediation of the benzo(a)pyrene present in the Landfill 17.

The best yields were achieved in the groups with double and triple combinations of microbial bioremediation, vermiremediation and phytoremediation if compared with

the individual treatments. In addition, intragroup variation was much lower in the groups where treatments were combined. The large variability observed in non-remediated soils or in soils remediated with only one bioremediation technique was reduced in the double and triple treatment due to the integration of all technologies. However, a phenomenon to be noted in the aforementioned variability is the negative yields found in some of the remediation treatments, especially in the individual ones. In the absence of any sludge input during the experimentation phase, the increase in pollutants in some plots can only be justified by either anthropic or animal removal of the soil; but can also be justified by the diffuse nature of pollution.

Besides, metal accumulation in earthworms increased significantly after bioremediation process. The large metal accumulation in earthworms maintained in soils with dual and triple treatments could be explained by a higher bioavailability induced by those remediation techniques. In conclusion, dual and triple bioremediation techniques exert a positive effect on metal mobilization and, hence in their accessibility for remediating organisms as already seen by Lacalle et al. (2020) under laboratory conditions. The high variability observed among remediation treatments overshadowed the differences between treatments making it impossible to choose one as optimal for Landfill 17 remediation. Therefore, the most responsive and accurate assays during phase I were newly applied to elucidate changes in toxicity.

The reproduction test performed after remediation showed a clear soil health improvement from non-treated group to treated ones. For instance, P+B+E showed the highest cocoon values accounted with significantly higher values than non-treated group, where the lowest values were achieved. Single and dual treatments showed also a trend to produce more cocoons than non-treated group. Furthermore, juvenile production followed the same pattern. P+B+E was again the treatment exhibiting the highest values (juveniles); showing statistical differences not only with the non-polluted group, but also with all the individual treatments and P+B dual treatment. Dual and triple treatments were the treatments with higher juvenile number comparing to single treatments and non-treated group. Biomass of the offspring showed same pattern. Overall, all reproductive endpoints appointed the triple treatment (P+B+E) as the best one to improve soil health.

Alfalfa has been described as a crop with potential phytoremediator of all types of contaminants: metals such as Cd, Cr, Ni, Pb and Zn, PAHs and mixed contamination (Bonfranceschi et al., 2009; Donnarumma et al., 2010; Ouvrard et al., 2011; Hamdi et al., 2012; Agnello et al., 2016). Present results showed that alfalfa cultivation by itself was not an effective treatment in the soil under study, although really none of the single treatments (vermiremediation, microbial remediation) showed to be effective, as mentioned above. However, when phytoremediation was combined with vermiremediation (P+E) or the three groups were combined (P+B+E) an increase in the root elongation of *C. sativus* and *L. sativa* was observed which implies a soil health improvement. Therefore, the most effective treatment was the one that combines the three groups of organisms (P+B+E) followed by treatment L, P+B and P+E. This beneficial effect of different taxa has already been described in the literature (Yu et al., 2005; Wang et al., 2006; Ruiz et al., 2009; Jusselme et al., 2012), although some authors showed that the combination of different organisms reduces the uptake of metals by plants in the phytoremediation processes (Aghababaei et al., 2014; Aghababaei and Raiesi, 2015). Thus, the benefit of combining different organisms in a remediation process depends on three features: i) the type of polluted soil; ii) type and concentration of pollutants and iii) type of organisms that are chosen for remediation. Besides, the root elongation bioassay showed a high sensitivity to the toxicity produced by the mixed contamination found in this soil, and again *C. sativus* was the species most sensitive to mixed contamination, as has already been observed in the bioassays prior to the application of the treatments. Furthermore, the root elongation values of the 3 species practically did not vary after 12 months of treatment, so the results of the ecotoxicological bioassays with plants were consistent over time.

In relation to the soil microbial parameters determined, the high variability of the data overlapped the differences between treatments. Moreover, as the soils were rich in OM with low levels of contamination, the observed microbial activity and richness values determined soil status as healthy (Epelde et al., 2008). The fact that contamination by sewage sludge was carried out decades ago meant that, i) the bioavailability of the contaminants was low and ii) the microbial communities were adapted to the presence of these contaminants showing no symptoms of being altered.

Unlike the ecotoxicological tests carried out with earthworms (allochthonous organisms), the tests carried out with bacteria were applied with autochthonous organisms. Thus, while slight variations were appreciable/detectable in earthworm and plant assays, remediation differences could not be appreciated on already adapted autochthonous bacteria. Indeed, the small decrease in soil contaminant values obtained after treatments was not reflected in an improvement of soil health as determined by microbiological indicators.

It must be remarked that B+E group showed unusually/significantly lower enzymatic values in comparison to the rest of the treatments and non-treated group. However, the explanation of these lower values could be on the flooding episodes suffered during winter period, when due to some intense raining journeys two of the three replicates of the B+E group got partially flooded. This episodes could reduce the available oxygen in soil, while could also kill and reduce bacteria colonies present in soil. For this reason, the mentioned values should not be taken into account in order to establish witch treatment is best suited.

It can be concluded that the best elimination yields were obtained after P+B+E treatment, as pointed out by the battery of ecotoxicological tests and bioassays performed with earthworms, plants and bacteria.

5.- CONCLUSIONS

After 3 year period (time between samplings) soils showed similar Cd, Pb, Cr, Ni, benzo(a)pyrene and dieldrin concentrations. Soils did not produce acute effects upon *E. fetida* earthworms; but chronic effects were noted by an evident reproductive impairment (cocoon, juvenile and biomass decrease). A significant inhibition upon *L. sativa, C. sativa* and *A. cepa* root elongation was observed; while microorganism diversity was reduced. After bioremediation, dieldrin was the pollutant with the highest degradation rates (50-78%) in almost all experimental groups, while for metals and benzo(a)pyrene, elimination rates were between 20-25%.

The best elimination yields, and lowest variabilities in the reduction of contaminants, were obtained in P+E, B+E and P+B+E treatments (dual and triple). However, ecotoxicological tests pointed out that P+B+E treatment were the ones

improving most the soil health due to their removal performance. After integrating the chemical and ecotoxicological endpoints, it can be concluded that P+B+E is the most accurate bioremediation treatment in order to significantly improve the soil health of the Gernika-Lumo Landfill 17 after long-term deposition of active sludges from a WWTP.

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SUPPLEMENTARY MATERIAL

Table 1SI. Analytic results for cadmium, chromium, nickel, lead, dieldrin and B(a)P in the 19 points sampled at Landfill 17 during the previous sampling campaings.

		MAN-1	MAN-2	MAN-3	MAN-4	MAN-5	MAN-6	MAN-7	MAN-8	MAN-9	MAN-10
Cadmium	mg/kgdw	4.3	26	6.9	7.4	6.9	0.9	0.57	3.5	8.7	8.9
Chromium	mg/kgdw	88	400	99	120	130	39	41	76	190	160
Lead	mg/kgdw	47	120	52	55	57	29	26	42	56	76
Niquel	mg/kgdw	44	170	55	69	76	67	43	70	96	93
BaP	mg/kgdw	0.033	0.11	0.0075	0.015	<0.009	<0.009	<0.009	0.22	0.091	0.22
Dieldrin	mg/kgdw	0.025	0.15	0.045	0.067	0.044	0.046	0.05	<0.001	0.043	0.0081
OM	%dw	7	19	6.2	8.4	8	11	10	11	11	11

		MAN-11	MAN-12	MAN-13	MAN-14	MAN-15	MAN-16	MAN-17	MAN-18	MAN-19	MAN-20
Cadmium	mg/kgdw	7.1	0.33	5.3	22	6.2	4.1	7.6	7.3	0.33	1
Chromium	mg/kgdw	130	41	52	43	68	63	180	330	68	64
Lead	mg/kgdw	61	27	32	27	37	34	66	120	38	43
Niquel	mg/kgdw	78	77	44	35	48	66	76	91	32	46
BaP	mg/kgdw	0.087	<0.001	0.047	0.046	0.037	0.05	0.44	0.15	0.03	0.027
Dieldrin	mg/kgdw	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
OM	%dw	12	6.2	5.4	9.3	6.4	7.9	8.3	8	7	7.4

Table 2SI. Concentrations (mg/kg soil) of cadmium, chromium, lead, nickel, dieldrin and Benzo(a)pyrene –B(a)P- in the 8 soils sampled during May 2018.

		MN1	MN2	MN3	MN4	MN5	MN6	MN7	MN8
Cadmium	mg/kgms	12	11	26	16	4.9	4.3	7.6	10
Chromium	mg/kgms	130	120	320	170	47	44	180	300
Lead	mg/kgms	62	60	150	96	38	41	66	89
Nickel	mg/kgms	53	48	100	64	28	28	78	120
BaP	mg/kgms	0.04	0.04	0.1	0.08	0.03	0.04	0.08	0.23
Dieldrin	µg/kgms	14	19	58	5.9	<1	<1	2.9	9.3
Pollution index		7.45	7.64	19.76	10.02	3.39	3.78	7.97	17.76

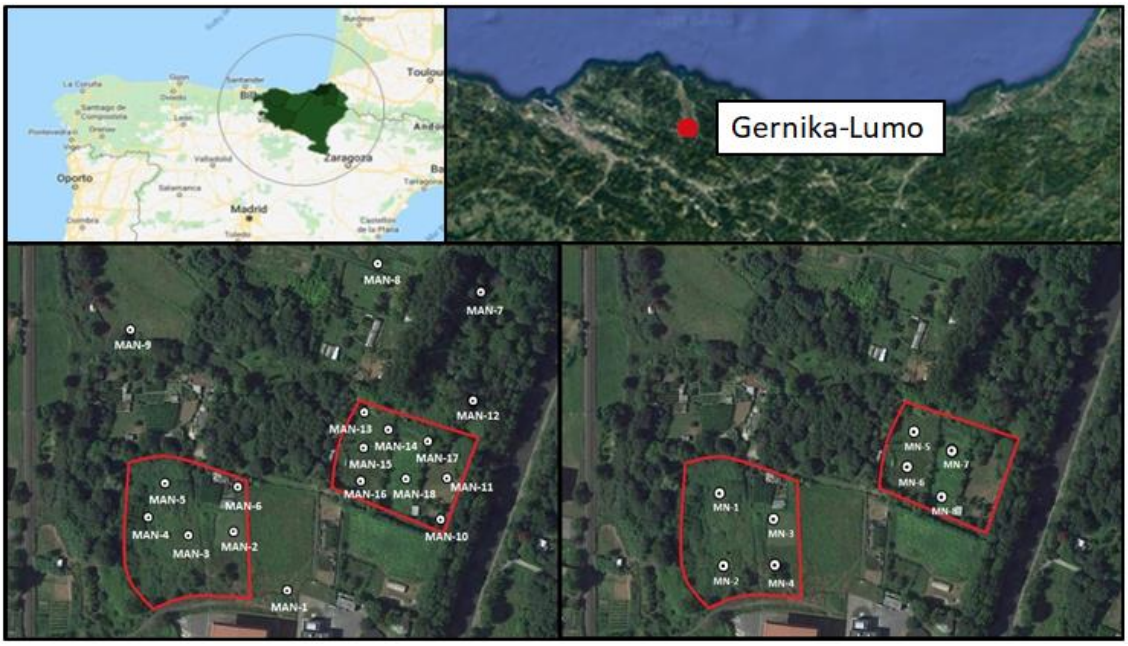

Figure 1SI. Location of (A) Basque Country, (B) Gernika-Lumo town, (C) points sampled in May 2015 and (D) points sampled in May 2018 at Landfill 17.

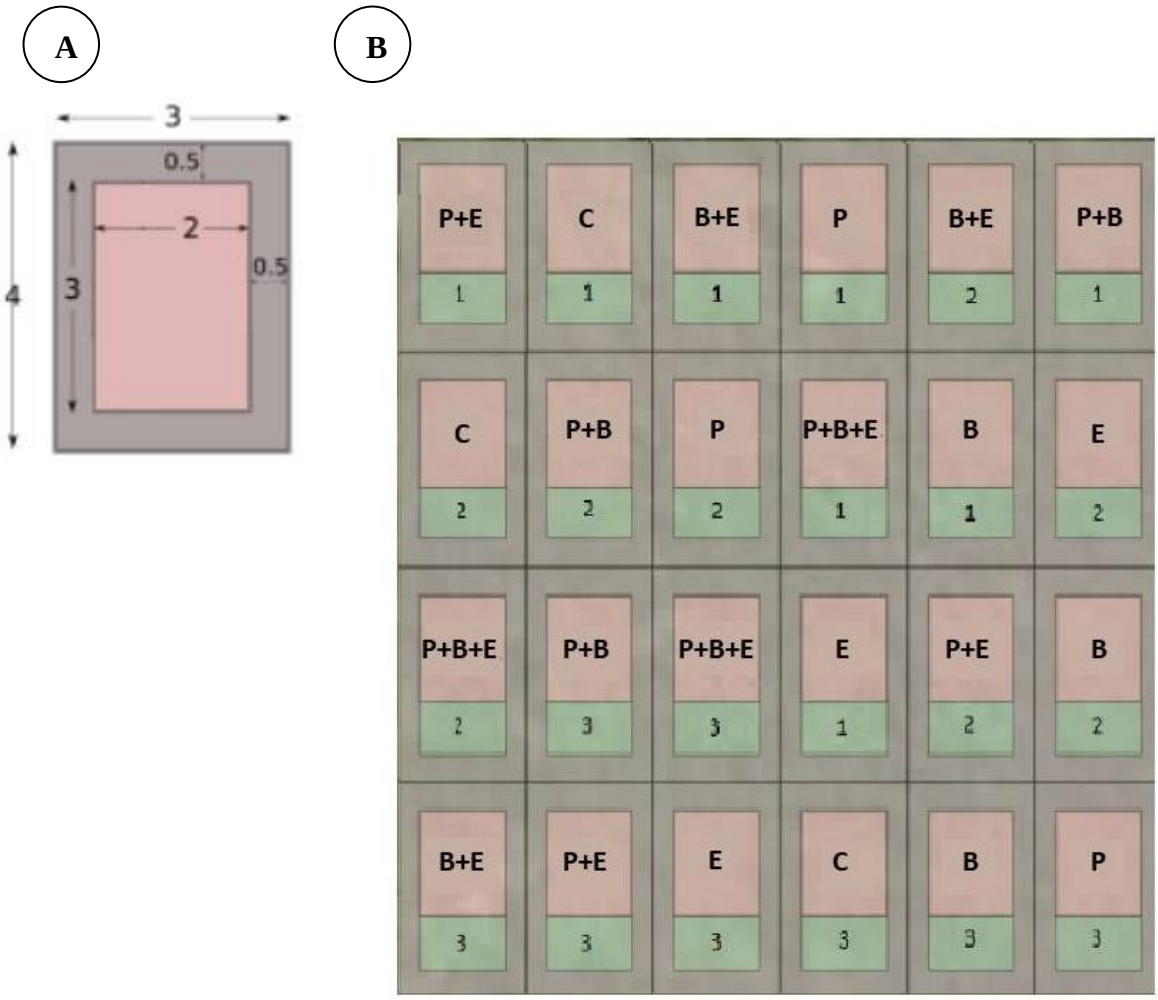

Figure 2SI. A) Dimensions (m) of each of the 24 soil subplots located in the area surrounding the worst case point of the landfill where bioremediation treatments were implemented. B) Treatment distribution (randomly) on the 24 subplots. Each acronym corresponds to: C-Non-treated; E-earthworms; B-bacteria; P-plants; P+B - plants + bacteria; P+E - plants + earthworms; B+E– bacteria + earthworms; P+B+E – plants + bacteria + earthworms. Number on the square below (Green) corresponds to the replicate number The centre of the plot corresponds to the selected point as worst case scenario.

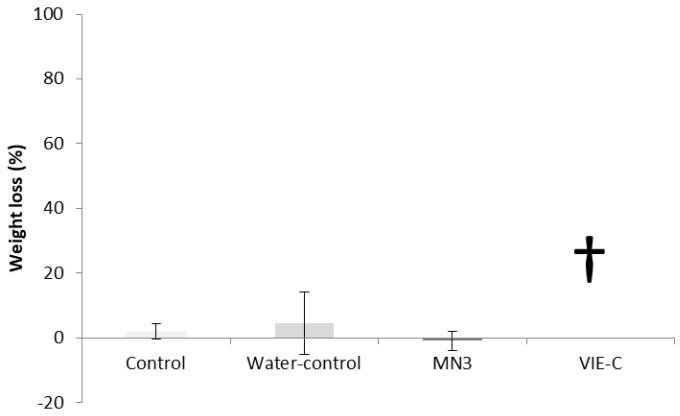

Figure 3SI. Weight loss (% respect to the initial weight) of *E. fetida* earthworms after 48 h exposure to: leachates of control OECD soil and MN3 soils, water control and a positive controls (incorporating VIE values of the Basque Government). Values indicate mean values and standard deviation.

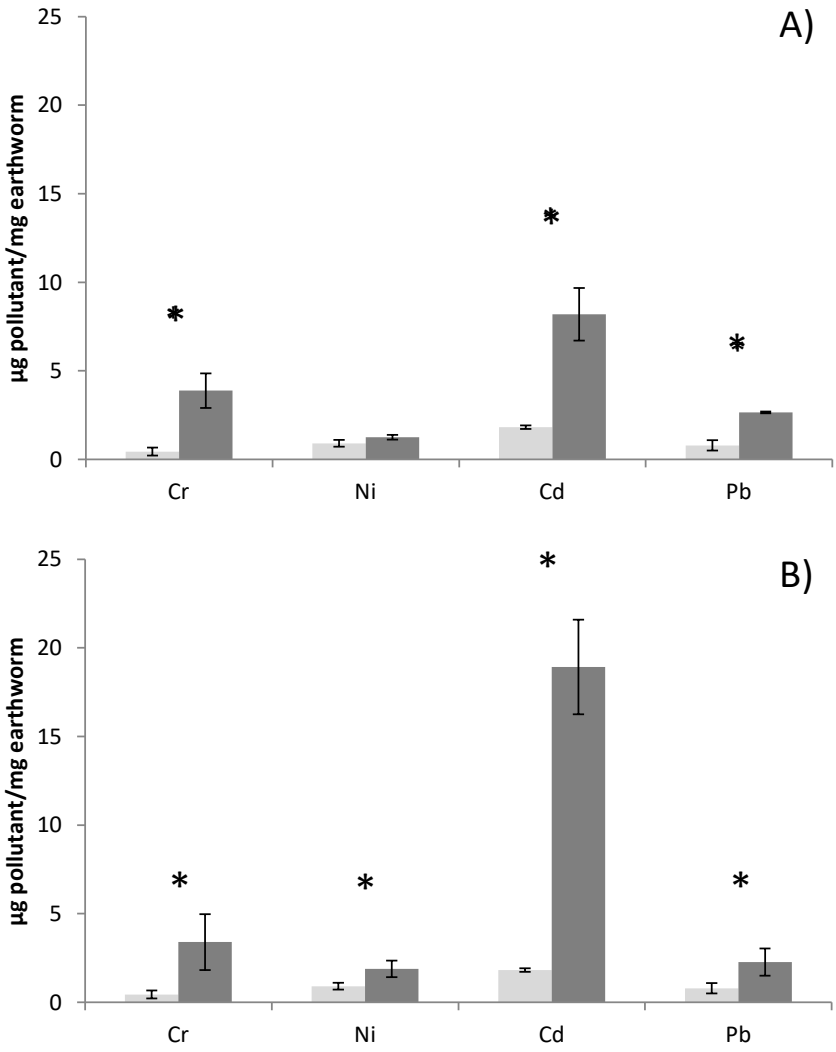

Figure 4SI. Chromium, nickel, cadmium and lead concentrations measured in *E. fetida* earthworms exposed to soils from Landfill 17 for 14 (A) and 28 (B) days. Control organisms (in clear gray) were grown in an OECD standard soil. Asterisk reflects significant differences ($p<0.05$) between treatments. Benzo(a)pyrene and Dieldrin measurements were under detection limits.

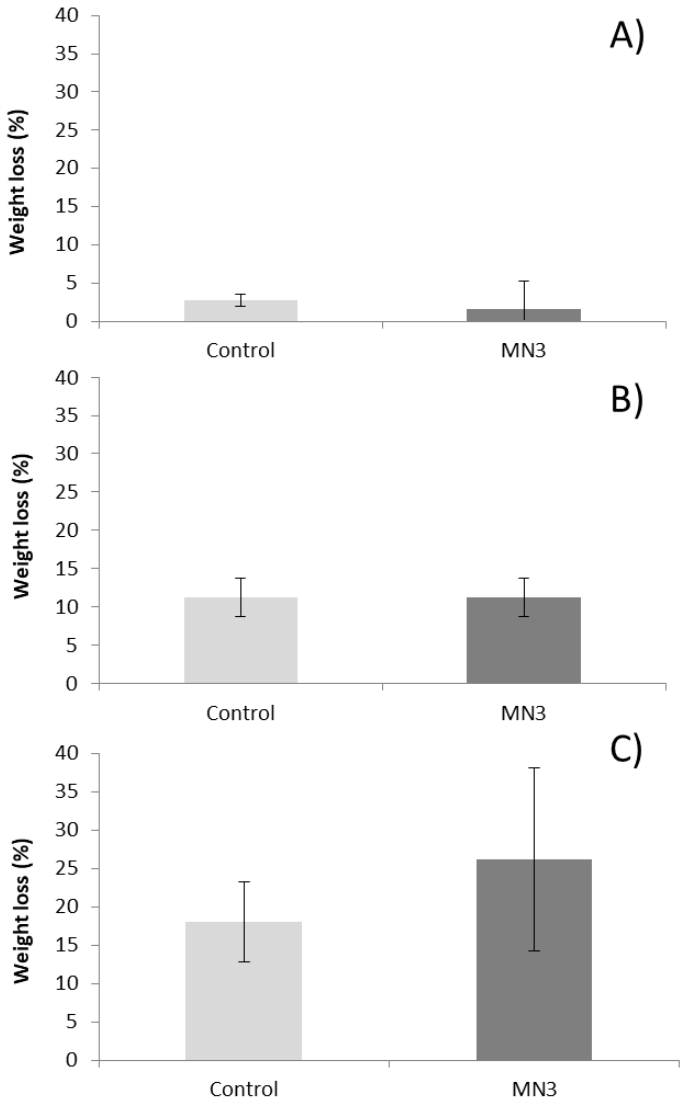

Figure 5SI. Weight loss (% respect to the initial weight) of *E. fetida* earthworms exposed to OECD and soils from Landfield 17 for 7 (A), 14 (B) (acute toxicity tests) and 28 (C) (reproduction test) days. Mean values and standard deviations for each treatment are shown. No mortality was observed in any of the treatments.

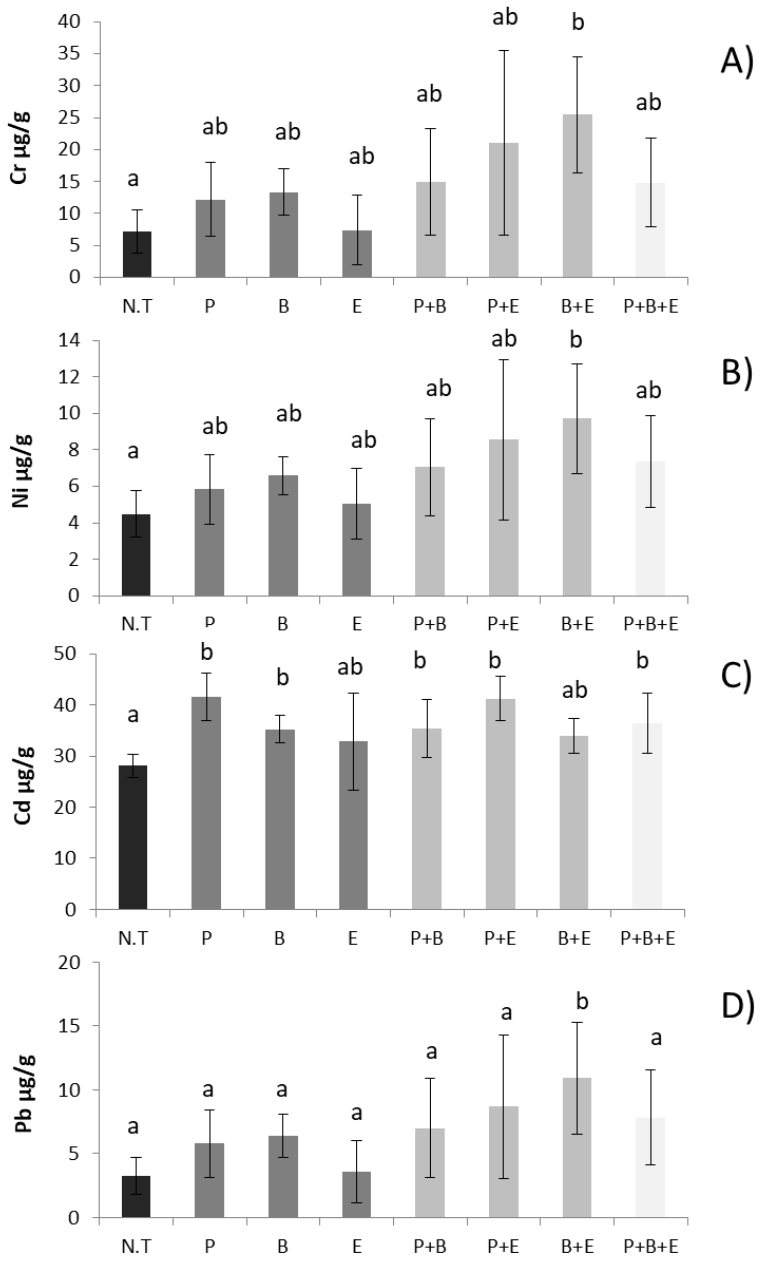

Figure 6SI. Seed germination bioassays of three plant species: *Lactuca sativa* (A), *Cucumis sativus* (B) and *Allium cepa* (C), exposed to OECD and MN3 soils (Pre-remediation); and remediated soils from Landfill 17 (Post-remediation ; N.T means non-treated). Treatments with different letters show significant differences according to Duncan's Test ($p<0,05$).

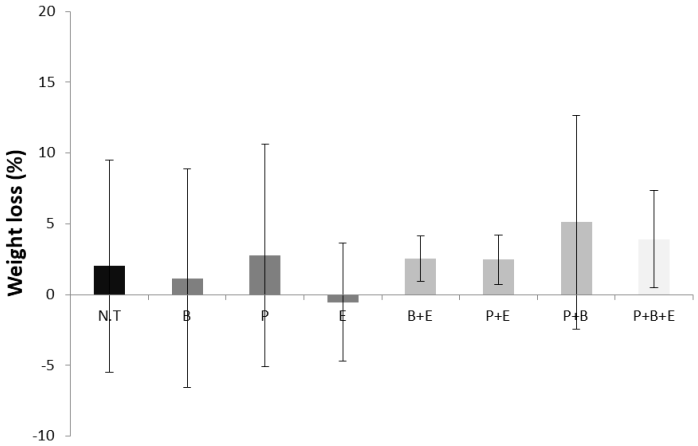

Figure 7SI. Chromium (A), nickel (B), cadmium (C) and lead (D) concentrations measured in *E.fetida* earthworms exposed to Landfill 17 remediated soils for 28 (N.T means non-treated). Asterisk reflects significant differences ($p<0.05$) between treatments. Benzo(a)pyrene and Dieldrin measurements were under detection limits.

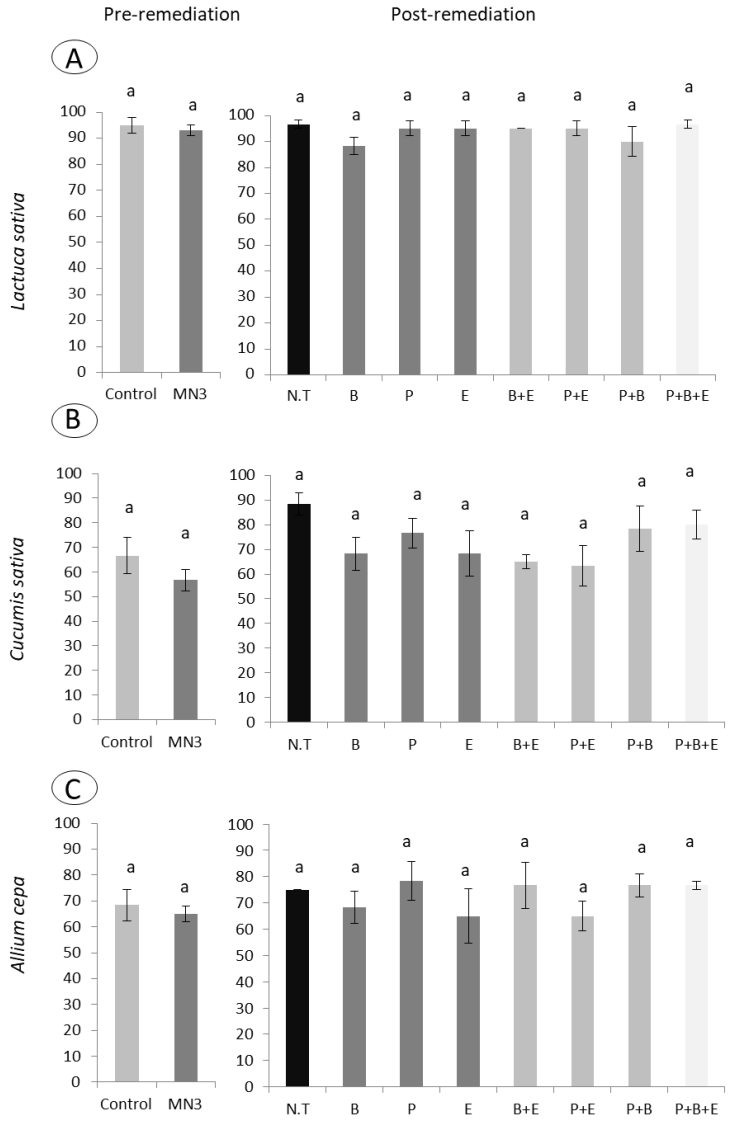

Figure 8SI. Weight loss (% respect to the initial weight) of *E. fetida* earthworms exposed to remediated soils from each Landfield 17 subplot for 28 days (reproduction test; N.T means non-treated). Mean values and standard deviations for each treatment are shown. No mortality was observed in any of the treatments

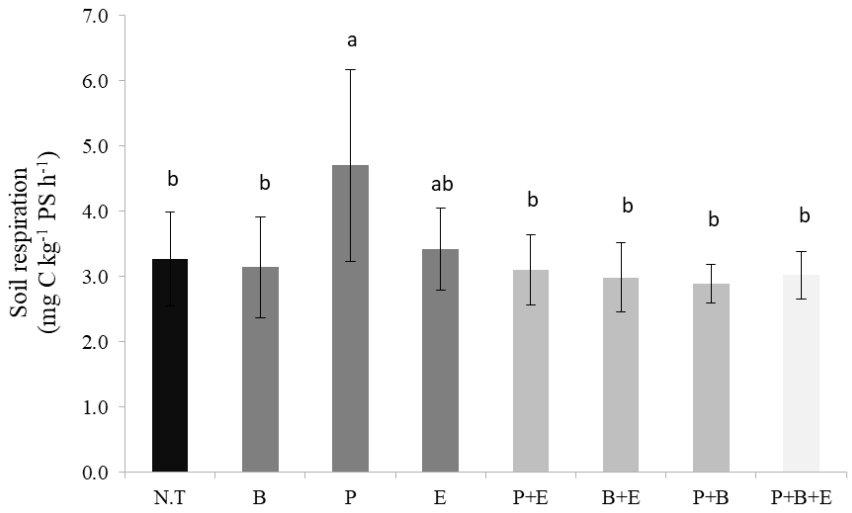

Figure 9SI. Soil respiration values obtained after the application of different bioremediation treatments in soils from Landfill 17 (N.T means non-treated). Treatments with different letters show significant differences according to Duncan's Test ($p<0.05$).

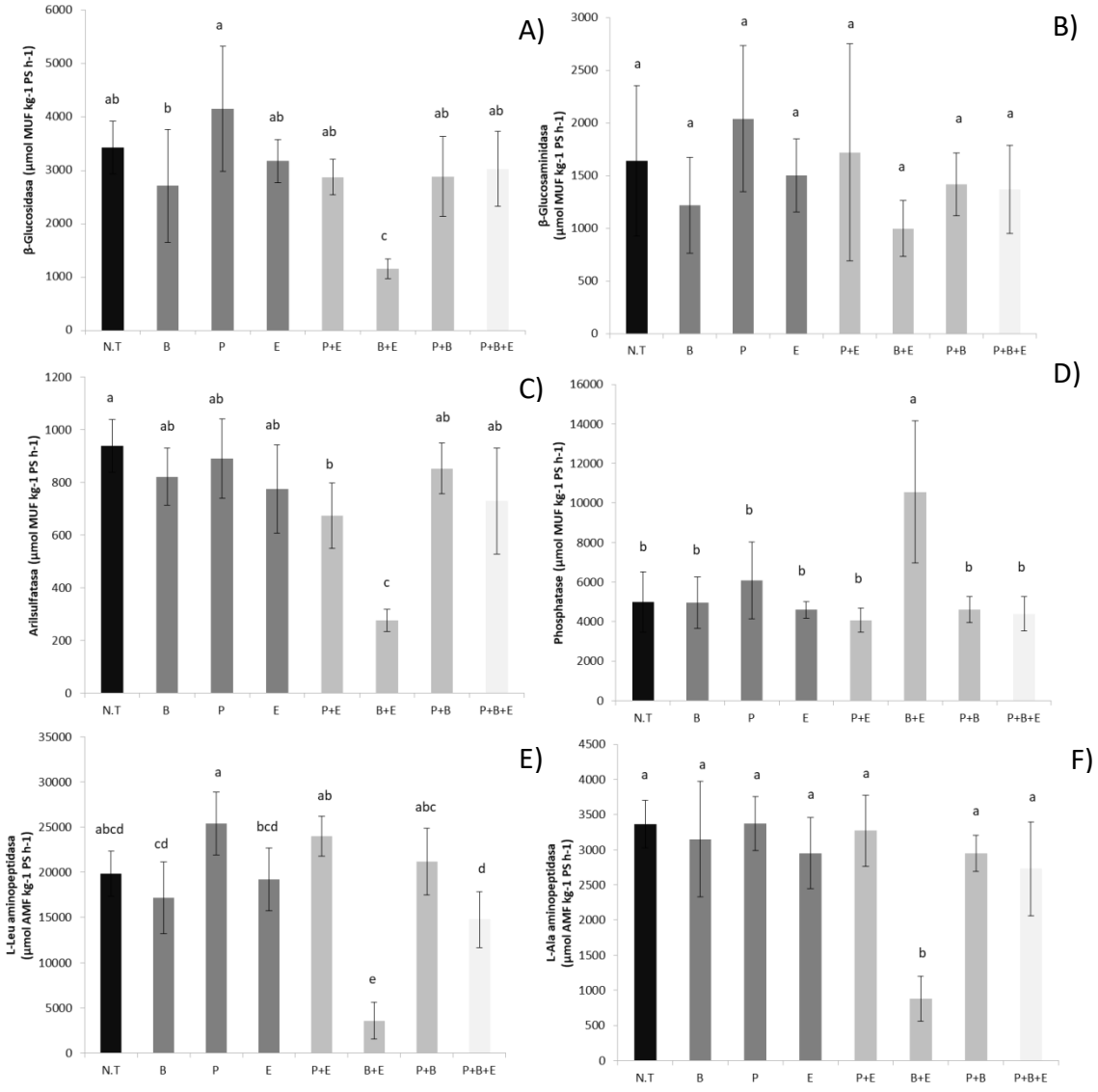

Figure 10SI. Enzymatic activities: β-Glucosidasa (A), β-Glucosaminidasa (B), Arilsulfatasa (C), Fosfatasa (D), L-Leu aminopeptidasa (E), L-Ala aminopeptidasa (F) obtained after the application of different bioremediation treatments in soils from Landfill 17 (N.T means non-treated). Treatments with different letters show significant differences according to Duncan's Test ($p<0.05$).

GENERAL DISCUSSION/EZTABAIDA OROKORRA

Estres faktore anitzen inpaktuak lurzoruetan berotze globalaren testuinguruan

Hemeretzigarren mende erdialdetik egunerarte biztanleria globalak pairatutako hazkuntza esponentzialak, mendebaldeko gizarteen dogma ekonomiko nagusiarekin batera, baliabide naturalen xahutze etengabe bat bultzatu du. Era berean, ondasunen fabrikazio eta kontsumo etengabeak, hondakin eta emisio desberdinak eragin izan dituzte; epe luzera, ingurune ezberdinak kaltetuz. Horrela, pestizida eta metal astunen isurpen eta metaketaren ondorioz lurzoruak degradatzen ari dira. Testuinguru honetan, bistakoa da lurzoru osasunaren adierazleak finkatzeko beharra; lurzoru kutsatuak karakterizatzea, ebaluatzea eta erremediatzea izanik datozen urteetarako ingurumen erronka nagusienetako batzuk.

Era berean, atmosfera zeharo kaltetu izan da azken mendeetan; batik bat, berotegi efektu gasen isurpen emendioagatik. Industriaurreko garaitik %147-ean igo izan dira CO_2 kontzentrazioak, 407.8 ppm-ak gaindituz (WMO, 2019). Emendio honek, lurrazaleko tenperatura gradu batean igotzea suposatu izan du; 2100-erako igoera hori 4°C-takoa izatera hel daitekeelarik (Sherwood et al., 2014). Berotze globalaren eszenario berriak lurzoruetan sortuko dituen efektuak aurreikuste aldera, test ekotoxikologiko konbentzionalen edo *in silico* modeloen erabilera gero eta sustatuago dago komunitate zientifikoan. Ikerketa toxikologiko gehienek, konposatu jakinen esposizioek organismotan eragiten dituzten efektuak ikertzen dihardute; gehienetan, estres faktore bakarrean eta laborategi baldintzetan fokatuz. Ingurumenean aldiz, elementu kimiko anitzen elkarrekintzak eta baldintza aldakorrak gertatzen dira. Horrela, elkarrekintza kimiko edota fisiko-kimikoek, konposatu kimikoaren toxikotasuna era gehigarri, sinergiko edo antagoniko batean eragin dezakete; bai konposatuaren estruktura edo eskuragarritasuna eraldatuz, bai lurraren ezaugarri fisiko-kimikoak (pHa, KTG-a, ATG-a, MO...) aldatuz, bai organismo esposatuen metabolismoa erasanez. Tenperatura altuek lurzoruko metalen eskuragarritasuna emendatu dezaketenez gero (Holmstrup?;Urionabarrenetxea et al., 2020), tenperatura kutsatzaileen toxikotasuna aztertzerako orduan kontuan izan beharreko faktorea da. Izan ere, mundu mailako tenperaturen emendioek, esparru ekologiko ezberdinetan bizi diren organismoak toxikoenganako erasokorragoak egingo dituen estres termiko

batera bultzatuko dituzte. Beraz, berebiziko garrantzia du entsegu toxikologikoen bitartez, estres termikoaren efektuak zein estres termikoak kutsatzaileen toxikotasunean izango zituzkeen efektuak ebaluatzea.

Tesi honetan, test ekotoxikologiko estandarrak eta maila biologiko ezberdinetan neurtutako biomarkatzaileak ikertu ziren *E. fetida* zizarean, estres termikoak kadmioaren toxikotasunean duen eragina aztertzeko (3. kapitulua). Zizare espezie merke, egoki eta eztabaida etiko bakoa izateak, esperimentaziorako modelo organismo oso hedatua bilakatu du *E. fetida* (Bilej et al., 2010; Shi et al., 2017). Gainera, beren izaera epigeikoak tenperaturaren efektuak kuantifikatzeko organismo aproposa bihurtzen du. Berotze globalaren eszenarioak aurreikusteko asmoarekin, zizareak, tokiko erregistroetako maximoen batez besteko tenperatura eta kadmiopean (Cd) mantendu ziren; efektuak konplexutasun maila ezberdinetan pairatuz. Epe laburrean, tenperatura emendioak Cd-aren metaketak igotzea eragin zuen. Gainera, estres termikopean Cd kontzentrazio ertain eta altuetara mantendutako zizareek, pisu galera esanguratsuak, heriotza tasa altuak, ugalketa parametroen beherakadak eta zelulen mintz desegonkortasunak erakutsi zituzten. Horrela, tenperatura altuen pean, efektu atalaseak tenperatura optimopean baino kontzentrazio baxuagoetan edota denbora baxuagoan gertatzen zirela ikusi zen. Emaitza hauek, metalen toxikotasuna tenperaturaren eraginpean dagoela frogatzen dute, tenperatura igoerarekin metalen inpaktua emendatuz. Horrela, beroketa globalak induzitutako eszenario berri bat iradoki daiteke; zeinetan toxikotasun zein arriskua ugariagoak izatea espero daitekeen. Testuinguru honetan, aldakortasun geografikoaren eragina (paisaia, nekazaritza, klimatologia, ezaugarri edafikoak, etab.) kutsatzaileen arriskua ebaluatzeko ezinbesteko faktorea da.

Europako paisaia aldakortasunaren araberako Landare-osasun Produktuen (PPPen) arrisku ebaluazioa lurzoruetan

Pestizidek eskualde mailan sortu ditzaketen arriskuak aurreikusteko EFSA autoritate Europearrak ezarritako *in silico* modeloak aplikatzen dira. Zorizko modeloen artean, lurzoruan eta lurzoruko ur fasean aurki daitezkeen produktuen, edota hauen azpiproduktuen kontzentrazio aurreikuspenak (PEC-ak) burutzeko modeloa da PERSAM. Software honek, egitura espaziala daukaten 62 datu-multzo erabiltzen ditu

kalkuluak burutzeko, esparru bakoitzeko lur-erabilera nagusian oinarrituz. Era honetan, posible da Europear eskualde desberdinetan pestizida baten aplikazioaz geroztiko kontzentrazioak estimatzea (lurzoru osoan/ fase likidoan) denbora (0, 7, 14, 21, 28 eta 56 egun) eta sakonera desberdinetara (1, 2.5, 5 eta 20 cm). Europear eskualde ezberdinen arteko aldakortasunak pestiziden iraunkortasunean, itu organismoak ez diren espezieenganako toxizitatean (*E. fetida* zizarea, *Folsomia sp.* kolenboloa), eta Ekosistema Zerbitzuen gaineko arriskuan daukaten eragina aztertze aldera, 4 pestiziden (2 fungizida -Picoxystrobin, Fenamidone- eta 2 intsektizida -Cyclaniliprole eta Esfenvalerate) inpaktua aztertu zen (4 eta 5. kapituloak). Pestiziden kontzentrazioen arteko ratioek (PEC_{ratio}; lurzoru osoaren eta lurzoruko ur interstizialaren artekoak) igoera nabarmena erakutsi zuten iparraldetik hegoaldera; hegoaldeko lurzoru poroetako uretan pestizida kontzentrazioa iparraldeko herrialdeetan baino altuagoa zela adieraziz. Joera horiek, hegoaldeko prezipitazio baxuago eta tenperatura altuagoekin erlazionatu daitezke batik bat, poro-ur kantitate baxuagoa eta pestizida kontzentrazioa altuagoa izanik. Gainera, hegoaldeko lurren MO kantitate baxuagoak, lurzoruaren aldetiko erretentzio baxuago bat faboratuko luke pestizidaren ur interstizialerako migrazioa ahalbidetuz. Aitzitik, iparraldeko lurretan espero litezkeen kontzentrazioak hegoaldean baino altuagoak izan ziren; iparraldeko lurretan MO kantitate altuagoek eta pH azidoagoek lurzoruaren erretentzio ahalmena baldintzatzearen ondorio. Paisaiaren aldagai hauek, pestizida kontzentrazioetan daukaten eragina ulertuta, posible da berotze globalak sor ditzakeen aldaketak aurreikustea. Berotze globalaren tenperatura emendioek, lurzoru poro-ur jaitsiera orokor bat eragingo lukete; batez ere hegoaldeko lurretan. Ondorioz, lurzoru poro-uretan egon litezkeen pestiziden kontzentrazioak are handiagoak lirateke, bertan bizi diren organismoenganako arriskua eta toxikotasun potentziala emendatuz. Gainera, tenperaturen emendioak, iparraldeko lurretako deskonposizio tasa igoko luke, MO-aren jaitsiera geldi bat bultzatuz eta lurzoruen erretentzio ahalmena kaltetuz. Hori dela eta, kutsatzaile gehiagok migratuko lukete lurreko fase likidora, bertako kontzentrazioa eta organismoenganako arriskua emendatuz.

Hala ere, badira lurzoruko baldintzez aparte, pestiziden lurreko kontzentrazioa, eta beraz toxikotasuna, baldintza dezaketen beste zenbait faktore; horregatik,

Euroerregio bakoitzeko nekazal praktikak (GAP eta uzta mota), konposatuen degradazio denbora, absortzio koefizientea (Kom) eta akzio mekanismoak (MoA) kontuan izan ziren arrisku analisian. Uztei dagokienez, PEC_{ratio} balio altuagoak lortu izan ziren tomate uztetan, patatekin alderatuz. Horrela, lurzoruko poro-urarekin kontaktuan dauden organismoak esposizio kontzentrazio altuagoak, eta hortaz, arrisku altuagoak pairatuko lituzkete tomate-landa batean baleude, patata-landa batean baino (batez ere hegoaldeko herrialdeetan). Ureztatze erregimenek edota landareen kimuek emandako itzalak, ebapotranspirazioa baldintzatu zezaketen, ur interstizialeko produktuaren kontzentrazioa eraginez.

PERSAM softwarean sartutako konposatu propietateen artean, *Kom*-a da pestiziden lurzoru konpartimenduen arteko distribuzioan eragiten duen ezaugarri garrantzitsuenetako bat. Izan ere, ur interstizialeko pestizida kontzentrazio altuenak *Kom* baxuko pestizidak aplikatzean ikusi ziren. Aldiz, *Kom* altudun pestiziden isuri osteko kontzentrazioak lurzoru totalean altuagoak ziren. Horrela, fenamidone (Kom: 225 L/kg) > cyclaniliprole (Kom: 280 L/kg) > picoxystrobin (Kom: 520 L/kg) > esfenvalerate (Kom: 145997 L/kg) izango lirateke PEC_{ratio} altuenak eta baxuenak hurrenez hurren.

PPPen kontzentrazioek, Europan zeharreko baldintza aldakorrekin batera, itu ez diren organismoak kaltetu ditzakete, azkenik lurzoruaren funtzioak eta zerbitzuak erasanduz. Lan honetan, aukeratutako PPP-ak itu organismoak nola erasaten zituzten ezagututa (MoA), itu ez diren organismoenganako efektuak aurreikusi ziren, gero ekosistemen gaineko arriskuaren ikerketa burutzeko.Aztertutako konposatuen artean, fungizidek *E. fetida* zizareetan eragindako efektuak, insektizidek eragindakoak baino errazago detektatzen direla ikusi zen (Bünemann et al.,2006; Jänsch et al., 2006; Pelosi et al.,2014). Aldiz, *Folsomia sp*. insektizidenganako sentikorragoa dela ikusi zen, batik bat, esposizio kronikoetan agertutako ugalketa kalteengatik .PPPen aplikazio ostean, habitat hornidura, lurzoru eraketa eta mantentzea, elikagaien ziklatzea, biodibertsitatea, higaduraren erregulazioa, lurzoru erremediazioa/hondakinen tratamendua eta izurrite/gaixotasunen erregulazioa izan ziren inpaktu gehien pairatutako Zerbitzu Ekosistemikoak. Europear nekazal lurren aldakortasun espazialak (pH, MO, tenperatura), PPPen ezaugarri fisiko-kimikoek (MoA, Kom,

disolbagarritasuna…) eta itu espezieak ez diren organismoen habitat, rol eta jokaerak PPPen toxikotasuna zeharo baldintzatu zuten; hortara, PPPen arrisku maneiuan kontuan izan beharreko faktoreak dira.

Araztegi lokatzak jasotako lurzoruaren analisi toxikologikoa zizare eta landareak erabiliz

Era berean, giza aktibitateen emendioak, isuri puntuen, betetze puntuen eta zabortegien ugaritzea suposatu du; lurzoru, ur masa eta aire kutsadura arrazoi handienetako bat izateraino. Euskal Herriko kutsatutako lurren inbentarioan 17 Zabortegia aurki daiteke, hainbat hamarkadetan zehar Gernika-Lumo araztegiko lokatzak jaso zituena. Jasotako lokatzek, araztegiak degradatu eziniko kutsatzaile organiko eta inorganikoak bereganatu zituen beronen materia organikoarekin batera. Araztegi lokatzen isurtzearen ondorioz, lurzorura heldutako kutsatzaileen analisi kimikoez gain, efektu biologikoak ikertu ziren *E. fetida* zizareak eta *Lactuca sativa* haziak erabiliz. Esposizioa burutzeko, zabortegiko gutxi- (MAN-17), erdi- (MAN-14) eta oso kutsatutako (MAN-7; ondorengo ikerketetan MN-3 izendatutakoa) lurrak aukeratu ziren neurketak denbora eta konplexutasun biologiko ezberdinetan burutuz. *L. sativa*-n neurtutako parametroetan ez zen aldaketarik sumatu esposizio denbora ezberdinetan. Aldiz, *E. fetida* organismoek efektu subletalak pairatu zituzten maila biologiko desberdinetan: maila zelularrean eta ugalketa parametroetan, besteak beste. Hala ere, talde esperimental ezberdinen arteko emaitzak erkatuz, ez zen kontzentrazioen araberako erantzun zuzenik nabari. Dosi araberako dependentzia falta horren erantzule, lurzoruaren ezaugarri fisiko-kimikoak eta kutsaduraren adina izango lirateke. Izan ere, lurzoruak erakutsitako MO kantitate altuak (19% P.L.) araztegiko kutsatzaile ezberdinekin lotura ezberdinak sortuko lituzke, isurtzetik pasatako urteetan zehar, kutsatzaileak egonkortuz eta metalen eskuragarritasuna ko-prezipitatuak sortzen doazen heinean jaitsiz (Martinez & McBride, 2001). Horregaitik, metalen bioeskuragarritasuna zein toxikotasuna jaistea espero da lurzoru zahartuetan; berriki kutsatutako lurzoruekin alderatuz (Smolders et al., 2009). Hau argi islatu zen, lurzoruetatik lixibiatuetara igarotako kutsadura maila baxuetan, eta hortara, urazengan eragindako efektu ez esanguratsuetan. Kontzentrazio baxu hauek, urazetan eragindako efektu sotilekin batera, kutsatzaileen poro-uretarako migrazioa baxua zela iradokitu

zuten. Era berean, organismoen heriotza-tasa ezak eta zizareetan efektu subletal eta kronikoen gertaerek, ezaugarri fisiko-kimikoek baldintzatutako toxikotasuna erakutsi zuten; MAN-7 eta MAN-14 lurzoruetan dauden ornogabeak arriskuan egon daitezkeela iradokituz.

Administrazioak, lurzoruen erabilera mantendu nahi zuela jakinda (*Beste Erabilerak* kategoria) zabortegiko lurren biorremediazioari ekin zitzaion, hauen osasun eta kalitatea berreskuratzeko xedez (7. Kapituloa). Bioremediazioa burutu zitekeen jakiteko, eta igarotako denboran, kutsaduraren eskuragarritasun eta toxizitatean aldaketarik eman zen ikertzeko, laginketa eta toxikotasun testen bigarren kanpaina burutu zen 17 Zabortegiko lurretan 3 urteren epea igarota. Lurzoruek, lehenagoko kanpainen Cd, Pb, Cr, Ni, BaP eta dieldrin maila antzekoak erakutsi zituzten; aldiz, azterketa ekotoxikologikoek desberdintasun esanguratsuak erakutsi zituzten erabilitako espezie behale ezberdinetan (zizare, landare eta mikroorganismoetan). Hiru urteren ostean, 17 Zabortegiko lurrek ez zuten efektu akutu nabarmenik eragin *E. fetida* zizareetan; aitzitik, ugalketa bezalako efektu kronikoak kalte nabarmenak pairatu zituzten, lehenagoko lanetan (6. kapitulua) ikusi bezala. Uraza, luzoker eta kipula landareen sustrai elongazioan inhibizio nabarmenak hauteman ziren eta lurzoru mikrooganismoen aktibitate zein dibertsitateak ere beherakada pairatu zuen; landare komunitate eta komunitate mikrobianoen afekzioa azpimarratuz.

Urtebetez , fito-, vermi- eta mikro- erremediazio tekniken aplikazio indibidual, dual, eta hirukoitzaren ostean, dieldrina izan zen eliminazio tasa altuenak pairatu zituen konposatua; 50-78% degradazioekin (7. Kapituloa). Era berean, metal astunek, eta BaP-ak 20-25% bitarteko eliminazio tasak pairatu zituzten (Cd: 15-35%, Cr: 7-39%, Pb:15-33%, Ni: 24-37%; BaP: 19,5-28%). Gainera, bioerremediazio osteko test ekotoxikologikoek erakutsi zutenez, zabortegiko lurrek ez zuten efektu toxiko akutu ez kronikorik eragin *E. fetida* zizareetan; ezta honen ugalketa ahalmenean ere. Landareekin burututako bioentseguek ere, lurzoru osasunaren emendioa aurreikusi zuten (tratamendurik jaso ez zuen kontrolarekin alderatuz). Mikroorganismoen kasuan biorremediazio osteko egoera hasierakoaren antzekoa izan zen.

Hala ere, eta bioerremediazio teknologien aplikazioa tratamendu guztietan onuragarria izan zen arren, errendimendu desberdinak ikusi izan ziren tratamendu

ezberdinen artean; bai lurzoru kalitateari, eta bai, osasunari dagokionez. Kutsatzaile eliminazio errendimendu altuenak eta aldagarritasun baxuenak, tratamentu bikoitz eta hirukoitzetan nabari izan ziren. Baina entsegu ekotoxikologikoei erreparatuz, landare, bakterio eta zizare tratamendu hirukoitzak erakutsi zuen lurzoru osasunaren hobekuntzarik altuena. Hortaz, eta emaitza kimiko eta ekotoxikologikoetan oinarrituz, Gernika-Lumoko 17 Zabortegiko kutsadura erremediatzeko tratamendu biologikorik aproposena tratamendu hirukoitza da; ezaugarri antzekodun lurretan aplikagarritasuna posible litzatekeela iradokituz.

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CONCLUSIONS AND THESIS/ONDORIOAK ETA TESIA

CONCLUSIONS

1- Effects of cadmium exposure in *Eisenia fetida* earthworms were enhanced at increasing temperature (from 19°C to 26°C) being effects more marked at longer exposure periods (14 d vs. 28 d). High mortality rates and reproductive impairment occurred at longer exposure periods at 26°C. Thus, elevated temperatures (in a possible future scenario of global warming) can enhance metal bioavailability and produce more marked toxic effects as a function of time at different levels of biological organization in soil macroinvertebrates.

2- The predicted environmental concentrations (PECs) of esfenvalerate, cyclaniliprole, picoxystrobin and fenamidone on crop soils varied depending on the soil compartment and spatial variability along Europe. The combination of low temperatures, low pH and high organic matter contents in total soil enhance pesticides risk for non-target organisms (*E. fetida*, *Folsomia* sp.); especially in northern soils. By the contrary, high pesticide concentrations in pore water exert toxic responses in non-target species; mainly in southern areas. Apart from the landscape variability, a strong relation was found between the estimated risk values and the treated crop type or the characteristics of Plan Protection Products (PPPs). Thus, a higher risk for pore water living organisms was noted on tomato crops comparing to potato crops; especially in southern soils. Similarly, pesticides with low K_{om} showed higher PECs on pore water; and therefore, higher risks.

3- Effects upon functions developed by non-target species were useful to identify the most impacted Ecosystem Services after esfenvalerate, cyclaniliprole, picoxystrobin and fenamidone worst case application. The most impacted Ecosystem Services for those cases were: habitat provision, soil formation & retention, nutrient cycling, biodiversity, erosion regulation, soil remediation/waste treatment and pest & disease regulation. Moreover, spatial variability among European agricultural soils (pH, organic matter –OM-, temperature), PPPs physicochemical properties (Mode of Action, K_{om}, solubility…), and non-target species behavior, habitat and role in ecosystem seemed to be the main factors to be taken into account for a correct PPP use management in crops.

4- Determining pH and OM quantities in soils receiving sewage sludges in Landfill 17 (Gernika-Lumo) was crucial to interpret properly the ecotoxicological results and explain differences among pollutants bioavailability. Earthworms maintained in the ladfills soils exhibited high levels of Cd, Cr, Ni and Pb that increased with time. Analysis based on cellular endpoints demonstrated the cytotoxic effect exerted by the soils onto coelomocyte viability. Therefore, it was confirmed that organisms exposed to soils exhibiting intermedium and high pollutant levels were subjected to chronic risk. Hence, the application of soil remediation technologies to minimise the risk was strongly recommended.

5- Landfill 17 soils sampled in 2018 showed similar Cd, Pb, Cr, Ni, BaP and dieldrin concentrations to those registered in 2015, concluding the high stability of these pollutants in these aged soils. Moreover, due to this reduced bioavailability soils sampled in both years did not produce acute effects upon *E. fetida* earthworms; but chronic effects were noted by an evident reproductive impairment (cocoon, juvenile and biomass decrease). A significant inhibition upon *Lactuca sativa*, *Cucumis sativa* and *Allium cepa* root elongation was observed; while the abundance of microorganisms community was reduced. After the application of bioremediation technologies, dieldrin was the pollutant with the highest degradation rates (50-78%) in almost all bioremediation treatments, while for metals and B(a)p, elimination rates were between 20-25%. The best elimination yields, and the lowest variabilities in the reduction of contaminants were obtained after dual and triple treatments with plants, earthworms and bacteria. However, ecotoxicological tests pointed out that the triple combination of plant, earthworms and bacteria was the best treatment to improve soil health of the Gernika-Lumo Landfill 17 after long-term deposition of active sludges from a WWTP. These bioremediation technologies could be applied at a higher scale in the Landfill 17, and are susceptible of successful application in other soils with similar characteristics regarding pollutant nature and concentrations.

THESIS

Ecotoxicological approaches and the discrimination of risks accounting for landscape variability based on *in vivo* and *in silico* endpoints are useful tools to carry out a realistic assessment of soil health status and to allow the implementation of accurate protection goals and the design of sustainable recovery strategies.

ONDORIOAK

1- Kadmio espozizioaren efektuak tenperaturaren igoerarekin batera emendatu ziren (19°C-tik 26°C-tara) *Eisenia fetida* zizareetan, espozizio denbora luzeetan indartuz (14 d vs. 28 d). Esposizio denbora luzeetara, heriotza tasa altuagoak eta ugalkortasun kalteak hauteman ziren 26°C pean. Horrela, tenperatura altuek (beroketa globalaren etorkizuneko eszenario baten aurki daitezkeenak), metalen bioeskuragarritasuna eta hauek makro-ornogabetan eragiten dituzten efektu toxikoak emendatu ditzakete denboran zehar.

2- Aurreikusitako esfenvalerate, cyclaniliprole, picoxystrobin eta fenamidone landa lurretako kontzentrazioak (PECak), lurzoru konpartimenduaren eta Europako lurren arteko aldagarritasunaren menpekoak zirela erakutsi zuten. Materia organiko kantitate altuek eta pH zein tenperatura baxuek, pestiziden itu ez diren organismoentzako (*E. fetida, Folsomia* sp.) arriskua emendatu zuten; batez ere iparraldeko lurretan. Aitzitik, lurzoru ur-porotako pestizida kontzentrazioak hegoaldeko lurretan izango lirateke bereiziki toxikoak itu ez diren organismoentzat. Arrisku balioek, paisaia aldakortasunaz gain, PPPen ezaugarriekiko edo tratatutako uzta motarekiko erlazio indartsua erakutsi zuten. Horrela, lurzoru poruan bizi diren organismoek arrisku gehiago pairatuko lukete tomate uzta batean baleude patata uzta batean baino; bereiziki hegoaldean. Era berean, K_{om} baxuko pestizidek PEC altuagoak eta arrisku altuagoak erakutsiko lituzkete poro-uretan.

3- Itu ez diren espezieek garatutako funtzioen gaineko efektuen iragarpena, Ekosistema Zerbitzu galera aurreikusteko modu egokia da. Kasurik txarreneko esfenvalerate, cyclaniliprole, picoxystrobin eta fenamidone aplikazio baten ostean: habitaten hornitzea, lurzoru eratzea eta heltzea, elikagaien ziklatzea, biodibertsitatea, higaduraren erregulazioa, lurzoru erremediazioa/hondakinen tratamendua eta izurrite/gaixotasunen erregulazioa izan ziren gehien inpaktutako zerbitzu ekosistemikoak. Era berean, Europako nekazal lurren arteko aldakortasuna (pH, MO, temperatura), PPPen ezaugarri fisikokimikoak (Akzio Mekanismoa, K_{om}, disolbagarritasuna) eta itu ez diren espezieen portaera, habitat, zein rola dira PPPen kudeaketa egoki batean kontuan izan beharreko faktore garrantzitsuenak.

4- Analisi ekotoxikologikoen interpretazio egoki bat egiteko eta kutsatzaileen bioeskuragarritasunean aurki daitezkeen desberdintasunak azaltzeko, MO kantitatea eta pH-a determinatzea kritikoa da. 17 Zabortegiko lurzoruetan jarritako zizareetan maila altuko metal metaketak (Cd, Cr, Ni eta Pb) ikusi ziren 28 egunen buruan; denboran zehar emendatuz. Parametro zelularretan oinarritutako analisiek, zabortegiko kutsatzaile anitzek eragindako efektu zitotoxikoa frogatu zuten zelomozitoen bideragarritasunean. Horrela, kutsatzaileen maila altu eta ertaina zituzten lurzoruetan zeuden ornogabeak arrisku kronikoan egon zitezkeela frogatu zen. Hori dela eta, lurzoruen arriskua txikitzeko erremediazio teknologiak aplikatzea gomendatzen da.

5- 2018.urtean zabortegiko lurzoruetan lagindutako Cd, Pb, Cr, Ni, BaP eta dieldrin mailak, 2015ean neurtutakoen antzekoak zirela erakutsi zuten, kutsatzaile hauen egonkortasuna azpimarratuz. Eskuragarritasun murriztu honen ondorioz ez zen efektu akuturik ikusi *E. fetida* zizareetan; baina, efektu kronikoak nabariak izan ziren ugalketa ahalmenaren kaltetze esanguratsuarekin (cocoon, jubenil eta biomasa jaitsiera). Gainera *Lactuca sativa*, *Cucumis sativa* eta *Allium cepa* haziek elongazioaren inhibizio esanguratsua pairatu zuten; mikroorganimo komunitateak murriztu egin ziren heinean. Bioremediazio ostean, dieldrina izan zen tratamendu gehienetan degradazio tasa altuenak pairatu zituen kutsatzailea (50-78%). Metalak eta BaP aldiz, 20-25% bitartean jaitsi ziren. Kutsatzaile eliminazio errendimendu altuenak eta aldagarritasun baxuenak tratamendu bikoitz eta hirukoitzetan nabari izan ziren. Hala ere, test ekotoxikologikoek landare, zizare eta bakterioen aldibereko tratamendua azpimarratu zuten 17 Zabortegiko lurzoru osasuna gehien hobetzen zuen tratamendu gisa. Bioerremediazio teknologia hauen aplikazioa 17 Zabortegiko lurrei eskala osora aplika dakieke, eta kutsatzaileen natura eta kontentrazioa antzekodun lurzoruetan aplikatzeko oso aproposa suerta daiteke.

TESIA

In-vivo eta in-silico parametroetan oinarritutako fokatze ekotoxikologiko zein paisaia aldakortasunaren araberako arrisku diskriminazioa, lurzoruaren osasun egoera ebaluazio errealistak, babes neurri zehatzak eta berreskuratze estrategia iraunkorrak diseinatzeko tresna baliagarriak dira

APPENDIX/GEHIGARRIAK

Zelomozito erauzketa

(Irizar et al., 2014b)

I. Ekipamendua

-Zentrifuga

-Argi mikroskopioa

-Laborategi balantza

-Beirazko Petri plakak

-9V-dun pilak

-Pasteur pipeta

-Matxardak

-15 eta 50 mL-tako falkon hodiak

-Pipetak

-Hemozitometro edo zelula kontagailua

II. Erreaktiboak

-Ur distilatua (dH2O)

-Na2HP4. 12H2O (71649 Sigma edo antzerakoa)

- Na2HP4. H2O (Panreac 131965 edo antzerakoa)

-NaCl (Sigma S-9888 edo antzeakoa)

-EDTA (Sigma E-6758 edo antzerakoa)

-Trypan blue soluzioa %0.4 (Sigma T8154 edo antzerakoa)

III. Soluzioen prestaketa

-PBS-*Phosphate buffer saline-, pH 7.4 (litro 1erako)*, tanpoia

NaCl……..2.32 g

Na2HPO4 ……..28.98 g

NaH2PO4 ……2.65 g

Sodio fosfatoak banan banan disolbatu, azkenik gatza gehitzeko. pHa, 7.3an izan arte doitu. Erabilera arte hozkailuan gorde; erabilera giro tenperaturan.

-Extruzio fluidoa, pH 7.3 (100 ml)

0.02% EDTA disolbatu PBSan, ondoren pHa 7.3ra doitzeko

Erabilera arte hozkailuan gorde; erabilera giro tenperaturan.

IV. Prozedura

a) Zizareak garbitu ur distilatuarekin izan ditzaketen lurzoru partikulak garbitze aldera
b) Eztiki masaiatu zizareak hesteetan izan ditzaketen edukiak askatzeko
c) 5 zizareetako taldea Petri plaka batean jarri 5 mL extruzio pean (1ml zizareko); jarraian, 9Vko pilarekin, shock elekriko laburren bitartez zelomozitoak poro dortsaletatik erauzteko.

Zelomozito kontaketa eta biderakortasuna (Trypan blue)

d) Zelula suspentsioa 15mL tako Falkon hodi batera transferitu
e) 4ºC pean, 1500 rpm-tan 10 minutuz zentrifugatu
f) Pelet-a, PBS 5mL-tan eseki (1ml zizareko)
g) Trypan blue soluzioa (%0.4), esekitako zelula suspentsioarekin nahastatu, hemozitometro bitartez zelula bizi eta hilak kontatzeko. Erabili 2ko diluzio faktorea

10 µl zelula soluzio + 10 µl trypan blue soluzio (0.04%).

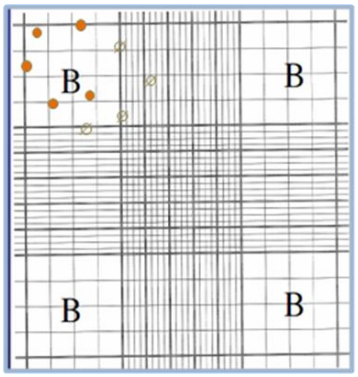
mL ko zelula kantitate totala:

Bizirik dauden zelulak/ml = (B1+B2+B3+B4)/4 x 10^4 x dilution factor*

+

Hilik dauden zelulak /ml= (B1+B2+B3+B4)/4 x 10^4 x dilution factor*

Viability % = Total nº of alive cells per ml/ Total number of cells per ml



Zelulen biderakortasunaren ebaluazioa mikroplaketan

a) Zelula soluzioa 10^6 zelula/mL-ko kontzentraziora doitu
b) Jarri $2*10^5$ zelula bizi mikroplakako (96 putzuetakoa) potzuetako bakoitzean. 8 erreplika taldeko
c) Utzi zelomozitoak estabilizatzen eta plakara itsasten 30 minututan giro temperaturan.
d) 4ºC pean, 1500 rpm-tan 10 minutuz zentrifugatu

Gorri neutroaren hartzea (NRU- Neutral red uptake, ingelesez)

(Irizar et al., 2014b)

I. Ekipamendua

-Zentrifuga, mikroplakentzako rotorearekin
-Argi mikroskopioa
-96 putzutako mikroplaka
-Pipetak (edo kanal anitzeko pipeta)
-96-putzuko mikroplakentzako spektofotometroa

II. Erreaktiboak

-Ur distilatua
-Gorri neutro tindagaia (Sigma N-7005 edo antzekoa)
-Etanola (Panreac 131086 edo antzekoa)
-Azido azetiko glaziala (Scharlau AC0343 edo antzekoa)

III. Soluzioen prestaketa

-Gorri neutro stock soluzioa (NRS) (10 mL)

5 mg gorri neutro hauts disolbatu 10 mL ur distilatutan (Gorri neutroa 0.5%)
Zentrifugatu 1500 rpm-tan 10 minutuz eta gainjariakina erabili
Iluntasunean mantendu eta egin berritan erabili

-Gorri neutro lanerako soluzioa (NRW) (20 mL)

Disolbatu 2mL NRS (Gorri neutro stock soluzioa) 18 mL PBStan (Gorri neutro 0.05%)

Iluntasunean mantendu eta egin berritan erabili

-Extrakzio/erauzketa *soluzioa (100 mL-tarako)*

Azido azetiko glaziala	1 mL
Ethanola	50mL
dH_2O	49mL

Erabilera arte hozkailuan gorde; erabilera giro tenperaturan.

IV. Prozedura

a) Gainjariakina xurgatze bidez kendu eta 200 µL NRW (0.05%) jarri potzu bakoitzean. Kontrol negativo gisa, 200 µL NRW jarriko dira hutsik dauden putzuetan.
b) Plaka giro tenperaturan eta iluntasunean inkubatu 30 minutuz
c) Zentrifugatu (G) 5 minutuz 4°C-tan
d) Garbitu plaka PBS-arekin, kontrol negatiboetan kolorerik ikusten ez den arte; horretarako, gainjariakina kendu eta 100µL PBSrekin ordezkatu.
e) Zelomozitoek gordetako tindagaia solubilizatu 100 µL erauzketa/extrakzio fluido gehituz. 5 minutuz giro tenperaturan inkubatu
f) Plaka irabiatu eta absorbantzia neurtu 540 nm-tan mikroplakentzako espektofotometroan

Kaltzeina AM bideragarritasun testa

I. Ekipamendua

-Zentrifuga, mikroplakentzako rotorearekin

-Argi mikroskopioa

-96 putzutako mikroplaka

-Pipetak (edo kanal anitzeko pipeta)

-96-putzuko mikroplakentzako spektofotometroa

II. Erreaktiboak

- Calcein AM (Molecular Probes® ThermoFisher Scientific)

***III.* Soluzioen prestaketa**

- *Kalcein AM lanerako soluzioa (2 mL)*
  5 µL Calcein AM in 1,995 mL PBS (2.5 µM Calcein AM)
  Iluntasunean mantendu eta egin berritan erabili

V. Prozedura

a) Gainjariakina xurgatze bidez kendu eta 100 µL Calcein AM lanerako soluzio jarri potzu bakoitzean (4 potzutan). Gainontzeko putzuetan, 100 µL PBS gehituko dira kaltzeina gehitu beharrean. Pausu honen bitartez, taldeen artean zelulen galera berdintsu bat eman dela bermatzea bilatzen da
b) Plaka giro tenperaturan eta iluntasunean inkubatu 40 minutuz
c) Zentrifugatu (G) 5 minutuz 4°C-tan
d) Garbitu plaka PBS-arekin; horretarako, gainjariakina kendu eta 100µL PBSrekin ordezkatu
e) Fluoreszentzia neurtu 490 ± 20 nm-tara kitzikapen filtroan eta 520 ± 20 nm-tara igorpen filtroan

Lurzoru kutsatuen bioerremediazio konbinatua burutzeko mapa kontzeptuala: 17 Zabortegiko lurzoruen erremediazioan jarraitutakoa

Jarraian, 17 Zabortegiko lurzoruen antzeko ezaugarriak dituzten lurrak erremediatzeko (teknika ez-oldarkorrak erabiliz) jarraitu beharreko gidalerroa aurkezten da (ikusi 1 irudia); era sistematiko eta protokolizatuan.

0- Lur esparrua eskokatu lurzoruaren lehortzea eta makineria astunaren (eremuaren agrokudeaketa burutzeko) sarrera ahalbidetzeko.

1- Lurzorua “chisel”edo golda bitartez (goldadun traktorea) goldatu, hau lehortu, aireatu eta partikula txikiagoatan birrindu dadin.

2- Bi astetan lehortzen eta aireatzen izan ondoren lurzorua berriz goldatu; horretarako, “rotabatoreak”, makineria ertaina eta aitzurrak erabiliz. Fase edo atalase hau, 15 egunetako tarteetan errepikatuko da (gutxi gorabehera) lurzoruaren lehortze, homogeneizazio eta disgregazio egokiak bermatu arte.

3- Erremediazio tekniken arrakasta bermatze aldera, lurzoruan dauden kutsatzaile kontzentrazioak maila letalak gainditzen dituzten edo ez konprobatu beharko da. Horretarako, lurzoruen analitika kimikoak bibliografiako emaitzekin erkatuko dira; subletalak direla egiaztatzeko. Lurzoruko kutsadura mailen analisirik izan ezean edo erabili beharreko organismoen biziraupena bermatzea ezinezkoa den kasuetan, zizare, landare eta bakterien bitazteko experimentu ekotoxikologikoak burutuko dira (ikusi 7. Kapitulua). Ez da inolaz gomendatzen prozesua datu hauek gabe hastea. Bioerremediazio tekniken aukeraketa egoki baterako, beharrezkoa da aurretiazko entseguak burutzea.

4- Fitoerremediaziorako, alfalfa (*Medicago sativa*) espeziea landatzea gomendatzen da (“Tierra de campos” barietatea). Ereite dosi gomendatua 20-30 kg/ha-koa da, ereite urtaldi gomendagarienak udaberri bukaera eta uda hasikera izanik (batez ere izozte berantiarrak saiheste aldera). Ereitea, ereiteko makinarekin egitea

gomendatzen da; hala ere, irisgarritasun zaileko landaguneetan eskuz erein daiteke, dosia 40 kg/ha-tara emendatuz.

5- Bioaumentaziorako, *Burkholderia xenovorans LB400* eta *Paenibacillus sp* bakterioen anduien lainoztatzea gomendatzen da, 1.9 eta 2.1 bitarteko dentsitate optikotan (1.25-eko dentsitate optikoa, 109 CFU mL^{-1} kontzentrazioaren baliokide da)

Horretarako anduiak bakarka haziko dira 2L eta 1.5L-tako Luria-Bertani medio esterilean (28°C eta irabiatze orbitalarekin). 72h igarota, hazkuntza kultiboa zentrifugatu (4500 rpm-tan 5 minutuz) eta 250mL-tako flaskoetara eramango dira, gainjariakina baztertuz. Zelulak ur desionizatuan ber-esekiko dira 2L-tako bolumenerarte. Ondoren, andui biak beste flasko handiago batean elkartuko dira landan aplikatzerarte.

Gomendatutako dentsitate zelularra bermatze aldera, dentsitate optikoa 660nm-tan neurtuko da espekrofotometro bitartez (Shimadzu UV-1800).

Behin zelaian, bakterio jariakina 10 alditan diluituko da, azkenik 1 L/m^2 kontzentrazioan lainoztatua izateko. Prozesu hau, 6 hilabetetako maiztasunarekin errepikatuko da, 3 astetan zehar, astean behineko aplikazioak burutuz; ahal bada, aktibitate mikrobiano altua dagoeneko hilabeteak aprobetxatuz (Udaberri-uda).

6- Bermierremediaziorako, lurzoruan 170 zizare/m^2-ko kontzentrazioa (pisuan 70g zizare/m^2 gutxi gorabehera) lortzea gomendatzen da. Horretarako, zizareak zenbatu edo pisatu egingo dira (bakarka edo osotasunean) aipatutako kontzentrazioa bermatze aldera. Zizareen zenbaketa edo pisaketa erremediatu beharreko esparruan bertan *in situ*, edo *ex situ* egin daiteke.

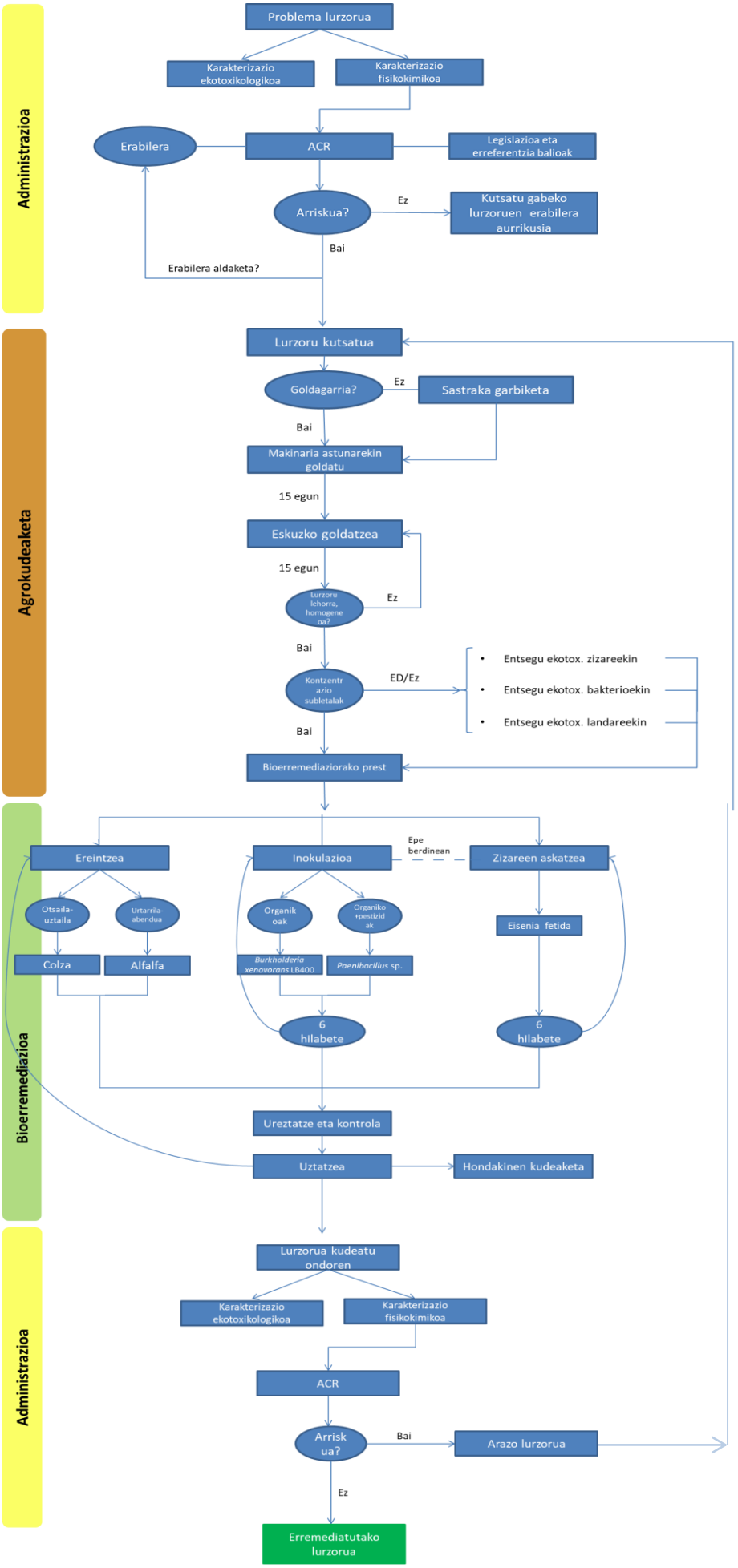
Zizareen askatzea eskuz egingo da homogeneoki; esparru osoan kontzentrazio berdintsu bat lortze aldera.

7- Uretatzea eta izurriteen kontrola astero burutuko da, ureztatzea eta hezetasunaren kontrola lehorte eta agorraldi denboraldietan indartuz.

8- Erremediazio prosezuak 6 hilabetero errepikatuko dira, denbora tarte hauetan burutako analisi kimikoek (monitorizazio analisiak), erremediazio errendimendua egonkortu arte edo kutsadura mailak desiratutako puntura heldu direla adierazi arte. Orduan, erremediazio jarduera bukatutzat emango da.

9- Landarediaren uztatzea lehen urtean zehar burutu ahal izango da; behin landareak behar bezala ezarri direnean eta gorpuzkera egokia daukatenean. Bigarren urtetik aurrera, ur erregimenen eta landareen hazkuntzaren araberako ebakiak egin ahal izango dira. Uzta ondoren, batutako materialaren analisi kimikoak burutu beharko dira; hauen arabera, biomasa hondakin erabilgarri bezala erabili ahal izateko; edo, hondakinak material kutsatu bezala kudeatu behar diren edo ez jakiteko.

1 irudia (hurrengo orrialdean). Gernika-Lumoko 17 Zabortegiaren antzeko ezaugarriak (kimiko, agronomiko eta klimatikoak) dituzten lurzoruen bioerremediazioa burutzeko mapa kontzeptuala.


Administrazioa
Problema lurzorua
Karakterizazio ekotoxikologikoa
Karakterizazio fisikokimikoa
Erabilera
ACR
Legislazioa eta erreferentzia balioak
Arriskua?
Ez
Kutsatu gabeko lurzoruen erabilera aurrikusia
Bai
Erabilera aldaketa?
Agrokudeaketa
Lurzoru kutsatua
Goldagarria?
Ez
Sastraka garbiketa
Bai
Makinaria astunarekin goldatu
15 egun
Eskuzko goldatzea
15 egun
Lurzoru lehorra, homogeneoa?
Ez
Bai
Kontzentrazio subletalak
ED/Ez
Entsegu ekotox. zizareekin
Entsegu ekotox. bakterioekin
Entsegu ekotox. landareekin
Bai
Bioerremediaziorako prest
Bioerremediazioa
Ereintzea
Inokulazioa
Epe berdinean
Zizareen askatzea
Otsaila-uztaila
Urtarrila-abendua
Organik oak
Organiko +pestizidak
Eisenia fetida
Colza
Alfalfa
Burkholderia xenovorans LB400
Paenibacillus sp.
6 hilabete
6 hilabete
Ureztatze eta kontrola
Uztatzea
Hondakinen kudeaketa
Administrazioa
Lurzorua kudeatu ondoren
Karakterizazio ekotoxikologikoa
Karakterizazio fisikokimikoa
ACR
Arriskua?
Bai
Arazo lurzorua
Ez
Erremediatutako lurzorua

ESKER ONAK

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