



# Valutazione ecotossicologica in acqua dolce

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## ACRONIMI

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ACQ - Alkaline Copper Quaternary (Quaternario di rame alcalino)  
 ASTM - American Society for Testing and Materials  
 CBA - Copper Borate Azole (Composti azolici di rame e boro)  
 CCA - Chromated copper arsenate (arsenato di rame cromato)  
 CLP - Classification, Labelling and Packaging (Regolamento europeo)  
 EC50 - Effect Concentration (Concentrazione Efficace per il 50% degli organismi utilizzati nei test di tossicità)  
 ISO -International Organization for Standardization  
 ITS - Integrated Testing Strategy (Strategia Integrata di Valutazione)  
 OECD - Organization for Economic Cooperation and Development  
 REACH - Registration, Evaluation, Authorisation and Restriction of CHemicals (Direttiva europea)  
 TM - Termically Modified (termicamente modificato)  
 TU50 - Toxicity Unit o Unità di Tossicità (TU50 = 100/EC50)  
 UC3 - Use Class 3 (classe d'uso del legno 3)  
 US-EPA - United States Environmental Protection Agency  
 UTW - UnTreated Wood (legno non trattato)

## SOMMARIO

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In questo studio è stato impiegato un approccio sperimentale basato su una batteria di indicatori in grado di fornire valutazioni del pericolo per il biota dulciacquicolo a diversi livelli trofici e di complessità biologica di alcuni prodotti usati per la protezione del legno. La valutazione della tossicità di lisciviati ottenuti a partire da provini di legno (trattato e non trattato) ha previsto l'adozione di una Integrated Testing Strategy (ITS), la cui struttura di tipo gerarchico permette la valutazione dell'impatto ambientale dei trattamenti che vengono impiegati per aumentare la

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durabilità del legno. In particolare, l'ITS si caratterizza per un aumento di sensibilità dei test ecotossicologici utilizzati nei tre livelli valutativi (TIER 1 - Test acuti; Tier 2 - Test sugli stadi più sensibili, Tier 3 - Test cronici) e valuta i possibili effetti e quanto questi siano "accettabili" su flora e fauna acquatica, permettendo così di identificare i trattamenti ecocompatibili. L'indagine è stata condotta con lo scopo di comprendere come i tempi di maturazione in acqua possano influenzare il processo di rilascio dal legno al fine di predisporre linee guida a supporto delle aziende per definire i tempi di maturazione necessari prima di impiegare un materiale in ambiente. L'applicazione del percorso ITS per l'ambiente dulciacquicolo ha previsto il confronto tra provini di legno trattato con il corrispondente non trattato (UTW) ed ha focalizzato l'indagine su provini di *Picea abies* modificati termicamente (termically modified, TM) e provini trattati con Silvanolin al fine di un suo impiego outdoor senza contatto con il suolo (UC3) (concentrazione di Cu nel trattamento pari allo 0.25%).

L'applicazione dei test di TIER I ha evidenziato come sulla base dei criteri adottati per la procedura di valutazione ITS predisposta per le acque dolci solo TM abbia fornito effetti tossici paragonabili, se non addirittura inferiori, al legno non trattato (UTW). UC3 al contrario non risulta un trattamento compatibile per l'ambiente acquatico, a causa dell'elevata tossicità acuta generata su batteri, alghe e crostacei indipendentemente dal tempo di maturazione. I risultati ottenuti in questo primo livello di valutazione hanno evidenziato pertanto come solo TM sia risultato eleggibile per il secondo step valutativo. Alla luce dei risultati ottenuti nel TIER II è stato possibile considerare il trattamento TM idoneo anche per la valutazione di livello III. Tuttavia gli effetti generati anche dal lisciviato del provino non trattato (UTW) nel caso dell'endpoint rottura della capsula nel gasteropode *L. auricularia* hanno evidenziato la necessità di un periodo di maturazione superiore a 15 giorni per rimuovere gli effetti tossici del lisciviato sullo sviluppo larvale. Considerati i risultati complessivi dei test cronici su molluschi *L. auricularia* e crostacei *Daphnia magna* del Tier III, il trattamento TM risulta equivalente in termini di impatti su entrambi gli indicatori acquatici impiegati al legno non trattato (UTW). Emerge tuttavia chiaramente, in particolare dai risultati dei test sulla riproduzione in *D. magna*, come sia necessario un periodo di maturazione di almeno 15 giorni prima dell'impiego del materiale, per limitare gli impatti sull'ambiente dulciacquicolo. Ai sensi della valutazione ITS proposta in questo progetto, quindi, il trattamento TM risulta ecocompatibile non comportando rischi aggiuntivi per l'ambiente, neanche dopo un'esposizione a lungo termine.

## 1 INTRODUZIONE

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L'obiettivo principale del progetto di ricerca è la valutazione ecotossicologica di lisciviati ottenuti da legni trattati termicamente e chimicamente, seguendo una strategia integrata di valutazione (ITS), la cui struttura di tipo gerarchico permette la valutazione dell'impatto ambientale dei trattamenti che vengono impiegati per aumentare la durabilità del legno. In particolare, la strategia ITS valuta i possibili effetti e quanto questi siano "accettabili" su flora e fauna di ambiente d'acqua dolce.

### 1.1 Ricerca bibliografica preliminare

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La ricerca bibliografica è stata condotta con lo scopo di definire quale sia lo stato dell'arte relativo alla valutazione della tossicità del lisciviato del legno naturale o

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trattato e ha messo in evidenza a) quali sono le procedure standard e quali sono invece sperimentali, b) il tipo di fluido lisciviante impiegato, c) eventuali pretrattamenti dei legni, d) i parametri chimico-fisici e il tipo di analiti chimici che si è andati a ricercare, e) organismi e tipi di test ecotossicologici impiegati nelle valutazioni, f) e infine i risultati degli studi con le relative criticità.

La ricerca bibliografica è stata sintetizzata in due allegati, il primo focalizzato sui parametri e le tecniche di lisciviazione che sono state impiegate (Allegato 1) e il secondo relativo alla caratterizzazione chimica ed ecotossicologica dei lisciviati (Allegato 2).

### **Tecniche di lisciviazione**

Lo scopo di questa parte della ricerca bibliografica è stato quello di capire quali sono i metodi che sono stati utilizzati per caratterizzare materiali di tipo legnoso che siano trattati o non trattati al fine di definire il disegno sperimentale.

Di fatto, dei 21 lavori presi in esame dieci sono riferiti a legni trattati con vari impregnanti a base di rame (Allegato 1). Il più studiato è l'arsenato di rame cromato (CCA, 5 lavori), ora vietato dall'UE, seguito dal quaternario di rame alcalino (ACQ) e dai composti azolici di rame e boro (CBA) (4 lavori ciascuno). Altri due tipi di trattamenti studiati, non a base di rame, sono la furfurilazione e l'applicazione di pentaclorofenolo. Sette lavori avevano lo scopo di definire la tossicità naturale del legno e anche in questo caso, come per i legni trattati, sono state usate delle tecniche di lisciviazione definite in maniera tale da essere riproducibili. Quattro studi invece prevedevano la raccolta di lisciviato direttamente in campo e generato dalle piogge stagionali.

Per quanto riguarda la procedura di lisciviazione, il fluido maggiormente usato è l'acqua distillata/deionizzata o di rubinetto (13 lavori) mentre l'acqua marina artificiale è stata usata in 3 studi. Alcuni studi come quello di Dubey et al. (2007) prevedono l'utilizzo di ben 8 acque naturali dolci, salate e salmastre con caratteristiche differenti tra loro, mentre Adeleye et al. (2016) hanno confrontato acque marine, estuarine e potabili.

Il test di lisciviazione di tipo statico è stato quello più usato dato che è stato impiegato in 12 lavori, mentre test dinamici sono stati usati solo cinque volte. Il rapporto solido:liquido (S/L) è molto variabile a seconda dell'autore e va da un minimo 1:5 ad un massimo di 1:40. Il maggior numero di esperienze è stato tuttavia condotto utilizzando un rapporto di circa 1:10. Svensson et al. (2012, 2014a,b) hanno utilizzato un rapporto S/L 1:40 nel caso specifico dei test su segatura, dal momento che quest'ultima assorbendo molta acqua non consentiva una lisciviazione ottimale con rapporti inferiori.

### **Caratterizzazione chimico-fisica ed ecotossicologica dei lisciviati**

Dalla ricerca bibliografica relativa ai lisciviati (Allegato 2) è stato possibile osservare che la maggior parte dei test sono stati condotti su acqua dolce, mentre solo 5 studi sono relativi all'acqua marina. Inoltre, nella maggior parte degli studi, alle analisi chimiche sono state affiancate analisi ecotossicologiche.

Per quanto riguarda i test ecotossicologici effettuati sui lisciviati, la maggior parte degli studi ha previsto l'utilizzo del batterio *Aliivibrio (Vibrio) fischeri* e del crostaceo *Daphnia magna*. Tra queste due specie, la più sensibile è sempre risultata essere *D. magna*. Per quanto riguarda *A. fischeri*, la bibliografia indica che il truciolo di legno produce lisciviati tossici indipendentemente dal tipo di lisciviazione utilizzato (Rex at

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al., 2016, Svensson et al., 2014a,b).

Altre specie test utilizzate per valutare la tossicità dei lisciviati sono il bivalve *Crassostrea gigas* e il crostaceo *Artemia salina* (Libralato et al., 2007; Svensson et al. 2012, 2014a), mentre tra gli organismi vegetali si segnala l'impiego di *Scirpus microcarpus* (Wolf et al. 2019). Il test di embriotossicità con *C. gigas* si è dimostrato più sensibile rispetto al test acuto con *Artemia salina* (Libralato et al., 2007) anche perché, come riportato da Svensson et al. (2014a), questo crostaceo non risente significativamente dell'effetto tossico del lisciviato. Il test di crescita con *S. microcarpus*, invece, ha consentito di rilevare che alti livelli di fenoli disciolti possono limitare l'accesso ai nutrienti per le piante (Wolf et al. 2019).

Per quanto riguarda, invece gli organismi terrestri, sono state condotte delle esperienze mirate a valutare il bioaccumulo di metalli e metalloidi e gli effetti su riproduzione e crescita nell'oligochete *E. fetida* esposto a suoli irrorati con lisciviati di legni trattati con CCA e ACQ (Leduc et al., 2008).

In generale è possibile affermare che le sostanze responsabili degli effetti tossici acuti osservati nei legni non trattati probabilmente differiscono secondo l'essenza presa in considerazione; ad esempio, la tossicità della quercia è molto probabilmente legata ai composti fenolici, mentre la tossicità del pino deriva probabilmente dai terpeni (Svensson et al., 2012). Dall'analisi della letteratura è inoltre emerso che l'elevata componente organica del percolato o la combinazione di composti organici e acidi resinici contribuisce in maniera sensibile alla tossicità (Rex et al., 2016). Per quanto riguarda i trattamenti utilizzati per la protezione del legno, invece, è stato riscontrato che la tossicità acquatica è correlata alla frazione di Cu labile presente nel lisciviato. La minore tossicità acquatica osservata nei percolati di legno trattati ottenuti con acque naturali rispetto alle soluzioni di lisciviazione di laboratorio è probabilmente legata alla presenza di maggiori quantità di leganti e chelanti nei medium naturali (Dubey et al., 2007, Kannepalli et al., 2016).

Anche per quanto concerne le caratteristiche chimico-fisiche dei lisciviati la letteratura ha messo in risalto rilevanti differenze legate all'essenza. Ad esempio, sulla base di prove di lisciviazione eseguite con il truciolo di quattro diverse specie (quercia, pino, acero e faggio) sono state osservate differenze statisticamente significative tra la quercia e le altre tre essenze per quanto riguarda quasi tutte le variabili considerate, ovvero carbonio organico disciolto (DOC), fenoli, richiesta biochimica di ossigeno (BOD5), pH, tannini, lignina e colore (Svensson et al., 2014b). Quando il legno viene tagliato in piccoli pezzi, il processo di lisciviazione e il trasporto degli inquinanti avvengono in un tempo molto più breve di quanto dovrebbe avvenire naturalmente. Secondo i risultati, la specie, la dimensione delle particelle e le differenze anatomiche del legno del materiale stoccato sembrano determinare il carico inquinante organico rilasciato nella fase acquosa quando il legno viene a contatto con l'acqua.

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## 2 MATERIALI E METODI

### 2.1 Preparazione del lisciviato per i test ecotossicologici

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Con riferimento alla letteratura scientifica e a precedenti esperienze per l'ambiente lagunare (UniVe-CVN, 2011; MAV, 2013), per l'esecuzione delle attività di progetto è stato messo a punto un metodo per la valutazione della tossicità dei provini di legno che prevede la lisciviazione al tempo zero (T0), previo lavaggio dei campioni in acqua

corrente con lo scopo di eliminare eventuali residui adesi alle superfici, e successivamente altre due lisciviazioni dopo 15 (T15) e 30 (T30) giorni di maturazione in acqua.

Il processo di maturazione è rappresentato schematicamente in Figura 1.

Ogni acquario è stato riempito con 30 litri di “hardwater” (US-EPA); all’interno di questi sono stati riposti i provini a seguito della prima lisciviazione per procedere alla maturazione. I provini di legno sono stati legati ad un peso per assicurare la loro completa immersione.

Ciascun acquario è stato allestito con pompe che garantiscono un riciclo di 25 L h<sup>-1</sup> per ricreare le condizioni ambientali a cui ci si si aspetta possano essere sottoposti i pali in ambiente, con rinnovo parziale settimanale. L’acqua è stata totalmente rinnovata al 15° giorno d’inizio del test. Ai tempi T<sub>0</sub>, T<sub>15</sub> e T<sub>30</sub> i provini di legno sono sottoposti alla procedura di lisciviazione rappresentata in Figura 2.

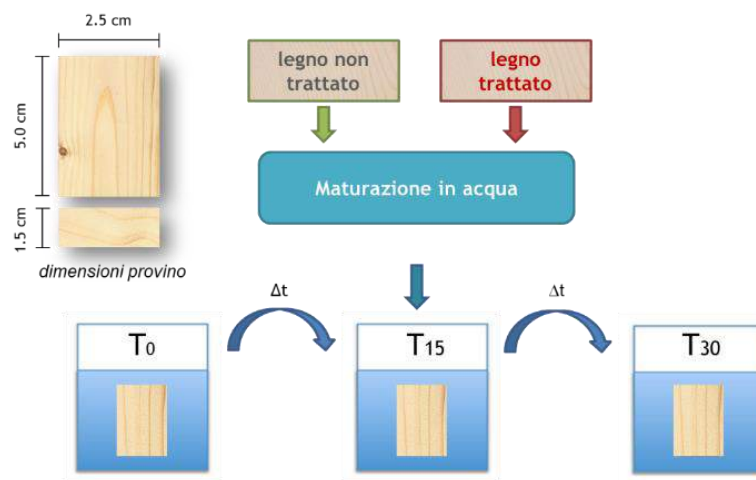


Figura 1. Processo di maturazione dei provini in legno di dimensioni 5.0 x 2.5 x 1.5 cm.

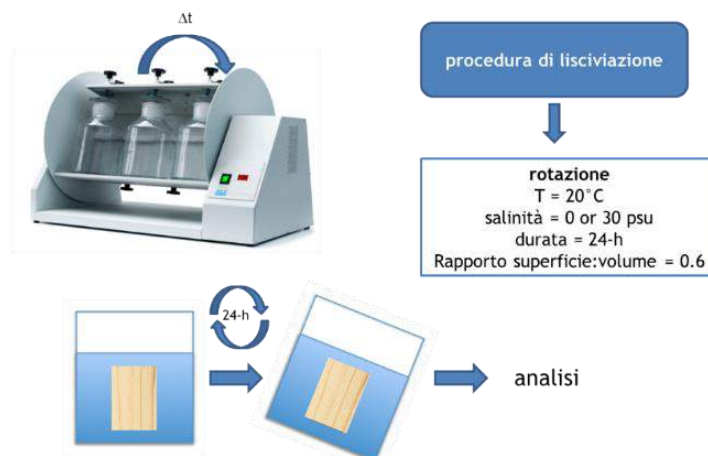


Figura 2. Strumento ROTAX sulla sinistra, parametri di lisciviazione sulla destra. In basso una rappresentazione della lisciviazione.

Per la lisciviazione è stato utilizzato un ROTAX (Velp scientifica) che prevede una rotazione verticale completa dei campioni posti all’interno di bocce chiuse ermeticamente. La lisciviazione è effettuata a 20°C per 24h in accordo con le linee guida MAV (2013). È stato scelto un rapporto superficie campione/volume d’acqua pari a 0.6 cm<sup>2</sup> cm<sup>-3</sup> (OECD, 1999; OECD 2007).

Tutti i provini di legno hanno dimensioni di 5.0 x 2.5 x 1.5 cm e sono stati prodotti secondo l'indicazione della norma europea EN 113-1:2020 in materia di durabilità del legno e dei prodotti a base di legno.

## 2.2 Integrated Testing Strategy (ITS)

La valutazione della tossicità dei lisciviati prodotti a partire dai provini di legno trattato e non trattato ha previsto l'adozione di una "Integrated Testing Strategy" (ITS), un approccio gerarchico in grado di guidare il processo decisionale relativo alla valutazione di impatto ambientale di nuovi prodotti presenti sul mercato per l'uso nell'industria, nel commercio e nell'edilizia (Figura 3).

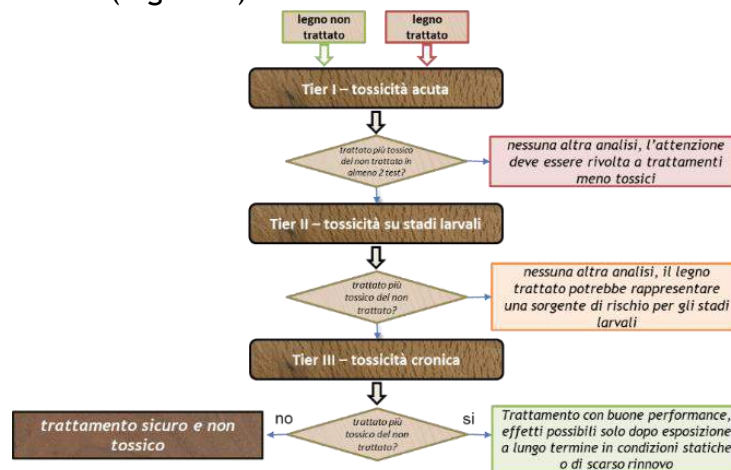


Figura 3. Approccio gerarchico dell'ITS previsto per il progetto Durasoft.

In questo progetto, l'ITS ha previsto tre livelli in cui viene valutata l'accettabilità di possibili effetti tossici su flora e fauna. Dal primo livello che prevede l'esecuzione dei test acuti, ad un secondo livello che va a valutare gli effetti sugli stadi più sensibili (embrio-larvali), al terzo ed ultimo livello con i test di tipo cronico, l'ITS si caratterizza per un aumento della sensibilità dei test utilizzati. In tutto il percorso valutativo, si sono confrontati i campioni di legno trattato con il corrispondente naturale non trattato e nel caso in cui il trattato sia risultato meno tossico (o se comunque presenta dei valori di  $TU < 1$ ) si passa allo step di valutazione successivo. L'ultimo livello valutativo, qualora non mostri positività ai test identifica un trattamento totalmente sicuro e compatibile e qualora mostri un certo effetto cronico identifica comunque un trattamento con buona compatibilità, per il quale effetti sono possibili ma solo in particolari condizioni di esposizione (es. condizioni statiche o di scarso rinnovo).

I test previsti per ogni step ("TIER"), hanno delle caratteristiche ben precise e, come si osserva in Figura 4, in ambiente dulciacquicolo sono stati suddivisi secondo il seguente schema:

- Il "TIER 1" valuta gli effetti acuti a breve termine attraverso il test di inibizione della bioluminescenza del batterio *A. fischeri*, il test di inibizione della crescita della clorofitea unicellulare *Raphidocelis subcapitata* e il test di immobilizzazione con il cladocero *Daphnia magna*.
- Il "TIER 2" valuta la tossicità sugli stadi di sviluppo più sensibili degli organismi acquatici, attraverso misure di sviluppo larvale. Per l'ambiente dulciacquicolo il metodo scelto è stato il test di sviluppo larvale con il gasteropode *Lymnaea (Radix) auricularia*.
- il "TIER 3" valuta l'effetto del lisciviato sull'intero ciclo di vita degli organismi del test; per l'ambiente dulciacquicolo sono stati previsti test di riproduzione

con *L. auricularia* e *D. magna*.

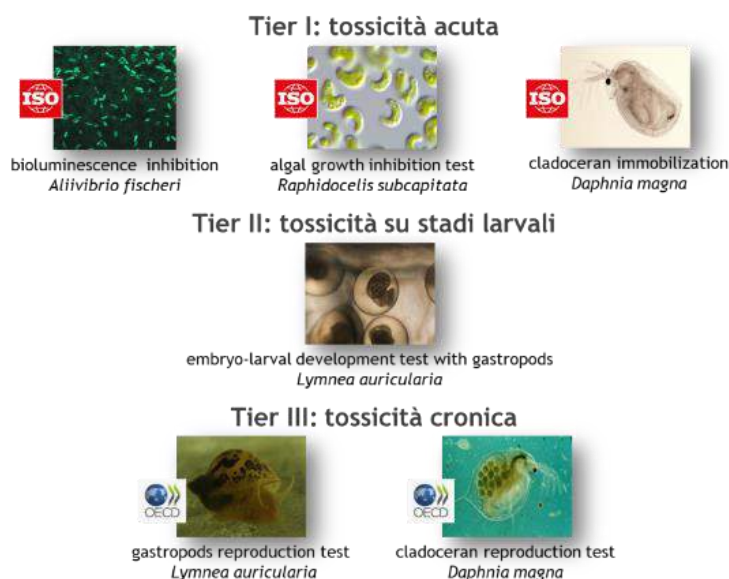


Figura 4. ITS per acque dolci.

Tra i metodi scelti per la ITS, si ricorda che il test con *R. subcapitata* ed i test acuto e cronico con *D. magna* sono riportati tra i metodi ecotossicologici da utilizzare per la valutazione degli effetti tossici di sostanze e composti dal Regolamento (CE) n. 1907/2006 del Parlamento europeo e del Consiglio (Regolamento REACH) che prevede la registrazione, valutazione, autorizzazione e restrizione delle sostanze chimiche, in particolare riguarda la registrazione di tutte le sostanze prodotte o importate nell'Unione Europea in quantità maggiori di una tonnellata per anno e dal Regolamento (CE) n. 1272/2008 del Parlamento Europeo e del Consiglio (Regolamento CLP).

### 2.3 Procedure dei test di tossicità per le acque dolci

#### Tier 1

##### **-Test di inibizione della bioluminescenza batterica in *A. fischeri* (Test Microtox®)**

Il test Microtox® è stato eseguito utilizzando un analizzatore M500 (Azur Environmental) e seguendo la procedura per campioni liquidi riportata nello standard ISO 11348 (2007). La riattivazione dei batteri liofilizzati (Microtox® Acute Reagent, Modern Water, lotto n°19K4239A) è stata effettuata aggiungendo la soluzione ricostituente (Reconstitution Solution, Modern Water) alla fiala del reagente acuto costituito da batteri liofilizzati, per ottenere una dispersione batterica concentrata. La cuvetta con la dispersione batterica è stata acclimatata nell'apposito pozzetto dell'analizzatore a 4 °C per la durata del saggio biologico, che è stato condotto a 15 °C. L'inibizione della bioluminescenza a 490 nm di *A. fischeri* è stata misurata dopo 5, 15 e 30 minuti di esposizione a diverse diluizioni di eluati, ottenuti con acqua dolce ed in acqua distillata, per formulazioni di prodotto da utilizzare in ambiente d'acqua dolce (UC3, TM e UTW). L'aggiustamento osmotico del campione è stato ottenuto sciogliendo 0,5 g di sale NaCl in 25 ml di campione (20‰). Secondo il protocollo test, il campione è stato analizzato come non diluito (1:1) e dopo diluizioni sequenziali distanziate di un fattore 3, ottenendo quindi le seguenti concentrazioni, in ordine decrescente: 100%, 33%, 10%, 3%, 1%, 0.3%. Come controllo negativo e mezzo di diluizione sono state utilizzate acqua marina artificiale ASTM. Il controllo positivo è stato eseguito



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utilizzando una soluzione di ZnSO<sub>4</sub> come tossico di riferimento e seguendo la procedura riportata nel metodo standard ISO per campioni liquidi utilizzando batteri liofilizzati (ISO 11348, 2007).

**-Test di inibizione della crescita algale con *R. subcapitata***

Il test di crescita algale è oggetto della procedura standard internazionale ISO 8692 (ISO, 2012). Prima dell'esecuzione del test, la coltura in crescita esponenziale è stata tenuta in costante agitazione grazie ad una piastra agitatrice, ad una temperatura compresa tra 21°C e 24°C. È stato necessario mantenere la coltura algale sempre nella condizione di crescita esponenziale rinnovandola con frequenza settimanale.

Il metodo prevede che delle aliquote di *R. subcapitata* siano inoculate all'interno dei campioni da testare, i quali sono stati precedentemente arricchiti con nutrienti. Il test ha una durata di 72 ore, al termine delle quali è stata misurata la densità cellulare in ogni trattamento, da cui si è successivamente calcolato il tasso di crescita specifico del campione.

Per ogni trattamento, i campioni sono stati analizzati eseguendo sei diluizioni in progressione geometrica in tre repliche per concentrazione (100, 33, 10, 3, 1, 0.3%). Per il controllo negativo sono state fatte 6 repliche. Le repliche di ogni trattamento sono state allestite in piastre monouso a sei pozzetti (Iwaki, Japan) ciascuno riempito con 10 mL di campione.

Prima di procedere all'inoculo delle alghe, viene calcolata la densità algale della coltura di crescita e successivamente preparato l'inoculo ad una concentrazione di 10<sup>4</sup> cell ml<sup>-1</sup>. Le piastre sono successivamente incubate a 20 ± 2 °C sotto luce continua (6000 lx) per 72 ± 2 h.

Al termine delle 72 h una stima della concentrazione algale all'interno dei pozzetti è ottenuta attraverso la conta diretta con camera di Bürker.

**-Test di immobilizzazione con *D. magna***

Questo saggio, che considera l'immobilizzazione del crostaceo, rientra tra quelli più utilizzati nelle valutazioni di sostanze immesse in ambiente, venendo adottato sia dalle linee guida nazionali (APAT-IRSA) che internazionali ISO 10706 (ISO, 2000), OECD 202 (OECD, 2004), nonché menzionato sia dalla Direttiva 98/8/CE (che concerne l'immissione sul mercato dei Biocidi) sia dal regolamento REACH per valutare la commerciabilità di sostanze chimiche.

Le colture mantenute in laboratorio vengono periodicamente rinnovate e devono essere conservate in condizioni controllate di temperatura e illuminazione; la temperatura deve essere mantenuta tra i 18°C e i 22°C mentre l'illuminazione segue un ciclo giorno-notte di 16-8 ore. Gli organismi vengono mantenuti in acqua M7 e sono nutriti somministrando periodicamente una soluzione contenente l'alga unicellulare *R. subcapitata*.

Il test ha una durata di 48 ore e, come per il test con *R. subcapitata*, per ogni trattamento, i campioni sono stati analizzati eseguendo sei diluizioni in progressione geometrica (100, 33, 10, 3, 1, 0.3%), il controllo negativo viene eseguito utilizzando solo medium M7. Il test è stato eseguito in piastre a 6 pozzetti da 10 mL, ognuno contenente 5 individui di *D. magna* di età inferiore a 24 ore. Dopo aver aggiunto gli individui a ciascun pozzetto, le piastre sono state incubate per 48h alle stesse condizioni di mantenimento della coltura. Al termine delle 48 ore si osservano i pozzetti e si procede alla conta degli organismi che, all'interno di ogni singolo pozzetto, non manifestano movimenti.

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## Tier 2

### **-Test di embriotossicità con il gasteropode *Lymnea (Radix) auricularia***

Il test di embriotossicità con il gasteropode polmonato *L. auricularia* è stato messo a punto dal gruppo di ricerca di ecotossicologia dell'Università di Venezia in accordo con metodologie già sviluppate per *Radix balthica* (Tills et al., 2013). Consiste nell'osservare lo sviluppo di placche ovigere esposte alle varie concentrazioni di lisciviato e verificare l'eventuale presenza di un ritardo nello sviluppo. Questa tipologia di test è importante perché indaga gli effetti sulla fase più sensibile di un organismo.

Gli organismi allevati in laboratorio vengono mantenuti in acquari riempiti con acqua del rubinetto dechlorata, dotati di filtro esterno ed impianto di areazione e vengono alimentati con insalata biologica. Gli acquari sono stati mantenuti in una cella termostata, ad una temperatura costante di 21°C ed un ciclo luce buio di 16-8 ore.

Per agevolare la raccolta e l'osservazione delle placche ovigere per l'esecuzione del test, vengono allestite delle vasche di vetro di volume 2 litri, riempite con l'acqua dell'acquario le cui pareti sono ricoperte da vetri da microscopio rimovibili.

Per il test vengono utilizzate placche ovigere deposte entro 24 ore dall'inizio dell'attività sperimentale (ovvero allo stadio di "velum"). Una volta prelevato il vetrino con la placca, quest'ultima viene osservata al microscopio per verificarne lo stadio di sviluppo e viene contato il numero di uova presenti all'interno. Una volta verificato lo stato di sviluppo, sono allestite 3 repliche per ciascuna diluizione di campione. Quindi, ciascuna placca viene posta all'interno di un becker da 100 mL contenente la soluzione da testare facendo particolare attenzione ad immergere totalmente la placca. Parallelamente si avviano tre controlli negativi composti solo da "medium *Radix*", che consiste in un medium artificiale di acqua dura per organismi dulciacquicoli caratterizzato da valori di pH, durezza e alcalinità adatti alle esigenze del gasteropode (U.S. EPA, 2002).

Una volta allestite tutte le repliche queste vengono poste nelle stesse condizioni controllate degli acquari. Dopo 8 giorni dall'avvio del saggio si effettua una prima lettura al microscopio invertito, prelevando il vetrino con adesa la placca e verificando lo stadio raggiunto da ciascun embrione presente al suo interno. In condizioni standard gli embrioni raggiungono lo stadio "shell formation" all'ottavo giorno.

Procedimento del tutto analogo è stato messo in pratica al tredicesimo giorno di test, tenendo conto che a questo punto lo stadio di sviluppo raggiunto è quello di "capsule rupture", in cui è fondamentale che venga verificata la presenza del guscio del gasteropode e la rottura dell'involucro dell'uovo.

Viene, infine, registrata la percentuale di embrioni che hanno raggiunto lo stadio corretto per valutare un eventuale ritardo dovuto all'esposizione al lisciviato.

## Tier 3

### **-Test cronico con *D. magna***

Per l'esecuzione di questo test si è fatto riferimento alla procedura OECD n. 211 (OECD, 2012).

Sono state predisposte 10 repliche per ciascuna diluizione del lisciviato e per il controllo negativo, costituito da solo medium acquoso M7. Come per il test acuto, organismi di età inferiore alle 24 ore vengono prelevati dalle colture di laboratorio e

posti singolarmente nei becker da 100 mL contenenti ciascuno 50 mL di soluzione. Sempre in analogia col test acuto, i becker vengono quindi incubati in un ambiente controllato, dove la temperatura viene mantenuta costante, in un range tra 18 e 22 °C. Le soluzioni in cui gli organismi sono esposti vengono rinnovate completamente tre volte a settimana, facendo attenzione a minimizzare il volume di soluzione trasferita con l'organismo stesso. Insieme alla procedura di rinnovo, tutti gli organismi vengono nutriti, aggiungendo inizialmente 0,5 mL di sospensione algale e poi, dopo il primo evento riproduttivo, un volume proporzionale all'aumento di taglia ed eventualmente del numero di prole prodotta, fenomeno che non avviene solitamente prima del nono giorno di test.

Alla fine del periodo di 21 giorni viene registrata la sopravvivenza dei genitori insieme al numero di prole viva prodotta per ogni genitore.

#### **-Test cronico con *L. auricularia***

Per l'esecuzione di questo test è stato preso come riferimento il protocollo OECD per il gasteropode *Lymnaea stagnalis* (OECD 243). Il test ha una durata di 28 giorni con rinnovo totale del mezzo ogni tre giorni.

Il test viene effettuato in becker da litro contenenti il campione da testare; per ciascun becker vengono selezionati 5 organismi aventi la taglia minima riproduttiva (7,5-8,5 mm di lunghezza) che vengono alimentati con lattuga biologica. Vengono allestite tre repliche per concentrazione e per il controllo negativo, mantenuti ad una temperatura costante di 21 °C ed un fotoperiodo di 16 ore di luce e 8 ore di buio.

Gli endpoints considerati sono la mortalità e la variazione del tasso di crescita degli organismi in termini di peso e di lunghezza.

#### **Espressione dei risultati dei test e analisi dei dati**

I dati di inibizione della bioluminescenza batterica, della crescita algale e della motilità di *D. magna* sono stati elaborati ai fini del calcolo delle EC<sub>50</sub> (concentrazioni di lisciviato che generano un effetto su 50% degli organismi esposti) e delle TU<sub>50</sub>, calcolate come 100/EC<sub>50</sub>. Nel caso dei trattamenti con effetti bassi o trascurabili, ovvero quando non viene raggiunta una inibizione del 50% nel campione non diluito, le TU<sub>50</sub> sono calcolate secondo l'equazione  $TU_{50} = Ex/50$ , dove Ex rappresenta la percentuale di effetto rilevata nel trattamento X normalizzata rispetto all'effetto rilevato nel controllo negativo. Le EC<sub>50</sub> sono state calcolate utilizzando il software Microtox Omni® per il test Microtox ed un programma sviluppato dalla Danmarks Tekniske Universitet di Lyngby (Christensen et al. 2009) per l'analisi dei dati continui prodotti dai test algali e dai test con *D. magna*.

Nel caso dei test di Tier II, la percentuale di larve che ha raggiunto lo stadio di "shell formation" e "capsule rupture" nel campione di lisciviato non diluito (100%) è stata confrontata statisticamente con i risultati del controllo negativo e del lisciviato ottenuto da legno non trattato (UTW) utilizzando l'analisi della varianza a una via (ANOVA a una via) accoppiata al test t di Tukey.

Nei test di Tier III, il numero di prole prodotta da ogni *Daphnia* esposta ai lisciviati ed i tassi di crescita di *L. auricularia* espressi come incremento giornaliero di peso (in mg) ed incremento giornaliero di lunghezza (in µm) sono stati analizzati utilizzando l'ANOVA ad una via accoppiata al test t di Tukey per rilevare eventuali differenze tra controllo negativo, legno non trattato (UTW) e trattamenti per la conservazione.

Prima di analizzare i dati dei test del Tier II e del Tier III, la normalità e la distribuzione omogenea delle varianze sono state verificate utilizzando i test di

Kolmogorov-Smirnov e di Levene, rispettivamente. Quando una delle due condizioni non è stata verificata, i dati sono stati analizzati utilizzando metodi non parametrici, ovvero Kruskal-Wallis ANOVA e test di Mann-Whitney per i confronti tra due gruppi indipendenti.

### 3 RISULTATI E DISCUSSIONE

#### 3.1 TIER I

I test del TIER 1 sono stati applicati ai lisciviati prodotti con acque dolci e sono stati condotti utilizzando provini di *P. abies* non soggetti ad alcun trattamento (untreated wood, UTW), provini modificati termicamente (termically modified, TM) e provini trattati con Silvanolin al fine di un impiego outdoor senza contatto con il suolo (UC3). Il test Microtox ha evidenziato una diminuzione della tossicità dei lisciviati all'aumentare del tempo di maturazione e TM è risultato il trattamento meno impattato sulla bioluminescenza batterica (Figura 5). A T0, TM era considerevolmente meno tossico per *A. fischeri* sia di UTW che di UC3. Sia TM che UTM hanno evidenziato una significativa riduzione della tossicità a T15 rispetto a T0. Al contrario, l'inibizione della bioluminescenza da parte di UC3 ha mostrato una diminuzione minima rispetto a T0 e la tossicità era ancora notevole. Dopo 30 giorni di maturazione, la tossicità di TM e UTW è ulteriormente diminuita mentre UC3 esercitava ancora una forte inibizione della bioluminescenza.

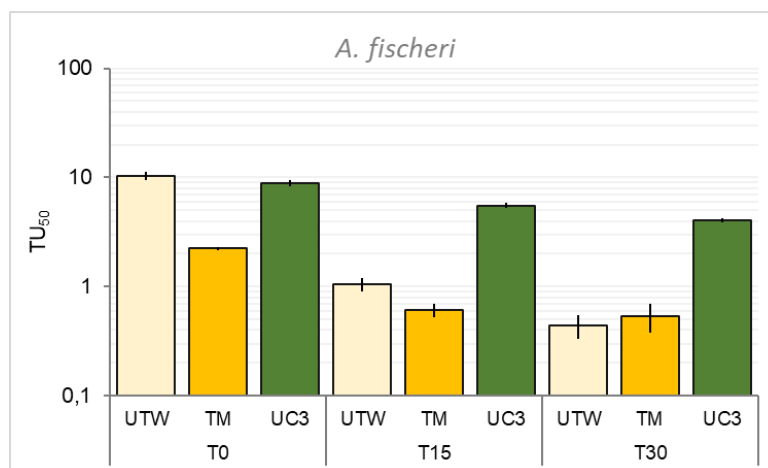


Figura 5. Tossicità dei lisciviati nei confronti di *A. fischeri*. Le barre di errore rappresentano i limiti di confidenza al 95% relativi alla TU50 media calcolata per le 3 repliche sperimentali.

Nel caso del test di crescita algale con *R. subcapitata*, i lisciviati ottenuti dai campioni UTW e TM hanno fornito risultati comparabili in tutte le condizioni testate, con una diminuzione dell'effetto tossico all'aumentare del tempo di maturazione (Figura 6). Inoltre, gli effetti tossici sono bassi a tutte le condizioni di maturazione testate. Al contrario, i lisciviati ottenuti dai campioni UC3 determinano una elevata inibizione del tasso di crescita algale, con un andamento ad U, caratterizzato da effetto massimo a T0, effetto minimo a T15 e da un significativo aumento della tossicità, dopo 30 giorni di maturazione.

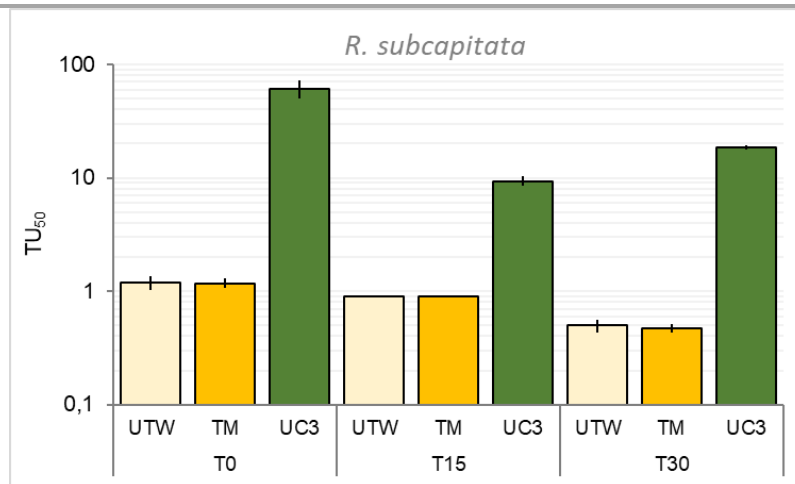


Figura 6. Tossicità dei lisciviati nei confronti di *R. subcapitata*. Le barre di errore rappresentano i limiti di confidenza al 95% relativi alla TU50 media calcolata per le 3 repliche sperimentali.

Il test acuto con *D. magna* ha confermato la tendenza osservata con *R. subcapitata* (Figura 7). I lisciviati dei campioni UTW e TM hanno mostrato effetti trascurabili o nulli su *D. magna*, mentre UC3 ha generato un effetto marcato sulla sopravvivenza, con effetti decrescenti da T0 a T15 e nessuna variazione della tossicità tra T15 e T30.

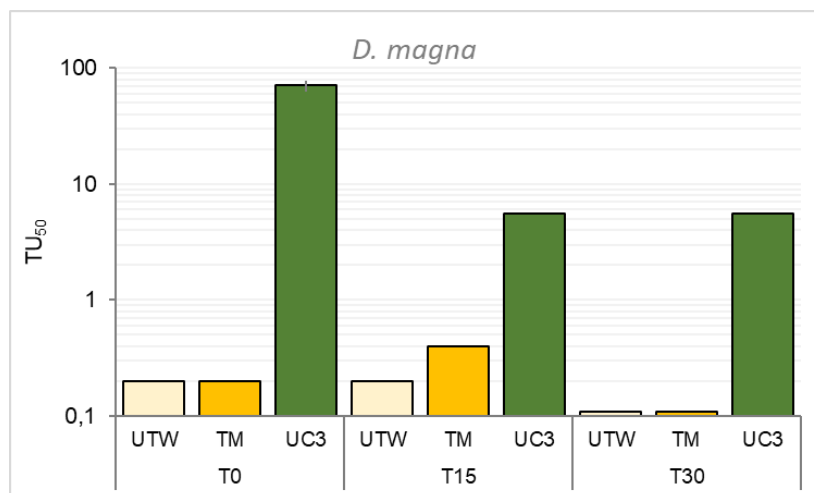


Figura 7. Tossicità dei lisciviati nei confronti di *D. magna*. Le barre di errore rappresentano i limiti di confidenza al 95% relativi alla TU50 media calcolata per le 3 repliche sperimentali.

In sintesi, il TIER I ha evidenziato come il solo TM abbia fornito effetti tossici paragonabili, se non addirittura inferiori, al legno non trattato (UTW). Al contrario, UC3 ha esercitato forti effetti acuti su batteri, alghe e crostacei indipendentemente dal tempo di maturazione. Inoltre, gli effetti esercitati da UC3 sono risultati in tutte le condizioni sperimentate nettamente superiori a quelli osservati per UTW, con l'unica eccezione a T0 per il test Microtox, condizione in cui UTW è risultato leggermente più tossico di UC3.

Di conseguenza, secondo i criteri adottati per la procedura ITS, UC3 non risulta un trattamento compatibile per l'ambiente acquatico, a causa dell'elevata tossicità acuta generata. Nel caso, ad esempio, del test con *D. magna*, i risultati ottenuti con i lisciviati prodotti dai provini UC3 eccedono addirittura la mortalità massima stabilita dalla legge

italiana per scarichi in acque superficiali, che non deve eccedere il 50%. Al contrario, i risultati ottenuti con TM lo rendono eleggibile per la valutazione di livello II.

### 3.2 TIER II

Tutti gli embrioni di *L. auricularia* esposti al lisciviato ottenuto da provini di legno non trattato (UTW) non sottoposto a maturazione (T0) sono morti; quindi, sia la formazione del guscio che la rottura della capsula non sono state valutate a T0 per UTW. Al contrario, dopo la maturazione in acquario, UTW non genera effetti negativi sulla formazione del guscio, così come effetti non sono stati osservati dopo l'esposizione ai lisciviati ottenuti da TM (Kruskal-Wallis ANOVA:  $H = 4,225$ ,  $p = 0,513$ ) (Figura 8).

L'endpoint rottura della capsula ha invece fornito risposte più variabili ed evidenziato differenze significative tra i trattamenti (Kruskal-Wallis ANOVA:  $H = 12,28$ ,  $p = 0,031$ ) (Figura 9). Per quanto riguarda UTW, l'aumento del tempo di maturazione ha generato un aumento significativo della percentuale di chioccioline che si sono schiuse dalla capsula dell'uovo da  $5,3 \pm 4,0\%$  a T15 a  $69,1 \pm 25,3\%$  a T30. Al contrario, la rottura della capsula ha mostrato un andamento inaspettato a forma di U per TM: la percentuale di chioccioline schiuse era equivalente al controllo negativo a T0 ( $85,0 \pm 7,6\%$ ), è scesa allo 0% a T15 ed è successivamente cresciuta a  $65,0 \pm 13,3\%$  a T30.

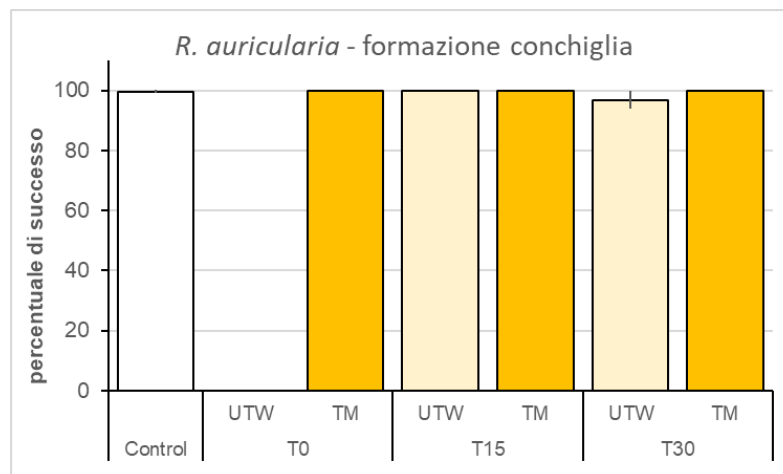


Figura 8. Percentuale di individui che hanno formato la conchiglia dopo 8 giorni di esposizione ai lisciviati. Le barre di errore rappresentano la deviazione standard delle repliche sperimentali.

Rispetto a UTW, i lisciviati ottenuti da TM hanno mostrato una percentuale di larve con formazione del guscio comparabile sia a T15 sia a T30, mentre la schiusa dalla capsula è risultata equivalente tra i due trattamenti solo a T30 ( $69,1 \pm 25,3\%$  e  $65,0 \pm 13,3\%$  rispettivamente per UTW e TM) (Mann-Whitney pairwise comparison test:  $U = 3,00$ ,  $p = 0,486$ ). Pertanto, il trattamento TM è stato considerato idoneo per la valutazione di livello III. Tuttavia, la tendenza a forma di U osservata per l'endpoint rottura della capsula ha evidenziato la necessità di un periodo di maturazione superiore a 15 giorni per rimuovere gli effetti tossici del lisciviato sullo sviluppo larvale di *L. auricularia*.

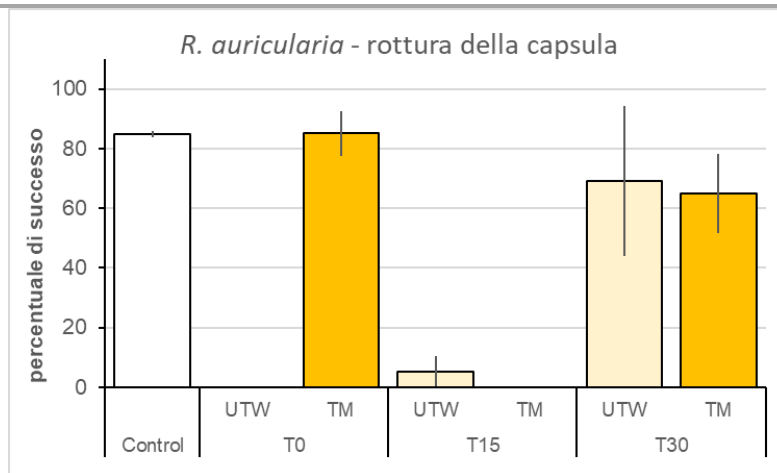


Figura 9. Percentuale di individui che sono usciti dalla capsula dopo 13 giorni di esposizione ai lisciviati. Le barre di errore rappresentano la deviazione standard delle repliche sperimentali.

### 3.3 TIER III

Il test cronico con *D. magna* ha confermato la necessità di un periodo di maturazione per eliminare gli effetti tossici dei lisciviati. A T0, tutti i cladoceri sono morti e non è stata osservata produzione di prole in UTW e TM, a fronte di una sopravvivenza del 90% ed una produzione prole di  $99,4 \pm 14,8$  individui per femmina osservata nel controllo negativo. La sopravvivenza è aumentata con il tempo di maturazione sia per UTW (70% a T15 e T30) che per TM (80% a T15 e T30). Allo stesso modo, la produzione di prole a T15 e T30 ha raggiunto livelli paragonabili o addirittura superiori ai criteri di accettabilità per buone prestazioni di controllo, vale a dire una media di 60 figli per femmina (Figura 10). Tuttavia, nessuno dei trattamenti testati si è avvicinato alla produzione di prole osservata nel controllo negativo (ANOVA a una via:  $F = 9,775$ ,  $p < 0,001$ ). A T15, TM ha fornito una produzione di prole leggermente superiore rispetto a UTW ( $75,5 \pm 13,7$  in TM;  $56,5 \pm 18,7$  in UTW), mentre a T30, i due trattamenti sono equivalenti ( $62,6 \pm 12,9$  e  $61,9 \pm 16,6$  rispettivamente per TM e UTW). Di conseguenza, non sono state osservate differenze significative tra UTW e TM a T15 (test di Tukey:  $Q = 3,393$ ,  $p = 0,142$ ) e T30 (test di Tukey:  $Q = 0,137$ ,  $p = 0,999$ ).

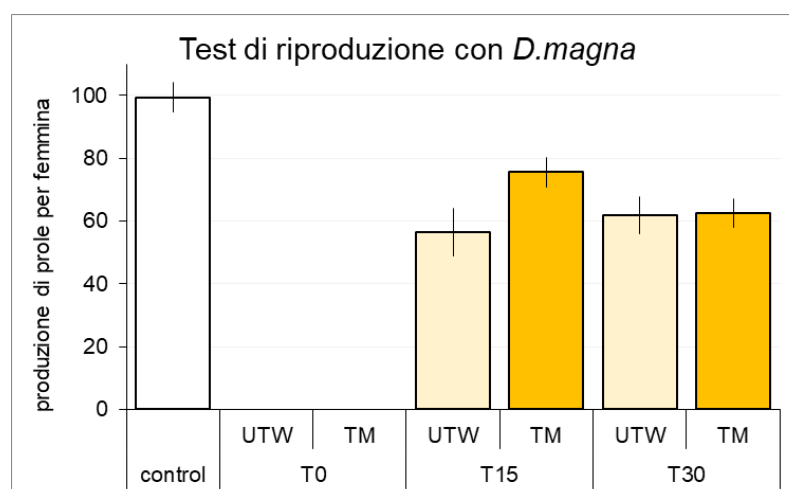


Figura 10. Numero di individui prodotti per femmina di *D. magna* durante il periodo di esposizione ai lisciviati (30 giorni). Le

barre di errore identificano l'errore standard.

Il test cronico con *L. auricularia* ha confermato l'esito del test a lungo termine con *D. magna*. L'incremento giornaliero in peso per UTW e TM è stato inferiore rispetto al controllo negativo (4,2 mg d<sup>-1</sup>) in tutte le condizioni saggiate (Figura 11) e i tassi di crescita più elevati sono stati misurati a T15 (2,3 mg d<sup>-1</sup> e 3,1 mg d<sup>-1</sup> rispettivamente per UTW e TM). Tuttavia, le analisi statistiche non hanno evidenziato alcuna differenza significativa tra i controlli negativi e il trattamento (ANOVA ad una via:  $F = 1,784$ ,  $p = 0,174$ ).

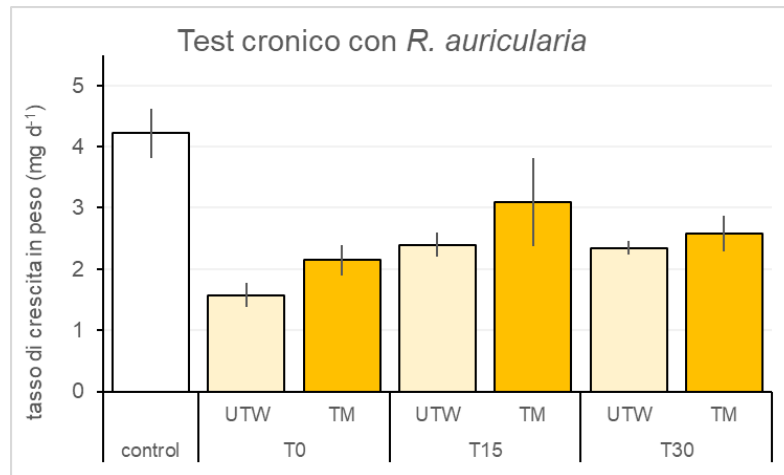


Figura 11. Crescita di *Lymnea (Radix) auricularia* espresso come incremento giornaliero in peso nel corso dei 21 giorni di esposizione. Le barre di errore identificano l'errore standard

Allo stesso modo, anche l'incremento giornaliero della lunghezza è stato inferiore in tutti i trattamenti rispetto al controllo negativo (105,7±44,7 μm d<sup>-1</sup>) e sia UTW che TM hanno fornito una curva dell'effetto a forma di cupola (Figura 12), con il valore più alto del tasso di crescita misurato a T15 (94,3 ± 43,7 μm d<sup>-1</sup> e 85,5 ± 10,9 μm d<sup>-1</sup> per UTW e TM, rispettivamente). Tuttavia, non è stata osservata alcuna differenza significativa tra controllo negativo e lisciviati (ANOVA a una via:  $F = 0,558$ ,  $p = 0,756$ ).

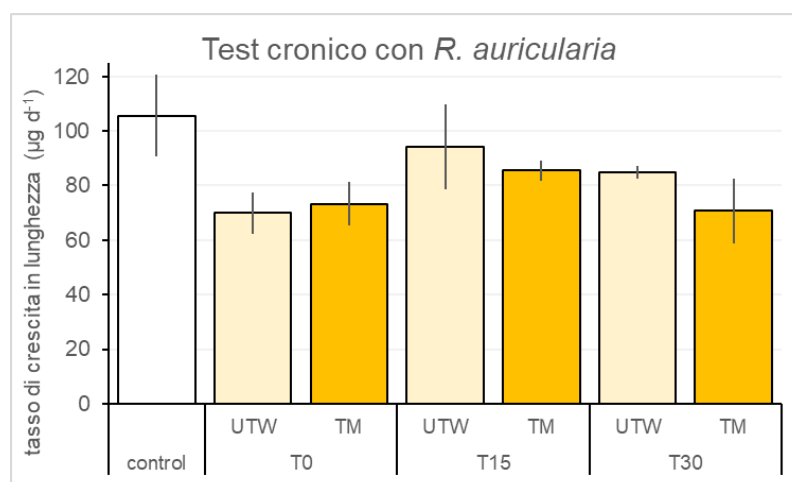


Figura 12. Crescita di *Lymnea (Radix) auricularia* espresso come incremento giornaliero in lunghezza nel corso dei 21 giorni di esposizione. Le barre di errore identificano l'errore standard.

Considerati i risultati del Tier III, il trattamento TM risulta complessivamente equivalente, in termini di impatti sugli indicatori acquatici, al legno non trattato.



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Emerge tuttavia chiaramente come sia necessario un periodo di maturazione di almeno 15 giorni prima dell'impiego del materiale, per limitare gli impatti sull'ambiente. Ai sensi della valutazione ITS proposta in questo progetto, quindi, il trattamento TM risulta sicuro e non comporta rischi aggiuntivi per l'ambiente, neanche dopo un'esposizione a lungo termine.

## CONCLUSIONI

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L'applicazione dei test di TIER I ha evidenziato come sulla base dei criteri adottati per la procedura di valutazione ITS predisposta per le acque dolci solo TM abbia fornito effetti tossici paragonabili, se non addirittura inferiori, al legno non trattato (UTW). UC3 al contrario non risulta un trattamento compatibile per l'ambiente acquatico, a causa dell'elevata tossicità acuta generata su batteri, alghe e crostacei indipendentemente dal tempo di maturazione. I risultati ottenuti in questo primo livello di valutazione hanno evidenziato pertanto come solo TM risulti eleggibile per il secondo step valutativo. Alla luce dei risultati ottenuti nel TIER II è stato possibile considerare il trattamento TM idoneo anche per la valutazione di livello III. Tuttavia gli effetti generati anche dal lisciviato del provino non trattato (UTW) nel caso dell'endpoint rottura della capsula nel gasteropode *L. auricularia* hanno evidenziato la necessità di un periodo di maturazione superiore a 15 giorni per rimuovere gli effetti tossici del lisciviato sullo sviluppo larvale. Considerati i risultati complessivi dei test cronici su molluschi e crostacei del Tier III, il trattamento TM risulta equivalente in termini di impatti su entrambi gli indicatori acquatici impiegati al legno non trattato (UTW). Emerge tuttavia chiaramente dai dati sperimentali, in particolare quelli ottenuti con il test sulla riproduzione in *D. magna*, come sia necessario un periodo di maturazione di almeno 15 giorni prima dell'impiego del materiale, per limitare gli impatti sull'ambiente dulciacquatico. Ai sensi della valutazione ITS proposta in questo progetto, quindi, il trattamento TM risulta ecompatibile non comportando rischi aggiuntivi per l'ambiente, neanche dopo un'esposizione a lungo termine.

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## ATTIVITÀ 15 /ACTIVITY 15

### Allegato 1. Analisi bibliografica relativa alle procedure di lisciviazione / Attachment 1. Bibliographic analysis related to leaching procedures

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Oak wood treated with commercial copper-based antifouling paint.	Tap water, estuarine water, and seawater	Wood was cut into 3 × 1 × 1 cm mini-bars and mini-bars were pre-treated and painted. Painted mini-bars were allowed to dry for 6 h, 24 h or 7 d, and then submerged in water. Each mini-bar was weighed before and after painting to determine mass of paint applied.	Wood mini-bars were submerged in 20mL of water and were kept in conditions simulating natural surface waters: cool white fluorescent lights (14:10 light:dark, 80–100 μmol/m <sup>2</sup> -s) at 20 °C with shaking (125 rpm). Release of Cu from wood and aluminum mini-bars was monitored as a time series for 180 d.		Adeleye A. S., Oranu E. A., Tao M., Keller A. A., 2016, <i>Release and detection of nanosized copper from a commercial antifouling paint</i> , Water Research, 102, 374-382.
Leachate from natural wood shavings from sawmill	Rainwater		Leachate was collected from within the heaps of wood shavings (Figure 1C) at five different points in the sawmill into a clean transparent 25 L plastic container to give homogenous mixture from the site.	Sieved (0.7 mm sieve) to remove wood debris.	Bakare A. A., Alimba C. G., Hassan T., Ajayi E. O., Sowunmi A. A., 2017, <i>Leachates biological treatment, toxicological characteristics and potential impacts on the environment</i> , Environmental research advances, 4.
Blocks wood treated with Chromated Copper Arsenate (CCA)	Synthetic Precipitation Leaching Procedure (SPLP) was used as the synthetic rainwater.		The wood was cut into 2 × 2 in blocks and extracted for 18 hrs with SPLP solution (pH=4.2) using a large mechanical rotator.	The resulting leachate was transferred to a pressure filtration apparatus and the solution was filtered through an acid-rinsed (20% HNO <sub>3</sub> ) filter (0.7 μm pore size).	Dagan R., Bitton G., Townsend T. G., 2006, <i>Metal Transport and Bioavailability in Soil Contaminated with CCA-Treated Wood Leachates</i> , Soil & Sediment Contamination, 15:1, 61-72.
Blocks of southern yellow pine treated with alkaline copper quaternary (ACQ) and copper boron azole (CBA), along with chromated copper arsenate (CCA)- treated wood	Eight natural waters: two rivers, three lakes, two wetlands, and the Atlantic Ocean. Deionized water and moderately hard water	Blocks of approximately 80 g (average surface area 140-150 cm <sup>2</sup> ) were obtained by cutting pieces of treated dimensional lumber using a power saw. Sawdust samples were collected for each treated wood type for subsequent total Cu measurements.	Immersion of the sample block (80 g block in this experiment) for 24 h within 1.6 L of leaching solution, resulting in a liquid to solid ratio (L/S) of 20:1.		Dubey B., Townsend T., Solo-Gabriele H., Bitton G., 2007, <i>Impact of Surface Water Conditions on Preservative Leaching and Aquatic Toxicity from Treated Wood Products</i> , Environmental Science Technology, 41, 3781-3786.
Leachate/runoff from wood recycling facilities	Rainwater		Samples of solid wood mulch and leachate/runoff were collected from three Class B wood recycling facilities in New Jersey over a 1.5 year period. A total of 26 leachate/runoff samples were collected either during or within 24 h of the occurrence of different rainfall events.		Kannepalli S., Strom P. F., Krogmann U., Subroy V., Gimenez D., Miskewitz R., 2016, <i>Characterization of wood mulch and leachate/runoff from three wood recycling facilities</i> , Journal of Environmental Management 182, 421-428

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Leachate from wood pressure-treated with micronized copper azole	Rainwater	Two pieces of lumber (originally 8' × 3.5" × 1.5" in size) were acquired and cut into three sizes: large (5 × 8,9 × 3,8 cm), medium (5 × 5,8 × 3,8 cm), and small (5 × 3 × 3,8 cm). All samples were weighed, and their exact physical dimensions measured.	Two Teflon straps (purchased from Industrial Netting), were affixed to each sample so that the samples could be secured in place over a rain-water runoff collection jar, thereby preventing sample soaking in accumulated rainwater. Samples were affixed such that the grain of the wood was perpendicular to incoming rainfall (top face exposed surface area 1 cm <sup>2</sup> ).		Lankone, R. , Goodwin, D. , Sung, L. , Ruggiero, E. , Vilsmeier, K. , Mueller, P. , Pulbere, S. , Challis, K. , Bi, Y. , Westerhoff, P. , Ranville, J. , Fairbrother, D. and Wohlleben, W., 2019, <i>Evaluating performance, degradation, and release behavior of a nanoform pigmented coating after natural and accelerated weathering</i> , NanoImpact
Wood spruce ( <i>Picea</i> sp.) treated with chromated copper arsenate (CCA) and alkaline copper quaternary (ACQ)	Synthetic Rainwater	<i>Picea</i> sp. lumber about 5 × 10 cm was treated for two weeks with ACQ or CCA following industry standards. The wood was shredded into small chips (about 0,2 × 0,2 × 0,2 cm) and approximately 0.6 kg of each wood type was placed into separate 20 L acid-washed glass containers.	Synthetic rain water was added about 6 L to each container to achieve a mixture with 10% (w/v) of wood:synthetic rainwater. The mixture was stirred to inundate all wood chips and incubated at 20 °C for 4 days.	Wood leachates were filtered (<0.2 µm under vacuum) and stored in acid-washed glass containers.	Leduc F., Whalena J. K., Sunaharab G. I., 2008, <i>Growth and reproduction of the earthworm Eisenia fetida after exposure to leachate from wood preservatives</i> , Ecotoxicology and Environmental Safety 69, 219–226
Natural wood: <i>Pouteria guianensis</i> , <i>Minquartia guianensis</i> , <i>Eschweilera</i> spp, <i>Quercus</i> spp, <i>Picea abies</i> .	Aerated artificial sea water	A predetermined ratio was maintained between wood block surface area (As) and water leaching solution volume (Vls), corresponding to the immersion of a wood block of 0.1m×0.1m×0.1m in 1 L of water (As/Vls = 0.6 cm <sup>-1</sup> ). The blocks were cut to the required dimensions (3 × 3 × 6,5 cm) with a circular saw.	The leaching procedure consisted of agitating the blocks fixed to a Jar Test at 60 rpm at 20 °C in a leaching medium consisting of aerated artificial sea water. The first cycle lasted 24 h, the leaching fluid was then completely renewed and the leaching procedure continued for a further 48 h (72 h cycle)	pH values below 7.5 were corrected by adding NaOH 0.1M aliquots	Libralato G., Losso C., Ghirardini A. V., 2007, <i>Toxicity of untreated wood leachates towards two saltwater organisms (Crassostrea gigas and Artemia franciscana)</i> , Journal of Hazardous Materials 144, 590–593
Wood chips of <i>Pinus resinosa</i> treated with pentachlorophenol (CBA);	sodium hydroxide solution (pH 10).	Wood chips were made from circular slices of Pentachlorophenol-Treated out-of-service poles. The approximate dimensions of the wood chips is longitudinal x radial x tangential = 5 x 0.5 x 0.5 cm.	A stock leachate solution was produced batchwise by recirculating a sodium hydroxide solution (pH 10) through a cylindrical plexiglass column (8.7 cm i.d. and 100 cm length) that was packed with PCP-treated wood chips. The pH of the leachate was controlled and the temperature was controlled at 90°C. The sodium hydroxide solution was recirculated for 48 h.		Mollah A. H., Allen D. G., 1999, <i>Biodegradation and Detoxification of Wood Leachate from Pentachlorophenol-Treated Poles</i> , The Canadian Journal of Chemical Engineering 77.
Blocks wood of <i>P. sylvestris</i> , <i>P. radiata</i> , <i>F. sylvatica</i> , <i>F. excelsior</i> , Southern yellow pine tretated with furfurylation process. Reference with chromated copper arsenate (CCA).	Demineralized water	After treatment the boards were cut into samples with dimensions 0,15x0,25x0,50cm for EN84 and Internal leaching procedure and 0,15x0,70x1,5cm for OECD procedure.	EN84: 10 water changes during 14 d in demineralized water with a water-over-wood volume equaling 5. Leaching water was collected after 1, 7, and 14 d. Internal leaching procedure: The specimens were submersed in demineralized water for 48 h with water changes and collection of leaching water after 0.5, 1, 6, 12, 24, and 48 h.  OECD Guideline 313: recommended ratio (0.4 cm <sup>2</sup> /ml) between the surface of the wood and the amount of water. The submersion test was continued for 32 d with demineralized water, during which the water was replaced eight times at set intervals. The leachates were collected after 0.25, 1, 2, 4, 6, 8, 15, 22, and 32d..	pH values below 7.5 were corrected by adding NaOH 0.5M aliquots	Pilgard A., Treu A., Zeeland V., Gosselink J. A., Westin M., 2010, <i>Toxic hazard and chemical analysis of leachates from furfurylated wood</i> , Environmental Toxicology and Chemistry 29, 1918–1924.

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Blocks wood of <i>Pinus radiata</i> , <i>Pinus sylvestris</i> treated with three different furfurylation process. Untreated <i>P. sylvestris</i> and <i>P. radiata</i> sapwood were used as reference material.	Demineralized water	Test specimens were larger than the standard recommendations and therefore have been cut at 0,15x0,70x1,5cm . The <i>P. radiata</i> material was planed prior to treatment, because the wood surface was suspected to have been treated with biocides (antisap-stain agents).	OECD Guideline 313: recommended ratio (0.4 cm <sup>2</sup> /ml) between the surface of the wood and the amount of water. The submersion test was continued for 32 d with demineralized water, during which the water was replaced eight times at set intervals. The leachates were collected after 0.25, 1, 2, 4, 6, 8, 15, 22, and 32d. The shower test: method was used to simulate rainfall. Every day, the specimens were sprayed with approximately 2 liters of freshwater over a time span of 1 h. This test was run for 2 weeks, and leachates were collected every day over a period of 10 d.	pH values below 7.5 were corrected by adding NaOH 0.1M aliquots	Pilgard A., Vetter L., Acker J.,z and Westin M., 2010b, <i>Toxic hazard of leachates from furfurylated wood: comparison between two different aquatic organisms</i> , Environmental Toxicology and Chemistry 29, 1067–1071.
Natural wood: <i>Populus tremuloides</i> , <i>Pinus contorta</i> , <i>Picea engelmannii</i> x <i>glauca</i> Parry, <i>Picea mariana</i> . As well as from two wood chipping sites using mixes of lodgepole pine and hybrid or black spruce.	1) Natural weather conditions 2) deionized water 3) deionized water		1) Tree species common to the Prince George Forest District mixed in two open 322-L containers at environmental condition for 23 month; 2)The static exposure consisted of placing 2 kg of wood chips in a polypropylene 1-cm opening mesh bag in 18 L of water for 28 days at room temperature and ambient light (9:1 water:wood). 3)A portable rainfall simulator was used to generate a heavy rainfall event of approximately 100 mm x h-1.		Rex J., Dubé S., Krauskopf P., Berch S., 2016, <i>Investigating Potential Toxicity of Leachate from Wood Chip Piles Generated by Roadside Biomass Operations</i> , Forests.
Natural wood: birch ( <i>Betula sp.</i> ) and spruce ( <i>Picea abies</i> ) woodchip, and their mixture with washed gravel (WG).	Tap water		Pure organic and mixed filling materials were used in one-stage batch leaching tests with a liquid to solid ratio of 10l·kg <sup>-1</sup> without size reduction. Using the calculated dry matter content of each material determined at the beginning, the equivalent mass of raw material (without any pre-treatment) was put into the 2l reagent bottles and filled with 1.6l of tap water. Reagent bottles were placed on the overhead shaker and mixed for 1–5 days at laboratory temperature with 5 RPM.	One sample of each material was decanted, centrifuged, and filtered using membrane paper (4 µm) on a daily basis.	Šereš Z., Došenović T., Šoronja-Simović D., Maravić N., Šaranović Z., Šereš L., Šćiban M., Djordjević M., 2019, <i>Modelling of cross-flow microfiltration coupled with bentonite treatment in sugar beet molasses purification</i> , The Canadian Journal of Chemical Engineering 97, 947-954.
Block of southern yellow pine treated with alkaline copper quaternary (ACQ), copper boron azole (CBA) and copper citrate (CC).	1) Toxicity Characteristic Leaching Procedure (TCLP) fluid 2) Synthetic Precipitation Leaching Procedure (SPLP) fluid, 3) deionized water, 4) synthetic seawater.	The samples used for the leaching analysis were cut into small blocks (2.5x5x10 cm) using a mill saw and then ground to particles less than 3 mm in diameter.	Treated wood samples were individually leached with four leaching fluids. Two liters of leaching fluids were combined with 100 g of ground wood (yielding a 1:20 solid/liquid ratio) in a glass container which was capped with a teflon-lined lid and placed on a rotary extractor for 18 ±2 hours.	The recovered leachates were filtered using a pressurized filtration apparatus with a 0,7 0 µm glass fiber filter.	Stook K., Dubey B., Ward M., Townsend T., Bitton G., Solo-Gabriele H., 2004, <i>Heavy Metal Toxicity of Pressure Treated Wood Leachates with MetPLATETM</i> , Bulletin Environmental Contamination and Toxicology 73, 987–994.

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Block of southern yellow pine treated with preservatives included chromated copper arsenate (CCA), alkaline copper quaternary (ACQ), copper boron azole (CBA), copper citrate (CC), and copper dimethyldithiocarbamate .	1) Toxicity Characteristic Leaching Procedure (TCLP) fluid 2) Synthetic Precipitation Leaching Procedure (SPLP) fluid, 3) deionized water, 4) synthetic seawater.	The samples used for the leaching analysis were cut into small blocks (2.5x5x10 cm) using a mill saw and then ground to particles less than 3 mm in diameter.	1)A 100-g, size-reduced, representative wood sample was placed into a 2.2-L Teflon-lined glass container. Two liters of the TCLP extraction fluid was added and the mixture was rotated for 18 (2 h). Other leaching tests were performed in the same manner as the TCLP, with the exception of the leaching fluid. The synthetic precipitation leaching procedure contains nitric and sulfuric acid at a 40/60 weight ratio. The synthetic seawater extraction solution was prepared by dissolving 0.5 cups of Instant Ocean in 3.8-L of deionized water; the resulting salinity of the solution was 3.2 psu.	The leachate was filtered through a 0.7-µm glass fiber filter	Stook K., Tolaymat T., Ward M., Dubey B., Townsend T., Solo-Gabriele H., Bitton G., 2005, <i>Relative Leaching and Aquatic Toxicity of Pressure-Treated Wood Products Using Batch Leaching Tests</i> , Environmental Science and Technology 39, 155-163.
Natural Sawdust and bark from: Pedunculate oak ( <i>Quercus robur</i> ), Scots pine ( <i>Pinus sylvestris</i> ), European larch ( <i>Larix decidua</i> ), Norway spruce ( <i>Picea abies</i> ) and European beech ( <i>Fagus sylvatica</i> )	Distilled water	The sawdust and the bark were obtained from sawmills located in Nybro, southern Sweden.	The leachate was produced according to the batch leaching Standard Method SS-EN 12457-2, Due to the very high water absorbing capacity of sawdust, which makes very difficult to run a leaching test with the ratio 10:1, a liquid to solid (L/S) ratio (40:1) four times the one recommended (10:1) was used. Leachate from bark of oak, pine and spruce where produced according to the same batch test method mentioned above. However, in these cases, the recommended L/S ratio of 10:1 was used.		Svensson H., Svensson B., Hogland W., Marques M., 2012, <i>Acute Toxic Effects Caused by Leachate from Five Different Tree Species on Artemia Salina and Vibrio Fischeri</i> , Journal of Biobased Materials and Bioenergy 6, 214–220.
1) water from pond that receives oak wood leachate generated due to log irrigation; 2) water from ditch that receives leachate generated due to rainfall contact with oak wood chip piles; 3) leachate generated via leaching test with oak sawdust under laboratory conditions.	1) irrigation water; 2) rainfall; 3) Distilled water	Vacuum filtration: filter paper grade 005 (20 µm), Munktell 00R (10 µm), and Watchman GF/C (1.2 µm); Next, with Millipore stirred Cell Model 8400 ultra-filtration discs Millipore 100 kDa (approximately 13 nm), 10 kDa (approximately 5 nm) and 1 kDa (approximately 2 nm). After each filtration step, 50 mL of water was removed and used for chemical analyses.	The leachate was produced according to the batch leaching Standard Method SS-EN 12457-2, Due to the very high water absorbing capacity of sawdust, which makes very difficult to run a leaching test with the ratio 10:1, a liquid to solid (L/S) ratio (40:1) four times the one recommended (10:1) was used. Leachate from bark of oak, pine and spruce where produced according to the same batch test method mentioned above. However, in these cases, the recommended L/S ratio of 10:1 was used.		Svensson H., Jani Y., Hogland W., Marques M., 2014, <i>Particle size characterization of oak wood leachate: chemical oxygen demand and toxicity distribution within different fractions</i> , Water Science & Technology 70.3
Natural sawdust obtained from oak ( <i>Quercus robur</i> ), maple ( <i>Acer platanoides</i> ), pine ( <i>Pinus sylvestris</i> ), beech ( <i>Fagus sylvatica</i> ) and wood chips from oak and pine.	Distilled water	Sawdust is wood debris with particles size 4 mm. Wood chips are larger than 4 mm and produced using a chipper that grinds wood to sizes up to 10 cm in diameter. 9 mg of sieved sawdust (particles 4 mm) were used to estimate the moisture content in the wood residues. The sawdust was dried in an oven for 24 h at 105°C.	The sawdust used in the leaching test was not dried in the oven, but the moisture content previously measured was taking into account for calculations purposes, as described in the Standard Method SS-EN 12457-2. Because of the very high absorption capacity of sawdust, the solid/liquid (S/L) ratio of 1 : 10 recommended by the Standard Method SS-EN 12457-2 after a preliminary trial was considered inappropriate, and S/L ratios of 1 : 20 (9 mg for 180 mL) and 1 : 40 (9 mg for 360 mL) were used instead in the batch tests.	The water phase was filtrated with a GF/C Whatman 0,45 µm microfiber filter	Svensson H., Marques M., Kaczala F., Hogland W., 2014, <i>Leaching patterns from wood of different tree species and environmental implications related to wood storage areas</i> , Water and Environment Journal.

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Natural chips wood of aspen ( <i>Populus tremuloides</i> )	Dechlorinated tap water	Five young healthy trees, ranging 28 to 64 years in age and 9.7 to 15.7 cm butt diameter (mean 13.6 cm), were cut at ground level with a chain saw. Trees were topped, limbed, and cut into 2.5-m (8-ft) lengths in the field and then reduced to chips (0.5 to 10 cm in length) with a commercial chipper.	50 kg fresh weight (about 28 kg dry weight) of aspen chips in 250 L of water in a 280-L vat lined with polyethylene. The ratio of aspen to water was thus 8.9:1 by dry mass at room temperature. Leaching continued for 35 d at room temperature and until chemical and physical characteristics ceased to show rapid changes.	0.45 mm microfiber filter	Taylor B. R., Goudey J. S., Carmichael N. B., 1996, <i>Toxicity of aspen wood leachate to aquatic life: laboratory studies</i> , Environmental Toxicology and Chemistry 15, 150–159.
Leachate from natural woodpiles of trembling aspen ( <i>Populus tremuloides</i> )	Rainwater or snowmelt	The logs were transported to the field site, where they were stacked in two ranks, making a triangular logpile 5.5 m long and 1.5 m high. The logpile comprised 174 aspen logs with a total volume of 17.8 m <sup>3</sup> .	A polyethylene tarpaulin beneath the logpile, supported around the perimeter by more logs, created a basin (depth, 40 cm) that captured any runoff from the logpile. Part of the bottom row of logs could be half-submerged when the catchbasin was full.	Temperature of the air and leachate were measured and the specific conductance, pH, and dissolved oxygen tension of the leachate.	Taylor B. R., Carmichael N. B., 2003, <i>Toxicity and chemistry of aspen wood leachate to aquatic life: field study</i> , Environmental Toxicology and Chemistry 22, 2048–2056.
Natural wood with bark chipped from a mixture of conifer tree species including <i>Abies concolor</i> , <i>Pinus lambertiana</i> , <i>Calocedrus decurrens</i> , <i>Pinus jeffreyi</i> , <i>Abies magnifica</i> , and <i>Pinus contorta</i> .	Deionized water		Separate batches of wood-only chips and bark-only chips of the same Sierra Nevada mixed conifer tree species were soaked in water to create wood and bark leachates. The wood and bark were oven dried and 1.75 kg each were separately submerged in 14 L DI water for 6 days at 20°C.	The leachate liquids were poured through a 0.152-mm screen to remove solids prior to use as phytometer treatments.	Wolf E. C., Rejmánková E., Cooper D. J., 2019, Wood chip soil amendments in restored wetlands affect plant growth by reducing compaction and increasing dissolved phenolics, Restoration Ecology 27, 1128–1136.



**Allegato 2. Analisi bibliografica relativa alla caratterizzazione ecotossicologica e chimico-fisica dei lisciviati. / Attachment 2. Bibliographic analysis relating to the ecotoxicological and chemical-physical characterization of the leachates.**

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
Oak wood treated with commercial copper-based antifouling paint.	Total Cu, dissolved Cu, nanosized Cu, bulk Cu.		Cu <sub>2</sub> O particles in commercial antifouling paints may be released into natural waters during use. These released particles may include those that are nanoparticulate in size.		Adeleye A. S., Oranu E. A., Tao M., Keller A. A., 2016, <i>Release and detection of nanosized copper from a commercial antifouling paint</i> , Water Research, 102, 374-382.
Leachate from natural wood shavings from sawmill	COD, BOD, dissolved oxygen (DO), conductivity, chloride, sulphate, nitrate, pH, temperature, lignin and tannin, heavy metals: Pb, Cd, As, Cr Cu.	<i>C. gariepinus</i> , Sub-Lethal Concentrations and Micronucleus Analysis.	The concentrations of some of the SWL constituents were above permissible NESREA and USEPA limits	Leachate induced a time dependent acute toxicity and concentration-dependent significant increase in the frequencies of micronucleated peripheral erythrocytes and nuclear abnormalities in <i>C. gariepinus</i> .	Bakare A. A., Alimba C. G., Hassan T., Ajayi E. O., Sowunmi A. A., 2017, <i>Leachates biological treatment, toxicological characteristics and potential impacts on the environment</i> , Environmental Reserch Advances, 4.
Blocks wood treated with Chromated Copper Arsenate (CCA)	Metal Analysis, ecotoxicological test	<i>Ceriodaphnia dubia</i> , MetPLATE™, <i>Selenastrum capricornutum</i>	Arsenic has the highest concentration compared to the other two metals in all three types of soil. It appears, however, to have greater mobility in the sandy soil.	Despite the presence of copper, chromium and arsenic in elutriates collected from all three soil types, no toxicity was observed in any of the fractions collected from these columns.	Dagan R., Bitton G., Townsend T. G., 2006, <i>Metal Transport and Bioavailability in Soil Contaminated with CCA-Treated Wood Leachates</i> , Soil & Sediment Contamination, 15:1, 61-72.
Blocks of southern yellow pine (SYP) treated with alkaline copper quaternary (ACQ) and copper boron azole (CBA), along with chromated copper arsenate (CCA)- treated wood	pH, total dissolved solids (TDS), alkalinity, hardness, chloride, total organic carbon (TOC), chemical oxygen demand (COD), turbidity (in NTU). Cu, As, Cr, B concentrations, labile Cu.	MetPLATE	Results show that ACQ- and CBA-treated wood leach approximately 10 and 20 times more total Cu relative to CCA-treated wood and that the presence of organic and inorganic ligands in natural waters lowered the labile fraction of Cu relative to that from laboratory generated leaching solutions.	Lower toxicity was observed with <b>seawater</b> samples for each wood type compared with the other leachates. In general, among the other nine leachates for each wood treatment, toxicity followed the pattern CBA > ACQ > CCA. For all three treated wood leachates, the highest toxicity was observed from the DI leachate. This coincides with the relatively high labile copper concentrations associated with DI water.	Dubey B., Townsend T., Solo-Gabriele H., Bitton G., 2007, <i>Impact of Surface Water Conditions on Preservative Leaching and Aquatic Toxicity from Treated Wood Products</i> , Environmental Science Technology, 41, 3781-3786.
Leachate/runoff from wood recycling facilities	pH, BOD, COD, TSS, settleable solids, total Kjeldahl nitrogen (TKN), total phosphate-phosphorus (TP), tannin/lignin/phenolic (T/L/P) compounds, fecal coliforms, ecotoxicological test.	<i>D. rerio</i>	BOD, COD, and TSS concentrations were comparable to those of untreated domestic wastewater (raw sewage), although both lower and higher individual values were observed. TKN, TP and fecal coliform values were usually slightly lower than is typical for untreated domestic wastewater. BOD, COD, TSS, TKN, and TP median values were all much higher than for typical urban stormwater runoff, while fecal coliform levels were lower.	The developmental effects observed were yolk sac edema, abnormal spine curvature, kink tail, and delay in development. Only delay in development regularly occurred at lower concentrations. little acute toxicity other than the one case attributable to low pH was observed in the mulch leachate/runoff samples, and no correlation was found between delayed development and T/L/P.	Kannepalli S., Strom P. F., Krogmann U., Subroy V., Gimenez D., Miskewitz R., 2016, <i>Characterization of wood mulch and leachate/runoff from three wood recycling facilities</i> , Journal of Environmental Management 182, 421-428

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
Wood spruce ( <i>Picea</i> sp.) treated with chromated copper arsenate (CCA) and alkaline copper quaternary (ACQ)	Metal concentration with ICP-OES system, ecotoxicological test	<i>E. fetida</i>		We found that earthworm tissue concentrations of 80 mg As kg <sup>-1</sup> , 89 mg Cr kg <sup>-1</sup> and 90 mg Cu kg <sup>-1</sup> were not lethal to <i>E. fetida</i> . There was Cu bioaccumulation in <i>E. fetida</i> exposed to soil contaminated with a single metal (Cu from ACQ-treated wood), but Cu was less bioavailable in alkaline than neutral soil conditions. Exposure to Cu alone did not affect <i>E. fetida</i> growth or reproduction, but exposure to multiple metals (As, Cr and Cu from CCA-treated wood) produced soil-specific growth and reproduction outcomes.	Leduc F., Whalena J. K., Sunaharab G. I., 2008, <i>Growth and reproduction of the earthworm Eisenia fetida after exposure to leachate from wood preservatives</i> , Ecotoxicology and Environmental Safety 69, 219–226
Natural wood: <i>Pouteria guianensis</i> , <i>Minuartia guianensis</i> , <i>Eschweilera</i> spp, <i>Quercus</i> spp, <i>Picea abies</i> .	pH values 3, 24 and 72 h, Dissolved oxygen (DO), chemical oxygen demand (COD), ecotoxicological test.	<i>Artemia franciscana</i> , <i>Crassostrea gigas</i>		Leachate toxicities showed that AB<MM≈RO<AC<PA at 24 h and AB≈AC≈MM≈RO< PA at 72 h. From 24 to 72 h, toxic effects were reduced by more than 50% for all samples except for PA.	Libralato G., Losso C., Ghirardini A. V., 2007, <i>Toxicity of untreated wood leachates towards two saltwater organisms (Crassostrea gigas and Artemia franciscana)</i> , Journal of Hazardous Materials 144, 590–593
Wood chips of <i>Pinus resinosa</i> treated with pentachlorophenol;	PCP concentration, chloride ion	<i>Daphnia magna</i>	Complete degradation of PCP was also achieved in the cases for 120 mg PCP/L and a 12 h cycle time or 50 mg PCPiL and a 6 h cycle time	<i>Daphnia magna</i> toxicity tests showed that the presence of PCP in leachate caused toxicity, and the biodegradation of PCP resulted in significantly reducing the effluent toxicity to a value similar to that of PCP-free nutrient solution.	Mollah A. H., Allen D. G., 1999, <i>Biodegradation and Detoxification of Wood Leachate from Pentachlorophenol-Treated Poles</i> , The Canadian Journal of Chemical Engineering 77.
Blocks wood of <i>P. sylvestris</i> , <i>P. radiata</i> , <i>F. sylvatica</i> , <i>F. excelsior</i> , Southern yellow pine treated with furfurylation process. Reference with chromated copper arsenate (CCA).	Furan compound analysis, Maleic acid analysis, Ecotoxicological test	<i>Daphnia magna</i> , <i>Vibrio fischeri</i> (Microtox®)	The present study shows that the difference in toxicity most likely cannot be attributed to maleic acid, furan, furfural, furfuryl alcohol, or 2-furoic acid. However, the difference could be caused by the two substances 5-hydroxymethylfurfural and 2,5-furandimethanol.	The leachates from furfurylated wood have low toxicity. Earlier studies have shown that leachates from wood treated with furfuryl alcohol prepolymers have higher toxicity to <i>Vibrio fischeri</i> than leachates from wood treated with furfuryl alcohol monomers.	Pilgard A., Vetter L., Acker J.,z and Westin M., 2010b, <i>Toxic hazard of leachates from furfurylated wood: comparison between two different aquatic organisms</i> , Environmental Toxicology and Chemistry 29, 1067–1071.
Blocks wood of <i>Pinus radiata</i> , <i>Pinus sylvestris</i> treated with three different furfurylation process. Untreated <i>P. sylvestris</i> and <i>P. radiata</i> sapwood were used as reference material.	Ecotoxicological test	<i>Daphnia magna</i> , <i>Vibrio fischeri</i> (Microtox®)		The results from the toxicity tests show that the bacteria were more sensitive than <i>D. magna</i> to furfurylated wood leachates. These results indicate that, with general furfurylated wood, leachates are not more toxic than untreated wood leachates, the only exception being <i>P. radiata</i> treated with treatment A.	Pilgard A., Vetter L., Acker J.,z and Westin M., 2010b, <i>Toxic hazard of leachates from furfurylated wood: comparison between two different aquatic organisms</i> , Environmental Toxicology and Chemistry 29, 1067–1071.

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
Natural wood: <i>Populus tremuloides</i> , <i>Pinus contorta</i> , <i>Picea engelmannii</i> x <i>glauca</i> Parry, <i>Picea mariana</i> . As well as from two wood chipping sites using mixes of lodgepole pine and hybrid or black spruce.	pH, True Color, Total Organic Carbon, Chemical Oxygen Demand, Ammonium, Resin Acids, Phenols, Microtox.	<i>Vibrio fischeri</i> (Microtox®)	Aspen chips produced the most acidic leachate with higher organic, phenolic, and ammonia concentrations compared to the coniferous and mixed samples. Coniferous samples showed some subtle differences with the spruce samples being more similar to each other than they were to pine.	All samples collected during the 580 days of exposure produced a toxic response within the 15 minute test period; all leachate samples exhibited a consistent toxicity response over the 4-week period, with each toxic sample at concentrations below 10% by volume. There was no significant difference in leachate toxicity between dry and wet exposure within tree species or between tree species. The dry chip Muldowan 18 leachate samples required a higher concentration to induce toxicity than the wet sample and all other samples.	Rex J., Dubé S., Krauskopf P., Berch S., 2016, <i>Investigating Potential Toxicity of Leachate from Wood Chip Piles Generated by Roadside Biomass Operations</i> , Forests.
Natural wood: birch ( <i>Betula sp</i> ) and spruce ( <i>Picea abies</i> ) woodchip, and their mixture with washed gravel (WG).	total organic carbon, dissolved organic carbon, phenol index.	<i>Daphnia magna</i> , <i>D. subspicatus</i> , <i>Lemna minor</i> , <i>Vibrio fischeri</i> (Microtox®)	Statistical analysis of the data showed no significant influence of the leaching period on the quality of effluent represented by concentrations of FNI, TOC, DOC and an inhibitory effect on aquatic organisms. The type of filling medium resulted in significantly different levels of the leachate quality, in terms of TOC, DOC and FNI. The pure organic material (BWCH and SWCH) resulted in a significantly higher concentration of FNI and organic carbon as well as higher inhibition of the tested aquatic organisms.		Šereš Z., Došenović T., Šoronja-Simović D., Maravić N., Šaranović Z., Šereš L., Šćiban M., Djordjević M., 2019, <i>Modelling of cross-flow microfiltration coupled with bentonite treatment in sugar beet molasses purification</i> , The Canadian Journal of Chemical Engineering 97, 947-954.
Block of southern yellow pine treated with alkaline copper quaternary, copper boron azole and copper citrate.		MetPLATE		Leachate from untreated southern yellow pine were found to be no-toxic to the MetPLATE test bacteria. The toxicity was greatest for CCA-treated wood. Higher EC50 values were calculated in the second part of the experience using larger wooden blocks.	Stook K., Dubey B., Ward M., Townsend T., Bitton G., Solo-Gabriele H., 2004, <i>Heavy Metal Toxicity of Pressure Treated Wood Leachates with MetPLATE™</i> , Bulletin Environmental Contamination and Toxicology 73, 987-994.
Block of southern yellow pine treated with preservatives included chromated copper arsenate, alkaline copper quaternary, copper boron azole, copper citrate, and copper dimethyldithiocarbamate.	Arsenic, chromium, and copper	<i>Ceriodaphnia dubia</i> , <i>Raphidocelis subcapitata</i> , <i>Vibrio fischeri</i> (Microtox®)	CCA-treated wood leached greater concentrations of arsenic and copper relative to chromium, with copper leaching more with the TCLP and synthetic seawater. Copper leached at greater concentrations from the arsenic-free preservatives relative to CCA.	All of the arsenic free alternatives displayed a greater degree of aquatic toxicity compared to CCA. Invertebrate and algal assays were more sensitive than Microtox.	Stook K., Tolaymat T., Ward M., Dubey B., Townsend T., Solo-Gabriele H., Bitton G., 2005, <i>Relative Leaching and Aquatic Toxicity of Pressure-Treated Wood Products Using Batch Leaching Tests</i> , Environmental Science and Technology 39, 155-163.
Natural Sawdust and bark from: Pedunculate oak ( <i>Quercus robur</i> ), Scots pine ( <i>Pinus sylvestris</i> ), European larch ( <i>Larix decidua</i> ), Norway spruce ( <i>Picea abies</i> ) and European beech ( <i>Fagus sylvatica</i> )	pH, TOC, phenolic compounds (reported as total poly-phenols)	<i>Artemia salina</i> , <i>Vibrio fischeri</i> (Microtox®)	The pH varied from very acid (for oak sawdust leachate pH 4.0) to neutral (for spruce sawdust leachate pH 7.2). Similar to TOC, within the same species, the phenols content was also significantly higher (p < 005) in bark than in sawdust regardless the tree species tested (oak, pine and spruce).	Microtox® test is more sensitive to all tested waters than the crustacean <i>A. salina</i> . Regarding <i>V. fischeri</i> , two of the tested sawdust leachates (pine and oak) and all three tested bark leachates (spruce, pine and bark) were highly toxic while minor toxic effects was observed for the leachate from other species (spruce, beech and larch sawdust).	Svensson H., Svensson B., Hogland W., Marques M., 2012, <i>Acute Toxic Effects Caused by Leachate from Five Different Tree Species on Artemia Salina and Vibrio Fischeri</i> , Journal of Biobased Materials and Bioenergy 6, 214-220.

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
1) water from pond that receives oak wood leachate generated due to log irrigation; 2) water from ditch that receives leachate generated due to rainfall contact with oak wood chip piles; 3) leachate generated via leaching test with oak sawdust under laboratory conditions.	pH, COD, total content of polyphenols (PP), ecotoxicological test	<i>Artemia salina</i> , <i>L. sativa</i> , <i>Vibrio fischeri</i> (Microtox®)	50% of the COD found in the samples collected in the field is in the 100 kDa fraction while only 10% of the COD is removed from the same water fraction after filtration of the laboratory leachate. The 100 kDa filter removes the largest amount of PP found in the field samples, while the amount of PP in the laboratory leachate is more evenly distributed along the fractions.	Only FSP 2 and LAB-L were toxic to <i>A. salina</i> and <i>V. fischeri</i> . No sample had a toxic effect on <i>L. sativa</i> germination. The toxic effect drops significantly for wood chips after the 100 kDa (over to) filter in both Microtox and Artemia tests, whereas the drop is not that significant for leachate water.	Svensson H., Jani Y., Hogland W., Marques M., 2014, <i>Particle size characterization of oak wood leachate: chemical oxygen demand and toxicity distribution within different fractions</i> , Water Science & Technology 70.3
Natural sawdust obtained from oak ( <i>Quercus robur</i> ), maple ( <i>Acer platanoides</i> ), pine ( <i>Pinus sylvestris</i> ), beech ( <i>Fagus sylvatica</i> ) and wood chips from oak and pine.	DOC, pH, conductivity, colour, phenols, tannins and lignin, biochemical oxygen demand (BOD).		The oak chips batch test released DOC up to 312 h of contact time when equilibrium was apparently reached. Based on sawdust leaching tests obtained from maple, oak, pine and beech, the organic compounds measured as DOC were mainly released during the first 24 h and the additional DOC release remains for up to 216 h of contact time. For pine sawdust, the DOC released was six times higher than that released from pine wood chips. For oak sawdust, the DOC released was 16 times higher than for oak wood chips. The pH range ranged from 4.1 (oak) to 6.8 (maple). Oak leachate color was found to be 8.7 and 15.2 times the color measured for pine, respectively. BOD7 / DOC ranged from 12 to 28% of the COD. Concentrations of phenol range from 12 to 77 mg / L. Unlike the total leaching of DOC per unit of dry matter, the DOC concentration (mg / L) in the aqueous phase is influenced by the S / L ratio. The DOC concentration in the leachate after a batch test decreased to approximately 40% after the second wash compared to unwashed sawdust.		Svensson H., Marques M., Kaczala F., Hogland W., 2014, <i>Leaching patterns from wood of different tree species and environmental implications related to wood storage areas</i> , Water and Environment Journal.
	Microtox, conductance, pH, DO, and absorbance (465 nm), 5-day BOD, COD, color, resin and fatty acids, nitrogen, total phenols, total organic carbon	<i>Daphnia magna</i> , <i>Oncorhynchus mykiss</i> , <i>Vibrio fischeri</i> (Microtox®)	The analyzes revealed an amber color, low pH (4.0), extremely high BOD (0.2.600 mg / L) and high conductivity (1140 mS / cm). The leachate was rich in phenols (30 mg / l), organic carbon (2,480 mg / l) and organic nitrogen (13 mg / l).	Median acutely toxic concentrations of leachate were consistently 1 to 2% of full strength for trout and <i>Daphnia</i> . Inhibition of bacterial metabolism began at concentrations below 0.3%. Leachate was less toxic to plant life but inhibited algal growth at concentrations of 12 to 16%.	Taylor B. R., Goudey J. S., Carmichael N. B., 1996, <i>Toxicity of aspen wood leachate to aquatic life: laboratory studies</i> , Environmental Toxicology and Chemistry 15, 150–159.
Leachate from natural woodpiles of trembling aspen ( <i>Populus tremuloides</i> )	pH, conductivity Color, BOD, COD, Phenols, TOC, Toxicity index	<i>Daphnia magna</i> , <i>Oncorhynchus mykiss</i> , <i>Vibrio fischeri</i> (Microtox®)	Aspen leachate was characterized by dark color, acid pH (5.0–6.5), high conductivity (200–500 mS / cm), high to very high BOD (500–5,000 mg / L) and concentrations of TOC (500–2,000 mg / L), varying levels of phenolic compounds (2–27 mg / L) and low dissolved oxygen voltages (.2 mg / L).	The leachate varied from weakly toxic (mean EC50 or LC50 . 10%) to very toxic (mean EC50 or LC50 , 1%). Leachate was produced in every season for two years and was chemically and toxicologically similar to that produced from wood chips in the laboratory (vedi Taylor, 1996)	Taylor B. R., Carmichael N. B., 2003, <i>Toxicity and chemistry of aspen wood leachate to aquatic life: field study</i> , Environmental Toxicology and Chemistry 22, 2048–2056.

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
Natural wood with bark chipped from a mixture of conifer tree species including <i>Abies concolor</i> , <i>Pinus lambertiana</i> , <i>Calocedrus decurrens</i> , <i>Pinus jeffreyi</i> , <i>Abies magnifica</i> , and <i>Pinus contorta</i> .	Growth test, phenolics concentration	<i>Scirpus microcarpus</i>		Leachate of mixed conifer bark contains approximately 20 times higher phenolics concentration than similarly prepared wood-only leachate. High levels of phenolics (211 mg/L) inhibited <i>S. microcarpus</i> growth; this growth inhibition was more pronounced in nutrient-limited conditions and in smaller individuals.	Wolf E. C., Rejmánková E., Cooper D. J., 2019, Wood chip soil amendments in restored wetlands affect plant growth by reducing compaction and increasing dissolved phenolics, <i>Restoration Ecology</i> 27, 1128–1136.

### Allegato 3: Analisi chimiche dei campioni di lisciviato. / Attachment 3: Chemical analyses of leachate samples.

Di seguito vengono riportate le analisi chimiche effettuate da Agri.Te.Co, sono riportate solo i valori relativi al rame in quanto per le altre sostanze analizzate (polifenoli e terpeni) non risulta essere presente una quantità rilevante. / Below are the chemical analyzes carried out by Agri.Te.Co, only the values relating to copper are reported as for the other substances analyzed (polyphenols and terpenes) there is not a significant quantity present.

Sample		Parameter	MU	Value
UTW	t0	Copper	µg/L Cu	30,32
	t1	Copper	µg/L Cu	23,7
	t2	Copper	µg/L Cu	23,5
TM	t0	Copper	µg/L Cu	7,89
	t1	Copper	µg/L Cu	34,1
	t2	Copper	µg/L Cu	47,5
UC3	t0	Copper	µg/L Cu	2780
	t1	Copper	µg/L Cu	883
	t2	Copper	µg/L Cu	630

# Interreg



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Standardni projekt sofinancira Evropski sklad za regionalni razvoj

1

# Ekotoksikološka ocena v sladki vodi

Različica: ŠT. 1

Povezan DS: DS3.3.2

Odgovorni partner: Univerza Cà Foscari (PP4)

Avtorji: Volpi Ghirardini Annamaria, Picone Marco, Russo Martina,  
Distefano Gabriele, Marchetto Davide

Datum: 31.julij 2022

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## OKRAJŠAVE

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ACQ - alkaline copper quaternary (alkalni bakreni kvaternar)

ASTM - American Society for Testing and Materials (Ameriško združenje za testiranje in materiale)

CBA- copper borate azole (bakrov-borov azol)

CCA - chromated copper arsenate (kromirani bakrov arzenat)

CLP - Classification, Labelling and Packaging (Evropska uredba o razvrščanju, označevanju in pakiranju)

EC50 - effect concentration (efektivna koncentracija za 50 % organizmov, uporabljenih pri toksikološkem preizkušnje)

ISO -International Organization for Standardization (Mednarodna organizacija za standardizacijo)

ITS - Integrated testing strategy (integrirana strategija testiranja)

OECD - Organization for Economic Cooperation and Development (Organizacija za gospodarsko sodelovanje in razvoj)

REACH - Registration, Evaluation, Authorisation and Restriction of CHemicals (Evropska direktiva o registraciji, evalvaciji, avtorizaciji in omejevanju kemikalij - REACH)

TM - termically modified (termična modifikacija)

TU50 - toxicity unit ali toksikološka enota (TU50 = 100/EC50)

UC3 - use class 3 (razred uporabe lesa 3)

US-EPA - United States Environmental Protection Agency (Agencija za varstvo okolja Združenih držav Amerike)

UTW - UnTreated wood (neobdelan les)

## POVZETEK

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Ta raziskava uporablja eksperimentalni pristop, sloneč na nizu kazalnikov, ki omogočajo oceno tveganja za sladkovodno bioto na različnih trofičnih ravneh ter biološke obremenitve nekaterih izdelkov za zaščito lesa. Pri oceni strupenosti izpirka iz lesnih vzorcev (obdelanih in neobdelanih) smo uporabili integrirano strategijo testiranja



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(Integrated testing strategy - ITS), katere hierarhično organizirana struktura nudi možnost proučevanja vpliva, ki ga obdelave za povečanje obstojnosti lesa imajo na okolje. ITS sicer zaznamuje povečana občutljivost ekotoksikoloških testov, ki se uporabljajo pri treh stopnjah testiranja (stopnja 1 - Akutni testi; stopnja 2 - Testi najbolj občutljivih stadijev, stopnja 3 - Kronični testi). S to metodo lahko ocenjujemo morebitne vplive in ugotovimo, koliko so le-ti »sprejemljivi« za vodno rastlinstvo in živalstvo, da bi tako določili okolju prijazno obdelavo. Cilj raziskave je bilo ugotoviti, kako bi obdobje izpostavljenosti v vodi vplivalo na sproščanje snovi iz lesa, z namenom priprave smernic, ki bi bile v pomoč družbam pri določanju potrebnega obdobja izpostavljenosti pred uporabo materiala v okolju. ITS postopek za sladkovodno okolje je predvidel primerjanje obdelanih vzorcev z neobdelanim (UTW), s posebnim poudarkom na oceni toplotno modificiranih vzorcev lesne vrste *Picea abies* (termically modified, TM) in vzorcev, obdelanih s Silvanolinom za uporabo na prostem, brez stika s tlemi (UC3) (0,25 % koncentracija CU v raztopini).

Iz testov TIER I izhaja, da je glede na sprejeta merila za ITS ocenjevanje za sladko vodo le obdelava TM pokazala enake ali celo nižje toksične učinke kot neobdelani les (UTW). Ugotovljeno pa je bilo, da obdelava UC3 ni prijazna do vodnega okolja zaradi visoke akutne strupenosti na bakterije, alge in rakce ne glede na čas izpostavljenosti. Pridobljeni rezultati prvonivojske ocene so pokazali, da je samo TM primerna za naslednjo drugo fazo ocenjevanja. Na podlagi rezultatov na ravni TIER II smo lahko ugotovili, da je obdelava TM primerna za oceno III ravni. Kljub temu so tudi splošni učinki izpirka neobdelanega preizkušanca (UTW) pri končni točki (endpoint) razpočenja ovojnice pri polžu *L. auricularia* pokazali potrebo po času izpostavljenosti, daljšem od 15 dni, s čimer je mogoče odpraviti toksično delovanje izpirka na razvoj ličink. Na podlagi splošnih rezultatov kroničnih testov stopnje III na školjkah *L. auricularia* in rakcih *Daphnia magna* je jasno razvidno, da ima obdelava TM enake učinke na oba vodna indikatorja, uporabljena za neobdelan les (UTW). Predvsem iz rezultatov testov o razmoževanju *D. magna* je bilo jasno ugotovljeno, da je treba pred uporabo materiala zagotoviti minimalni 15-dnevni čas izpostavljenosti, s čimer zaježimo vpliv na sladkovodno okolje. Po predlagani oceni ITS je bilo tako ugotovljeno, da je obdelava TM okolju prijazna, saj niti pri daljši izpostavljenosti ne povzroča dodatnega tveganja za okolje.

## 1 UVOD

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Glavni cilj te raziskave je bila ekotoksikološka ocena izpirkov iz toplotno-kemično obdelanega lesa skladno z integrirano strategijo testiranja (ITS), katere hierarhično organizirana struktura nudi možnost proučevanja obremenjenosti okolja, ki jo povzročajo obdelave za povečanje obstojnosti lesa. Strategija ITS sicer ocenjuje morebitne učinke in prouči, koliko so le-ti »sprejemljivi« za sladkovodno živalstvo in rastlinstvo.

### 1.1 Predhodni pregled objav

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Namen pregleda objav je bilo ugotoviti trenutno stanje raziskav na področju toksikološke ocene izpirkov iz neobdelanega in obdelanega lesa; proučili smo namreč: a) standardne in eksperimentalne postopke, b) vrsto uporabljene izpiralne količine, c) morebitno predhodno obdelavo lesa, d) fizično-kemične parametre in vrsto kemičnih

analiz, ki jih je treba proučiti, e) organizme in vrste ekotoksikoloških testov, uporabljenih pri ocenah f) in nazadnje rezultate raziskave ter povezane težave.

V dveh prilogah k temu poročilu je strnjeno prikazan pregled proučenih objav; v prvi prilogi so prikazani uporabljeni parametri in tehnike izpiranja, v drugi pa kemične in ekotoksikološke značilnosti izpirkov.

### **Tehnike izpiranja**

V tem delu pregledovanja raziskav smo želeli razumeti, katere metode se uporabljajo za opredelitev lastnosti obdelanega in neobdelanega lesenega materiala, na podlagi katerih se določi eksperimentalni načrt.

Deset od 21 proučenih objav obravnava les, impregniran s sredstvom na osnovi bakra (Priloga 1). Najbolj analiziran je kromirani bakrov arzenat (CCA, 5 raziskav), ki je danes prepovedan v EU, mu sledijo alkalni bakreni kvaternar (ACQ) in azolne spojine bakra in bora (CBA) (4 raziskave za vsako snov). Drugi dve proučeni obdelavi, ki nista na osnovi bakra, sta furfurizacija in uporaba pentaklorfenola. Cilj sedmih študij je bila opredelitev naravne toksičnosti lesa, kjer so bile tako kot za obdelan les uporabljene tehnike izpiranja, ki se lahko ponovijo. Štiri raziskave pa predvidevajo, da se izpirek, nastal med sezonskimi padavinami, neposredno odvzame na licu mesta.

Glede samega postopka izpiranja najbolj pogosto uporabljena tekočina je destilirana/deionizirana voda oz. voda iz pipe (13 raziskav), umetna morska voda pa je bila uporabljena pri 3 raziskavah. Nekatere študije, kot Dubey in sod. (2007), celo predvidevajo uporabo 8 naravnih sladkih, slanih in brakičnih voda z različnimi značilnostmi, Adeleye in sod. (2016) pa so primerjali morsko in pitno vodo ter tisto ob rečnih ustjih.

Statični test izpiranja je bil najbolj pogosto uporabljen (v dvanajstih študijah), dinamični test pa je bil opravljen petkrat. Razmerje tekoče:trdno (S/L) se močno razlikuje glede na avtorja, in sicer od minimalne vrednosti 1:5 do maksimalne 1:40. Največ testov je bilo izvedenih z uporabo razmerja približno 1:10. Razmerje S/L 1:40 so Svensson in sod. (2012, 2014a,b) uporabili pri posebnem primeru, tj. preizkušanju na žagovini, ker zaradi absorpcije velike količine vode ni bilo mogoče zagotoviti optimalnega izpiranja pri nižjih razmerjih.

### **Kemično-fizikalne in ekotoksikološke lastnosti izpirkov**

Pri pregledu objav o izpirkih (Priloga 2) smo lahko ugotovili, da je bila večina testov opravljena na sladki vodi, le 5 zadeva morsko. Pri glavnina študij pa je bila poleg kemijske izvedena tudi ekotoksikološka analiza.

Pri ekotoksikoloških testih na izpirkih je večina analiz uporabila bakterijo *Aliivibrio (Vibrio) fischeri* in rakca *Daphnia magna*, ki se je med dvema vrstama izkazal za najbolj občutljivega. Glede vrste *A. fischeri* iz literature izhaja, da iver proizvaja strupene izpirke ne glede na vrsto uporabljenega postopka izpiranja (Rex in sod., 2016, Svensson in sod., 2014a,b).

Za oceno strupenosti izpirkov se kot testne vrste uporabljata školjka *Crassostrea gigas* in rakec *Artemia salina* (Libralato in sod., 2007; Svensson in sod. 2012, 2014a), med uporabljenimi rastlinskimi organizmi pa velja omeniti vrsto *Scirpus microcarpus* (Wolf in sod. 2019). Test škodljivosti na zarodkih vrste *C. gigas* je pokazal večjo občutljivost kot akutni test z vrsto *Artemia salina* (Libralato in sod., 2007) predvsem zaradi dejstva, ki so ga izpostavili tudi Svensson in sod. (2014a), da ta rakec bistveno ne občuti strupenega učinka izpirka. Test rasti vrste *S. microcarpus* pa je pokazal, da visoke ravni

raztopljenih fenolov lahko omejijo možnost rastlin za dostop do hranil (Wolf in sod. 2019).

O kopenskih organizmih so bile sicer opravljene ciljne raziskave za oceno biokopičenja kovin in metaloidov ter vpliva na razmnoževanje in razvoj na maloščetincu *E. fetida*, izpostavljenem tlem, poškrabljenim z izpirkom iz lesa, obdelanega z CCA in ACQ (Leduc in sod., 2008).

Na splošno lahko trdimo, da bi se snovi, ki povzročijo akutne strupene učinke na neobdelanem lesu, razlikovale glede na proučeno lesno vrsto; na primer je toksičnost hrasta po vsej verjetnosti odvisna od fenolnih spojin, toksičnost bora pa domnevno od terpenov (Svensson in sod., 2012). Iz pregleda literature tudi izhaja, da visok organski delež izcedne vode ali kombinacija organskih spojin in smolnih kislin lahko občutno pripomorejo k nastanku toksičnosti (Rex in sod., 2016). Glede uporabljene obdelave za zaščito lesa pa je bilo ugotovljeno, da je strupenost za vodno okolje povezana z nestalnim deležem Cu, prisotnim v izpirku. V primerjavi z laboratorijskimi izpirnimi tekočinami je nižja vodna strupenost, izmerjena v izcedni vodi iz obdelanega lesa, pridobljeni z naravnimi vodami, verjetno povezana s prisotnostjo večje količine veziv in kelatov v naravnih medijih (Dubey in sod., 2007, Kannepalli in sod., 2016).

V literaturi so bile izpostavljene pomembne razlike v kemijsko-fizikalnih lastnosti izpirkov glede na vrsto lesa. Na podlagi testov izpiranja na iverju štirih različnih lesnih vrst (hrast, bor, javor in bukev) so bile na primer beležene statistično pomembne razlike med hrastom in drugimi tremi vrstami pri skoraj vseh proučenih spremenljivk, to so raztopljen organski dušik (DOC), fenoli, biokemijska potreba po kisiku (BOD5), pH, tanini, lignin in barva (Svensson in sod., 2014b). Ko se les razreže na manjše kose, postopek izpiranja in prenosa onesnažil potekata veliko hitreje, kot bi naravno. Na podlagi rezultatov je videti, da bi vrsta, velikost delcev in anatomske razlike lesa skladiščenega materiala vplivale na obremenjenost z organskim onesnaževalom, ki se sprošča v vodni fazi, ko je les v stiku z vodo.

## 2 MATERIALI IN METODE

### 2.1 Priprava izpirkov za ekotoksikološko testiranje

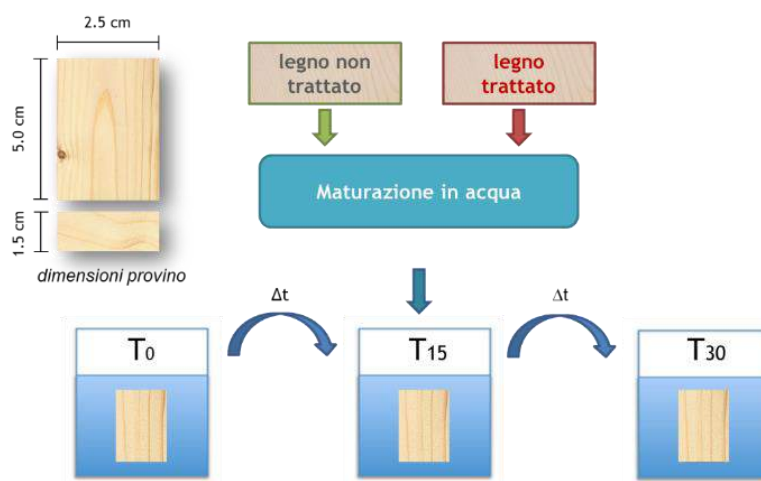
Glede na znanstveno literaturo in predhodne izkušnje v lagunskem okolju (UniVe-CVN, 2011; MAV, 2013) smo za izvajanje te projektne aktivnosti razvili posebno metodo za oceno toksičnosti lesnih vzorcev, pri kateri se vzorci predhodno operejo pod tekočo vodo, da se s površine odstranijo morebitni ostanki, nato se najprej izvede izpiranje pri času nič (T0), nato še dva dodatna izpiranja po 15 (T15) in 30 (T30) dneh izpostavljenosti v vodi.

Na sliki 1 je shematsko prikazan postopek izpostavljenosti vzorcev.

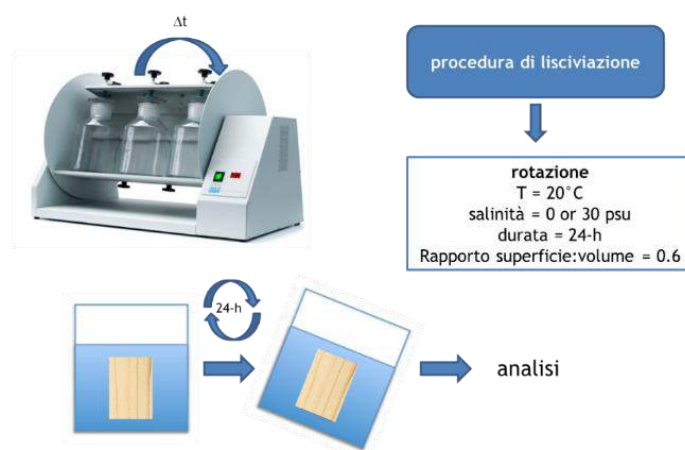
Vsak akvarij smo napolnili s 30 litrov »hardwater« (US-EPA); preizkušance smo po prvem izpiranju namestili v akvarij, da bi jih izpostavili okolju. Lesne vzorce smo privezali na utež, da bi zagotovili popolno potopljenost v vodi.

Vsak akvarij je bil opremljen s črpalkama, ki poskrbita za kroženje 25 l h<sup>-1</sup>, na tak način se simulirajo okoljski pogoji, katerim bodo domnevno izpostavljeni koli v naravi. Tedensko smo vodo delno zamenjali.

Voda je bila v celoti zamenjana na 15. dan od začetka preizkušanja. Pri T0, T15 in T30 smo na lesnih vzorcih izvedli postopek izpiranja, kot je prikazan na sliki 2.



Slika 1. Postopek izpostavljenosti lesnih vzorcev velikosti 5,0 x 2,5 x 1,5 cm.



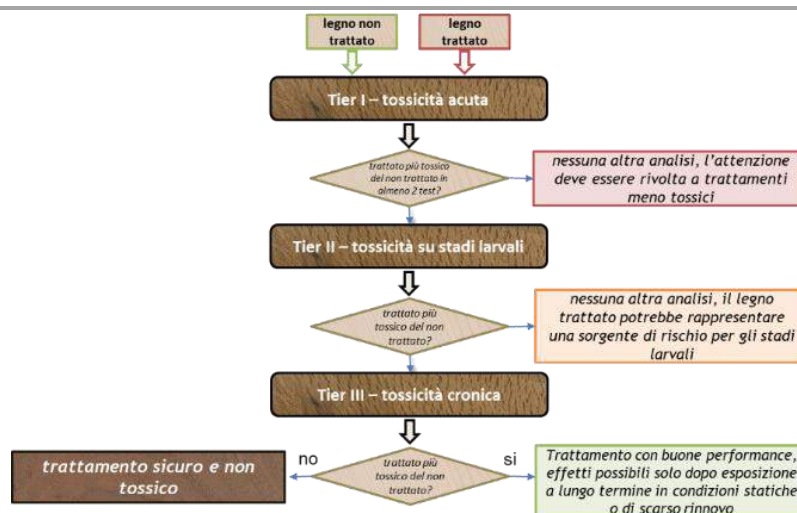
Slika 2. Levo je naprava ROTAX, desno pa parametri izpiranja. Spodaj je prikazan postopek izpiranja.

Za izpiranje smo uporabili napravo ROTAX (podjetja Velp scientifica), ki zagotavlja popolno navpično vrtenje vzorcev, ki so shranjeni v zaprtih steklenicah. Izpiranje poteka pri 20 °C za 24 h skladno s smernicami MAV (2013). Izbrali smo razmerje površina vzorca/količina vode 0,6 cm<sup>2</sup> cm<sup>-3</sup> (OECD, 1999; OECD 2007).

Vsi leseni vzorci so bili dimenzionirani na 5,0 x 2,5 x 1,5 cm in oblikovani po navodilih evropskega standarda EN 113-1:2020 o trajnosti lesa in lesnih izdelkov.

## 2.2 Integrated testing strategy (ITS) - Integrirana strategija testiranja

Za oceno strupenosti izpirkov iz obdelanih in neobdelanih lesnih vzorcev smo uporabili »integrirano strategijo testiranja« (ITS); na tem hierarhično organiziranem pristopu sloni odločitveni postopek ocene vpliva novih izdelkov na okolje, ki so namenjeni rabi na področju industrije, trgovanja in gradbeništva (slika 3).

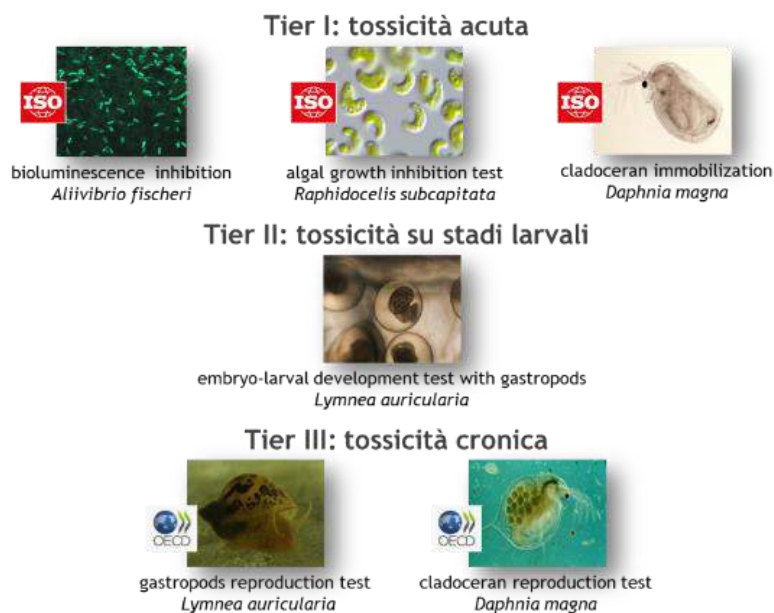


Slika 3. Hierarhično organiziran pristop ITS, ki je bil oblikovan za projekt Durasoft.

V tem projektu metodo ITS sestavljajo tri stopnje ocenjevanja sprejemljivosti morebitnih toksičnih učinkov na rastlinstvo in živalstvo. Prva stopnja predvideva izvajanje akutnih testov, druga oceno učinkov na najbolj občutljive stadije (embrionalni in larvalni), zadnja in tretja stopnja pa zajema kronične teste. Za ITS postopek je značilno stopnjevanje občutljivosti preizkušanja. Skozi celoten ocenjevalni postopek smo obdelane lesene vzorce primerjali s pripadajočim neobdelanim lesom. Če se je obdelani vzorec izkazal za manj strupenega (ali so vrednosti TU <1), preidemo na naslednjo fazo testiranja. Če testi niso pokazali pozitivnih rezultatov, zadnja ocenjevalna stopnja pomeni, da je obdelava popolnoma varna in skladna z okoljem, če pa je bil ugotovljen kronični učinek, to vsekakor pomeni, da ima zaščitna obdelava dobro raven kompatibilnosti in da je nastanek učinkov možen, a le pod posebnimi pogoji izpostavljenosti (npr. statični pogoji ali nezadostna izmenjava vode).

Vsaka stopnja, imenovana »TIER«, predvideva teste z jasno določenimi značilnostmi za sladkovodno okolje, ki so organizacijsko urejeni, kot je prikazano spodaj (slika 4):

- »TIER 1« ocenjuje kratkoročne akutne učinke s preskusom zaviranja bioluminescentnega sevanja bakterije *A. fischeri*, preskusom zaviranja rasti enocelične zelene alge *Raphidocelis subcapitata* in preskusom negibnosti vodne bolhe *Daphnia magna*.
- »TIER 2« ocenjuje strupenost na najbolj občutljive razvojne stadije vodnih organizmov z uporabo testa razvoja ličink. Za sladkovodno okolje je bil izbran preizkus larvalnega razvoja polža *Lymnaea (Radix) auricularia*.
- »TIER 3« ocenjuje učinek izpirka na celotni življenjski cikel testnih organizmov; za sladkovodno okolje smo izvedli test razmnoževanja vrst *L. auricularia* in *D. magna*.



Slika 4. ITS za sladke vode.

Izmed izbranih ekotoksikoloških metod za ITS preskušanje velja pripomniti, da se test z vrsto *R. subcapitata* ter akutni in kronični testi z vrsto *D. magna* uporabljajo za ovrednotenje strupenih učinkov snovi in spojin po Uredbi (ES) št. 1907/2006 Evropskega parlamenta in Sveta (Uredba REACH) o registraciji, evalvaciji, avtorizaciji in omejevanju kemikalij, ki zlasti ureja evalvacijo vseh snovi, ki so letno proizvedene ali uvožene v Evropsko unijo v količini ene tone ali več, in po Uredbi (ES) št. 1272/2008 Evropskega parlamenta in Sveta (Uredba CLP).

## 2.3 Postopki preizkušanja strupenosti za sladko vodo

### Tier 1

#### -Test zaviranja luminescence bakterije *A. fischeri* (Test Microtox®)

Preizkus Microtox® smo izvedli z uporabo analizatorja M500 (Azur Environmental) po postopku za tekoče vzorce, skladno s standardom ISO 11348 (2007). Liofilizirane bakterije (Microtox® Acute Reagent, Modern Water, št. serije 19K4239A) smo ponovno aktivirali z dodajanjem rekonstitucijske tekočine (Reconstitution Solution, Modern Water) v ampulo z akutnim reagentom, ki vsebuje liofilizirane bakterije, da smo tako pridobili suspenzijo bakterij. Ampulo s suspenzijo bakterij smo za čas biološkega testa, ki se je izvajal pri 15 °C, namestili v odprtino analizatorja pri temperaturi 4 °C, da se aklimatizira. Zaviranje oddajanja svetlobe vrste *A. fischeri* pri 490 nm smo izmerili po 5, 15 in 30 minutah izpostavljenosti različnim razredčenim eluatom, ki so bili pridobljeni iz sladke vode in v destilirani vodi, za formulacije izdelkov, ki so namenjeni rabi v sladkovodnem okolju (UC3, TM in UTW). Ozmotski popravek vzorca smo dosegli s raztopitvijo 0,5 g soli NaCl v 25 ml vzorca (20‰). Skladno s testnim protokolom smo najprej analizirali nerazredčen vzorec (1:1), nato s pomočjo serije razredčitev v razmerjih, ki se stopnjevalno razlikujejo v razmiku faktorja 3, in sicer 100 %, 33 %, 10 %, 3 %, 1 %, 0,3 %. Za negativno kontrolo in sredstvo redčenja morskih vzorcev je bila uporabljena umetna morska voda ASTM. Pozitivna kontrola je bila opravljena z uporabo raztopine ZnSO<sub>4</sub>, kot referenčno strupeno raztopino, skladno s postopkom, navedenim v standardu ISO za tekoče vzorce z uporabo liofiliziranih bakterij (ISO 11348, 2007).

### **-Test zaviranja rasti alg vrste *R. subcapitata***

Test rasti alg je predmet postopka mednarodnega standarda ISO 8692 (ISO, 2012). Pred preizkušanjem smo kulturo v eksponentni fazi rasti nenehno tresli s pomočjo ploščnega tresalnika pri temperaturi med 21 °C in 24 °C. Kulturo alg smo ohranjali v eksponentni rasti tako, da smo jo obnavljali vsak mesec.

Po predmetni metodi se deleži *R. subcapitata* inokulirajo v testne vzorce, ki so bili predhodno obogateni s hranili. Preizkus traja 72 ur, po preteku katerih smo izmerili celično gostoto v vsaki obdelavi, na podlagi katere smo izmerili stopnjo rasti posameznega vzorca.

Za vsako obdelavo smo ocenili vzorce s serijo šest razmerij redčenja v geometričnem zaporedju za tri replikacije posamezne koncentracije (100, 33, 10, 3, 1, 0,3 %). Za negativno kontrolo smo uporabili 6 replikacij. Replikacije vsake obdelave smo vstavili v vsako od šestih vdolbin na ploščah za enkratno rabo (Iwaki, Japan), vsako od katerih smo napolnili z 10 ml vzorca.

Pred inokulacijo alg smo izmerili gostoto alg v rastnem mediju, nato smo pripravili inokulum koncentracije 104 cell ml<sup>-1</sup>. Plošče smo inkubirali pri 20 ± 2 °C ob stalni osvetlitvi (6000 lx) za 72 ± 2 h.

Po preteku 72 ur smo oceno koncentracije alg v vdolbinah opravili z neposrednim štetjem s pomočjo kamere Bürker.

### **-Test negibnosti *D. magna***

Preizkus o nezmožnosti gibanja vodne bolhe velja za eden od najbolj uporabljenih metod za ocenjevanje snovi, vnesenih v okolje, saj ga predpisujejo tako državne smernice (APAT-IRSA) kot mednarodni standardi ISO 10706 (ISO, 2000), OECD 202 (OECD, 2004), opredeljen je tudi v Direktivi 98/8/ES (o dajanju biocidnih pripravkov v promet) in v Uredbi REACH za evalvacijo tržnosti kemikalij.

V laboratoriju shranjene kulture redno obnavljamo in hranimo pod kontroliranimi pogoji temperature in osvetlitve; temperatura mora biti med 18 °C in 22 °C, dnevno-nočni cikel 16-8 ur. Organizmi, izpostavljeni v vodi M7, redno hranimo s tekočino, ki vsebuje enocelično algo *R. subcapitata*.

Preizkus traja 48 ur; tako kot za test z vrsto *R. subcapitata* smo za vsako obdelavo analizirali vzorce s serijo šest razmerij redčenja v geometričnem zaporedju (100, 33, 10, 3, 1, 0.3%). Za negativno kontrolo smo uporabili le medij M7. Test smo izvedli na ploščah s šestimi vdolbinami po 10 ml, vsaka od katerih je vsebovala 5 osebkov *D. magna*, starih največ 24 ur. Potem ko smo v vsako vdolbino vnesli po en osebek, smo plošče inkubirali 48 ur na enakih pogojih za ohranjanje kulture. Po preteku 48 ur smo analizirali vsako vdolbino in prešteli negibne organizme.

## **Tier 2**

### **-Test toksičnosti za zarodke polža *Lymnea (Radix) auricularia***

Preizkus toksičnosti za zarodke vodnega pljučnega polža *L. auricularia* je razvila raziskovalna skupina Univerze v Benetkah skladno z metodo, ki je bila že zasnovana za vrsto *Radix balthica* (Tills in sod., 2013). S testom opazujemo razvoj skupka jajčec, ki so bila izpostavljena različnim koncentracijam izpirka, ter ugotavljamo morebitne razvojne zaostanke. Tovrstni preizkus je zelo pomemben, ker proučuje učinke na najbolj občutljive faze razvoja organizma.

V laboratoriju gojene organizme pustimo v akvarijih z deklorirano vodovodno vodo, zunanjim filtrom in sistemom za prezračevanje. Hranimo jih z biološko pridelano solato. Akvariji so nameščeni v termostatski komori pri stalni temperaturi 21 °C z dnevno-nočnim ciklom 16-8 ur.

Za lažje nabiranje in opazovanje skupka jajčec, ki se bodo uporabljala za preizkus, pripravimo 2-litrsko steklene posode, napolnjene z vodo iz akvarijev, na stene katerih so postavljena snemljiva mikroskopska stekelca.

Za preizkus uporabljamo skupke jajčec, ki so bila odlagana v 24 urah po začetku preizkušanja (ki so sicer v stadiju tančice oz. »velum«). Odvzeto stekelce s skupkom jajčec opazujemo z mikroskopom, da ugotovimo razvojni stadij in seštejemo število prisotnih jajčec. Po ugotavljanju razvojnega stadija pripravimo 3 replikacije za vsako redčenje vzorca. Vsak skupek jajčec vstavimo v 100 ml beaker posodo s testno raztopino, pri tem pa pazimo, da so jajčeca v celoti potopljena. Vzporedno opravimo tri negativne kontrole z »medijem Radix«, to je sicer umetni medij trde vode za sladkovodne organizme z ustreznimi vrednostmi pH, trdnosti in alkalnosti za polže (U.S. EPA, 2002).

Pripravljene replikacije pustimo na enakih kontroliranih pogojih kot za akvarije. 8 dni po začetku testiranja opravimo prvo opazovanje z invertnim mikroskopom tako, da odstranimo stekelce, na katerega je pritrje skupek jajčec, in ugotavljamo dosežen stadij vsakega prisotnega zarodka. V običajnih pogojih zarodki dosežejo stadij »tvorjene lupine« (»shell formation«) na 8. dan.

Enak postopek ponovimo na 13. dan preizkusa, ko bo dosežen stadij »razpočenja ovojnice« (»capsule rupture«); pri tem je treba nujno preveriti, ali ima polž lupino in je ovojnica počila.

Na koncu ugotavljamo odstotek zarodkov, ki so se normalno razvili in dosegli ustrezen razvojni stadij, ter morebitni razvojni zaostanek, ki je posledica izpostavljenosti izpirku.

### Tier 3

#### - *Kronični test z vrsto D. magna*

Ta preizkus smo izvedli skladno s postopkom OECD št. 211 (OECD, 2021).

Pripravili smo 10 replikacij za vsako razredčitev izpirka in negativno kontrolo, ki vsebuje le vodni medij M7. Tako kot smo storili za akutni test, smo iz laboratorijske kulture odvzeli organizme, stare največ 24 ur, in po enega vnesli v 100 ml beaker posodo, ki vsebuje 50 ml raztopine. Tako kot za akutni test smo beaker posode inkubirali v kontroliranem okolju pri stalni temperaturi med 18 in 22 °C.

Raztopine, kjer so organizmi izpostavljeni, v celoti zamenjamo trikrat tedensko, pri tem pa moramo paziti, da čim bolj omejimo volumen prenesene raztopine z organizmom. Poleg navedenega postopka zamenjave vse organizme hranimo tako, da najprej dodamo 0,5 ml suspenzije alg, po prvem razmnoževanju - ta običajno ne poteka pred devetimi dnevi preizkušanja - pa količino, ki je sorazmerna povečanju velikosti in, po potrebi, številu izleženih ličink.

Po 21 dneh ugotavljamo preživele starše in število živih potomcev, ki jih je vsak odrasel osebek izlegel.

#### - *Kronični test z vrsto L. auricularia*

Ta preizkus smo opravili po protokolu OECD za polža *Lymnaea stagnalis* (OECD 243). Test traja 28 dni, medij v celoti zamenjamo vsake tri dni.



Uporabljamo enolitrske beaker posode, ki vsebujejo testni vzorec; za vsako beaker posodo izberemo 5 organizmov minimalne velikosti za razmnoževanje (7,5–8,5 mm dolžine), ki jih hranimo z biološko pridelano solato. Pripravimo 3 replikacije na koncentracijo in negativno kontrolo in jih pustimo na stalni temperaturi 21 °C z obdobjem osvetljenosti 16 ur svetlobe in 8 ur teme.

Proučene končne točke sta smrtnost in sprememba stopnje rasti organizmov glede na težo in dolžino.

### Prikaz testnih rezultatov in analiza podatkov

Obdelava podatkov o zaviranju luminescence bakterij, rasti alg in smrtnosti vrste *D. magna* je služila izračunu vrednosti  $EC_{50}$  (koncentracije izpirka, ki imajo učinek na 50 % izpostavljenih organizmih) in  $TU_{50}$ , ocenjeno kot  $100/EC_{50}$ . Za zaščitne obdelave z nizkim ali zanemarljivim učinkom ali pri katerih ne pride do 50 % zaviranja v nerazredčenem vzorcu, se vrednost  $TU_{50}$  izračuna po enačbi  $TU_{50} = Ex / TU_{50}$  kjer  $Ex$  predstavlja beležen odstotek učinka v obdelavi  $X$ , ki je normaliziran glede na učinek, ugotovljen pri negativni kontroli. Za preizkus Microtox smo vrednosti  $EC_{50}$  določili z uporabo programske opreme Microtox Omni®, za oceno dinamičnih podatkov iz testov alg in vrste *D. magna* pa s pomočjo programske opreme, ki jo je razvila univerza Danmarks Tekniske Universitet v kraju Lyngby (Christensen in sod. 2009).

Pri preizkušanju Tier II, smo odstotek ličink, ki so dosegle stadij »shell formation« in »capsule rupture« v nerazredčenem vzorcu izpirka (100%) statistično primerili z rezultati negativne kontrole in izpirka neobdelanega lesa (UTW) po metodi enosmerne variance (enosmerna ANOVA) skupaj s Tukeyjevim t-testom.

Pri preizkusih Tier III sta bila število potomcev vsakega odraslega osebka vrste *Daphnia*, ki je bil izpostavljen izpirkom, in stopnja rasti *L. auriculara*, izražena kot dnevno povečanje telesne teže (v mg) in dolžine (v  $\mu\text{m}$ ), ocenjena po metodi enosmerne analize ANOVA skupaj s Tukeyjevim t-testom, da bi tako ugotovili morebitne razlike med negativno kontrolo, neobdelanim lesom (UTW) in obdelavami za zaščito lesa.

Pred analizo podatkov iz testov Tier II in Tier III smo normalno porazdelitev in enakost varianc preverili s Kolmogorov-Smirnovim in Levenovim testom. Ko enega od omenjenih pogojev ni bilo mogoče ugotoviti, smo podatke analizirali z neparametričnimi metodami, to je z metodo Kruskal-Wallis ANOVA in testom Mann-Whitney za primerjavo dveh neodvisnih vzorcev.

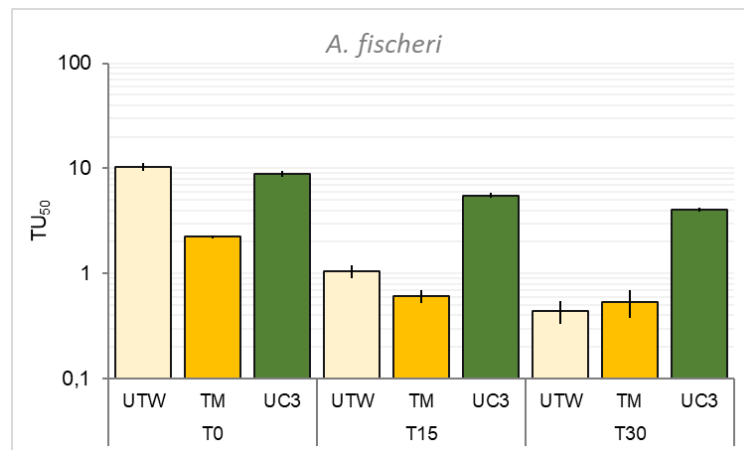
## 3 REZULTATI IN RAZPRAVA

### 3.1 Tier I

Preizkuse TIER 1 smo uporabljali za izpirke, nastale s sladko vodo, in smo jih izvdeli na neobdelanih vzorcih lesne vrste *P. abies* (untreated wood, UTW), termično obdelanih vzorcih (termically modified, TM) in vzorcih, obdelanih s Silvanolinom za uporabo na prostem brez stika s tlemi (UC3).

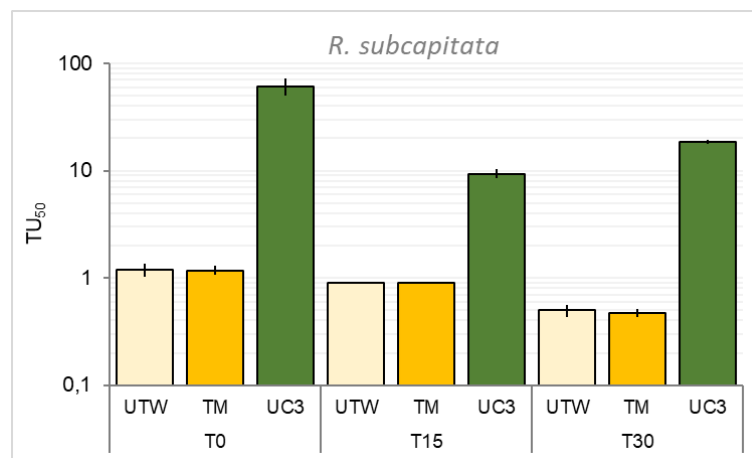
Test Microtox je pokazal zmanjšanje toksičnosti vseh izpirkov ob povečanju časa izpostavljenosti; TM je imela najmanjši učinek na luminescenco bakterij (slika 5). Pri T0 se je TM izkazala za veliko manj strupeno za *A. fischeri* v primerjavi z UTW in UC3. Tako TM kot UTM sta beležili znatno zmanjšanje strupenosti pri T15 v primerjavi s T0, zaviranje luminescence UC3 pa je privedla do minimalnega zmanjšanja strupenosti v primerjavi s T0, saj je stopnja strupenosti ostala dokaj visoka. Po 30 dneh izpostavljenosti se je

strupenost TM in UTW dodatno znižala, UC3 pa je še imela močan zaviralni učinek na luminescenco.



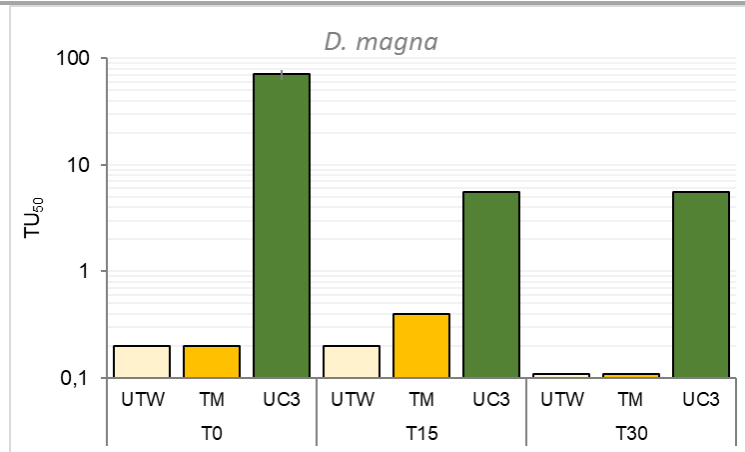
Slika 5. Toksičnost izpirkov za *A. fischeri*. Stopnje napak predstavljajo 95 % meje zanesljivosti za povprečno vrednost  $TU_{50}$  treh testnih replikacij.

Pri testu rasti alg vrste *R. subcapitata* so bili rezultati izpirkov iz vzorcev UTW in TM enaki pri vseh preizkušanih pogojih, strupeni učinek pa se je zmanjšal ob povečanju časa izpostavljenosti (slika 6). Pri vseh preizkušanih pogojih izpostavljenosti so bili ugotovljeni nizki strupeni učinki. Izpirki vzorcev UC3 pa povzročajo visoko zaviranje stopnje rasti alg s trendom v obliki U, pri čemer je najvišji zaviralni učinek ugotovljen pri T0, najnižji pri T15, po 30 dneh izpostavitve pa je mogoče opaziti znatno povišanje strupenosti.



Slika 6. Toksičnost izpirkov za *R. subcapitata*. Stopnje napak predstavljajo 95 % meje zanesljivosti za povprečno vrednost  $TU_{50}$  treh testnih replikacij.

Aktuni test z *D. magna* je potrdil enak trend, ki je bil ugotovljen pri testu z *R. subcapitata* (slika 7). Izpirki vzorcev UTW in TM niso povzročili nobenega učinka na *D. magna* oz. so bili učinki zanemarljivi, medtem ko je UC3 znatno vplivala na preživetje z vse manjšim učinkom med T0 in T15 in nobeno spremembo toksičnosti med T15 in T30.



Slika 7. Toksičnost izpirkov za *D. magna*. Stopnje napak predstavljajo 95 % meje zanesljivosti za povprečno vrednost TU<sub>50</sub> treh testnih replikacij.

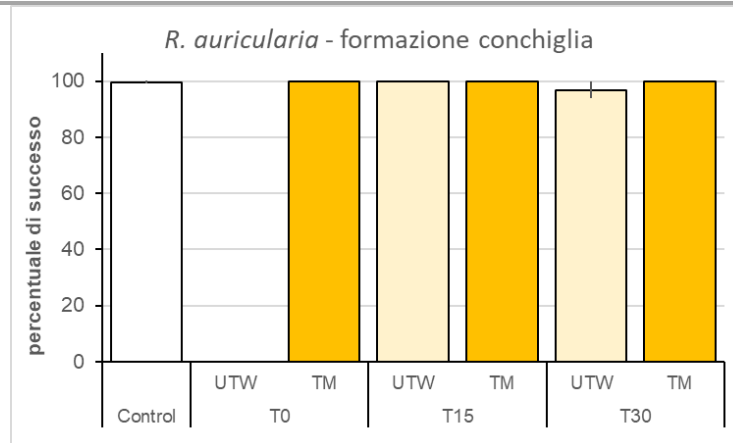
Skratka je TIER I pokazal, da le TM ima strupene učinke, ki so primerljivi ali celo nižji od tistih neobdelanega lesa (UTW). Obdelava UC3 pa je povzročila visoke akutne učinke na bakterije, alge in rake ne glede na čas izpostavljenosti. Prav tako so bili učinki UC3 pri vseh preizkušanih pogojih znatno višji od tistih, beleženih pri UTW, z edino izjemo pri T0 za Microtox test, kjer se je UTW izkazala za rahlo bolj strupeno kot UC3.

Na podlagi navedenega in ob upoštevanju meril za postopek ITS je bilo ugotovljeno, da obdelava UC3 ni prijazna do vodnega okolja zaradi visoke akutne strupenosti. Pri testu z *D. magna*, na primer, so rezultati izpirkov iz vzorcev UC3 celo presegali najvišji prag smrtnosti, ki ga določa italijanska zakonodaja za izlive v površinske vode, to je največ 50 %. Nasprotno se je TM na podlagi pridobljenih rezultatov izkazala za primerno za oceno II stopnje.

### 3.2 TIER II

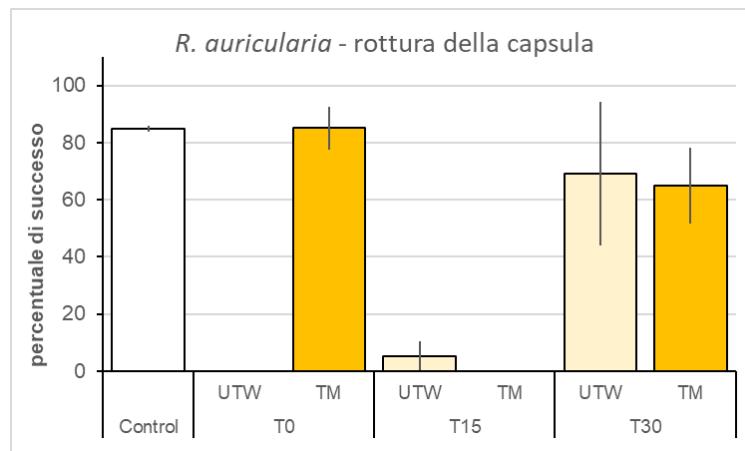
Vsi zarodki *L. auricularia*, izpostavljeni izpirku vzorcev neobdelanega lesa (UTW), so pred začetkom izpostavljenosti (T0) umrli; tvorjenje lupine in razpočenje ovojnice pri T0 nismo zato ocenili glede UTW. Nasprotno, po izpostavitvi v akvariju UTW ne povzroča negativnih učinkov na tvorjenje lupine, niti po izpostavitvi izpirkom TM (Kruskal-Wallis ANOVA:  $H = 4,225$ ,  $p = 0,513$ ) (slika 8).

Končna točka razpočenja ovojnice je podala bolj spremenljive odzive in večje razlike med obdelavami (Kruskal-Wallis ANOVA:  $H = 12,28$ ,  $p = 0,031$ ) (slika 9). Glede UTW je podaljšanje časa izpostavljenosti privedlo do znatnega povečanja odstotnega deleža polžev, ki so se izlegli iz ovojnice, in sicer  $5,3 \pm 4,0\%$  pri T15 ter  $69,1 \pm 25,3\%$  pri T30. Pri TM pa je razpočenje ovojnice pokazalo nepričakovani trend v obliki črke U: odstotek izleženih polžev je bil enak tistemu iz negativne kontrole pri T0 ( $85,0 \pm 7,6\%$ ), nato se je pri T15 znižal na 0 %, pri T30 pa se je potem zvišal na  $65,0 \pm 13,3\%$ .



Slika 8: Odstotek osebkov, ki so tvorili lupino po 8 dneh izpostavljenosti izpirkom. Stopnje napak predstavljajo standardni odklon testnih replikacij.

V primerjavi z UTW so izpirki obdelave TM beležili primerljivi odstotek ličink z ovojnico tako pri T15 kot pri T30, medtem ko je bila izvalitev iz ovojnice za obe obdelavi enaka pri T30 ( $69,1 \pm 25,3$  % za UTW in  $65,0 \pm 13,3$  % za TM) (Mann-Whitney pairwise comparison test:  $U = 3,00$ ,  $p = 0,486$ ). Obdelava TM se je potemtakem izkazala za primerno za oceno stopnje III. Trend v obliki črke U, ugotovljen pri končni točki razpočenja ovojnice, je namreč nakazal na potrebo po času izpostavljenosti, daljšem od 15 dni, da se tako odpravijo toksični učinki izpirka na razvoj ličink *L. auricularia*.

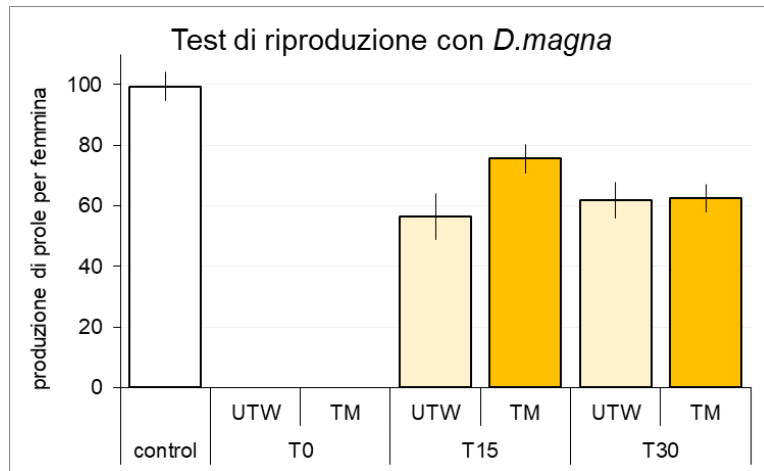


Slika 9: Odstotek osebkov, ki so se izvalili iz ovojnice po 13 dneh izpostavljenosti izpirkom. Stopnje napak predstavljajo standardni odklon testnih replikacij.

### 3.3 TIER II

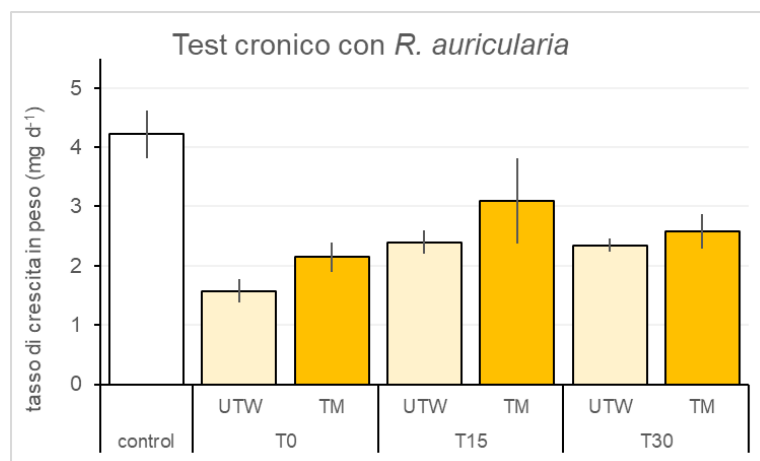
Kronični test z vrsto *D. magna* je potrdil potrebo po ustreznem času izpostavljenosti, da se odpravijo strupeni učinki izpirkov. Pri T0 so vse vodne bolhe umrle in ni bil ugotovljen nastanek potomcev pri UTW in TM, na drugi strani pa je bila pri negativni kontroli beležena 90 % stopnja preživetja in  $99,4 \pm 14,8$  ustvarjanja potomcev na samico. Stopnja preživetja se je povečala s časom izpostavljenosti tako za UTW (70 % pri T15 in T30) kot za TM (80 % pri T15 in T30). Prav tako je ustvarjanje potomcev pri T15 in T30 doseglo enake ali celo višje ravni od meril sprejemljivosti za dober izid kontrole, to je povprečno 60 potomcev na samico (slika 10). Kljub temu se ni nobena preizkušena obdelava približala stopnji ustvarjanja potomcev, izmerjeni pri negativni kontroli (enosmerna

ANOVA:  $F = 9,775$ ,  $p < 0,001$ ). Pri T15 je bilo ustvarjanje potomcev s TM rahlo višje od tistega pri UTW ( $75,5 \pm 13,7$  in TM;  $56,5 \pm 18,7$  in UTW), pri T30 pa sta obe obdelavi beležili enake rezultate ( $62,6 \pm 12,9$  za TM in  $61,9 \pm 16,6$  za UTW). Na podlagi navedenega niso bile ugotovljene pomembne razlike med obdelavama UTW in TM pri T15 (Tukeyjev test:  $Q = 3,393$ ,  $p = 0,142$ ) in T30 (Tukeyjev test:  $Q = 0,137$ ,  $p = 0,999$ ).



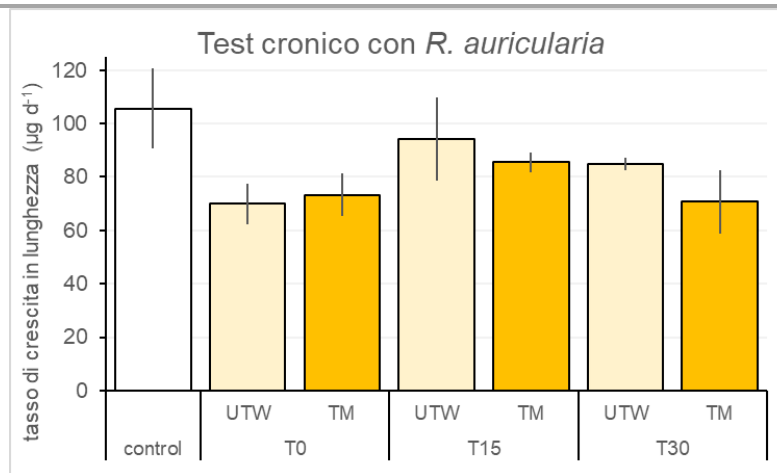
Slika 10. Število ustvarjenih osebkov na samico *D. magna* v času izpostavljenosti izpirkom (30 dni). Stopnje napake predstavljajo standardno napako.

Kronični test z vrsto *L. auricularia* je potrdil izide dolgoročnega testa z vrsto *D. magna*. V vseh preizkuženih pogojih je bilo dnevno povečanje teže za UTW in TM nižje od negativne kontrole ( $4,2 \text{ mg d}^{-1}$ ) (slika 11), najvišje stopnje rasti pa so bile beležene pri T15 ( $2,3 \text{ mg d}^{-1}$  za UTW in  $3,1 \text{ mg d}^{-1}$  za TM). Sicer statistične analize niso pokazale večjih razlik med negativnimi kontrolami in obdelavo (enosmerna ANOVA:  $F = 1,784$ ,  $p = 0,174$ ).



Slika 11. Rast *Lymnea (Radix) auricularia*, izražena kot dnevno povečanje teže v času 21-dnevnega obdobja izpostavljenosti. Stopnje napake predstavljajo standardno napako

Prav tako je bilo dnevno podaljšanje dolžine nižje pri vseh obdelavah v primerjavi z negativno kontrolo ( $105,7 \pm 44,7 \text{ } \mu\text{m d}^{-1}$ ); UTW in TM pa sta pokazali učinek s trendom v obliki kupole (slika 12), pri katerem je bila najvišja stopnja rasti izmerjena pri T15 ( $94,3 \pm 43,7 \text{ } \mu\text{m d}^{-1}$  za UTW in  $85,5 \pm 10,9 \text{ } \mu\text{m d}^{-1}$  za TM). Sicer niso bile opažene pomembne razlike med negativno kontrolo in izpirki (enosmerna ANOVA:  $F = 0,558$ ,  $p = 0,756$ ).



Slika 12. Rast *Lymnea (Radix) auricularia*, izražena kot dnevno podaljšanje dolžine v času 21-dnevnega obdobja izpostavljenosti. Stopnje napake predstavljajo standardno napako.

Na podlagi rezultatov ocene tretje stopnje je bila obdelava TM po učinku na vodne indikatorje v celoti enaka neobdelanemu lesu. Sicer se izkaže potreba po vsaj 15-dnevnem času izpostavitve pred uporabo materiala, da se tako omeji obremenitev okolja. Po predlagani oceni ITS je bilo tako ugotovljeno, da je obdelava TM varna, brez dodatnega tveganja na okolje, niti pri daljši izpostavljenosti.

## ZAKLJUČKI

Iz testov TIER I izhaja, da je glede na sprejeta merila za ITS ocenjevanje za sladko vodo le obdelava TM imela toksične učinke, ki so enaki ali celo nižji od tistih, beleženih pri neobdelanem lesu (UTW). Ugotovljeno pa je bilo, da UC3 ni prijazna do vodnega okolja zaradi visoke akutne strupenosti na bakterije, alge in rakce ne glede na čas izpostavljenosti. Pridobljeni rezultati prvonivojske ocene so pokazali, da je samo obdelava TM primerna za drugo fazo ocenjevanja. Na podlagi rezultatov na ravni TIER II smo lahko opazili, da je obdelava TM primerna za oceno III ravni. Kljub temu so tudi splošni učinki izpirka neobdelanega preizkušanca (UTW) pri končni točki razpočenja ovojnice pri polžu *L. auricularia* pokazali potrebo po času izpostavljenosti, daljšem od 15 dni, s čimer je mogoče odpraviti toksično delovanje izpirka na razvoj ličink. Na podlagi splošnih rezultatov kroničnih testov stopnje III na školjkah in rakcih je jasno razvidno, da ima obdelava TM enake vplive v smislu obeh vodnih indikatorjev, ki sta bila uporabljena za neobdelan les (UTW). Iz rezultatov preizkuševanja, predvsem tistih iz testa o razmnoževanju *D. magna*, je bilo jasno ugotovljeno, da je treba pred uporabo materiala zagotoviti minimalni 15-dnevni čas izpostavljenosti, s čimer zajezimo vpliv na sladkovodno okolje. Predlagana ocena ITS je tako pokazala, da je obdelava TM okolju prijazna, saj niti pri daljši izpostavljenosti ne povzroča dodatnega tveganja za okolje.

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## ATTIVITÀ 15 /ACTIVITY 15

### Allegato 1. Analisi bibliografica relativa alle procedure di lisciviazione / Attachment 1. Bibliographic analysis related to leaching procedures

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Oak wood treated with commercial copper-based antifouling paint.	Tap water, estuarine water, and seawater	Wood was cut into 3 × 1 × 1 cm mini-bars and mini-bars were pre-treated and painted. Painted mini-bars were allowed to dry for 6 h, 24 h or 7 d, and then submerged in water. Each mini-bar was weighed before and after painting to determine mass of paint applied.	Wood mini-bars were submerged in 20mL of water and were kept in conditions simulating natural surface waters: cool white fluorescent lights (14:10 light:dark, 80–100 μmol/m <sup>2</sup> -s) at 20 °C with shaking (125 rpm). Release of Cu from wood and aluminum mini-bars was monitored as a time series for 180 d.		Adeleye A. S., Oranu E. A., Tao M., Keller A. A., 2016, <i>Release and detection of nanosized copper from a commercial antifouling paint</i> , Water Research, 102, 374-382.
Leachate from natural wood shavings from sawmill	Rainwater		Leachate was collected from within the heaps of wood shavings (Figure 1C) at five different points in the sawmill into a clean transparent 25 L plastic container to give homogenous mixture from the site.	Sieved (0.7 mm sieve) to remove wood debris.	Bakare A. A., Alimba C. G., Hassan T., Ajayi E. O., Sowunmi A. A., 2017, <i>Leachates biological treatment, toxicological characteristics and potential impacts on the environment</i> , Environmental research advances, 4.
Blocks wood treated with Chromated Copper Arsenate (CCA)	Synthetic Precipitation Leaching Procedure (SPLP) was used as the synthetic rainwater.		The wood was cut into 2 × 2 in blocks and extracted for 18 hrs with SPLP solution (pH=4.2) using a large mechanical rotator.	The resulting leachate was transferred to a pressure filtration apparatus and the solution was filtered through an acid-rinsed (20% HNO <sub>3</sub> ) filter (0.7 μm pore size).	Dagan R., Bitton G., Townsend T. G., 2006, <i>Metal Transport and Bioavailability in Soil Contaminated with CCA-Treated Wood Leachates</i> , Soil & Sediment Contamination, 15:1, 61-72.
Blocks of southern yellow pine treated with alkaline copper quaternary (ACQ) and copper boron azole (CBA), along with chromated copper arsenate (CCA)- treated wood	Eight natural waters: two rivers, three lakes, two wetlands, and the Atlantic Ocean. Deionized water and moderately hard water	Blocks of approximately 80 g (average surface area 140-150 cm <sup>2</sup> ) were obtained by cutting pieces of treated dimensional lumber using a power saw. Sawdust samples were collected for each treated wood type for subsequent total Cu measurements.	Immersion of the sample block (80 g block in this experiment) for 24 h within 1.6 L of leaching solution, resulting in a liquid to solid ratio (L/S) of 20:1.		Dubey B., Townsend T., Solo-Gabriele H., Bitton G., 2007, <i>Impact of Surface Water Conditions on Preservative Leaching and Aquatic Toxicity from Treated Wood Products</i> , Environmental Science Technology, 41, 3781-3786.
Leachate/runoff from wood recycling facilities	Rainwater		Samples of solid wood mulch and leachate/runoff were collected from three Class B wood recycling facilities in New Jersey over a 1.5 year period. A total of 26 leachate/runoff samples were collected either during or within 24 h of the occurrence of different rainfall events.		Kannepalli S., Strom P. F., Krogmann U., Subroy V., Gimenez D., Miskewitz R., 2016, <i>Characterization of wood mulch and leachate/runoff from three wood recycling facilities</i> , Journal of Environmental Management 182, 421-428

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Leachate from wood pressure-treated with micronized copper azole	Rainwater	Two pieces of lumber (originally 8' × 3.5" × 1.5" in size) were acquired and cut into three sizes: large (5 × 8,9 × 3,8 cm), medium (5 × 5,8 × 3,8 cm), and small (5 × 3 × 3,8 cm). All samples were weighed, and their exact physical dimensions measured.	Two Teflon straps (purchased from Industrial Netting), were affixed to each sample so that the samples could be secured in place over a rain-water runoff collection jar, thereby preventing sample soaking in accumulated rainwater. Samples were affixed such that the grain of the wood was perpendicular to incoming rainfall (top face exposed surface area 1 cm <sup>2</sup> ).		Lankone, R. , Goodwin, D. , Sung, L. , Ruggiero, E. , Vilsmeier, K. , Mueller, P. , Pulbere, S. , Challis, K. , Bi, Y. , Westerhoff, P. , Ranville, J. , Fairbrother, D. and Wohlleben, W., 2019, <i>Evaluating performance, degradation, and release behavior of a nanoform pigmented coating after natural and accelerated weathering</i> , NanoImpact
Wood spruce ( <i>Picea</i> sp.) treated with chromated copper arsenate (CCA) and alkaline copper quaternary (ACQ)	Synthetic Rainwater	<i>Picea</i> sp. lumber about 5 × 10 cm was treated for two weeks with ACQ or CCA following industry standards. The wood was shredded into small chips (about 0,2 × 0,2 × 0,2 cm) and approximately 0.6 kg of each wood type was placed into separate 20 L acid-washed glass containers.	Synthetic rain water was added about 6 L to each container to achieve a mixture with 10% (w/v) of wood:synthetic rainwater. The mixture was stirred to inundate all wood chips and incubated at 20 °C for 4 days.	Wood leachates were filtered (<0.2 µm under vacuum) and stored in acid-washed glass containers.	Leduc F., Whalena J. K., Sunaharab G. I., 2008, <i>Growth and reproduction of the earthworm Eisenia fetida after exposure to leachate from wood preservatives</i> , Ecotoxicology and Environmental Safety 69, 219–226
Natural wood: <i>Pouteria guianensis</i> , <i>Minquartia guianensis</i> , <i>Eschweilera</i> spp, <i>Quercus</i> spp, <i>Picea abies</i> .	Aerated artificial sea water	A predetermined ratio was maintained between wood block surface area (As) and water leaching solution volume (Vls), corresponding to the immersion of a wood block of 0.1m×0.1m×0.1m in 1 L of water (As/Vls = 0.6 cm <sup>-1</sup> ). The blocks were cut to the required dimensions (3 × 3 × 6,5 cm) with a circular saw.	The leaching procedure consisted of agitating the blocks fixed to a Jar Test at 60 rpm at 20 °C in a leaching medium consisting of aerated artificial sea water. The first cycle lasted 24 h, the leaching fluid was then completely renewed and the leaching procedure continued for a further 48 h (72 h cycle)	pH values below 7.5 were corrected by adding NaOH 0.1M aliquots	Libralato G., Losso C., Ghirardini A. V., 2007, <i>Toxicity of untreated wood leachates towards two saltwater organisms (Crassostrea gigas and Artemia franciscana)</i> , Journal of Hazardous Materials 144, 590–593
Wood chips of <i>Pinus resinosa</i> treated with pentachlorophenol (CBA);	sodium hydroxide solution (pH 10).	Wood chips were made from circular slices of Pentachlorophenol-Treated out-of-service poles. The approximate dimensions of the wood chips is longitudinal x radial x tangential = 5 x 0.5 x 0.5 cm.	A stock leachate solution was produced batchwise by recirculating a sodium hydroxide solution (pH 10) through a cylindrical plexiglass column (8.7 cm i.d. and 100 cm length) that was packed with PCP-treated wood chips. The pH of the leachate was controlled and the temperature was controlled at 90°C. The sodium hydroxide solution was recirculated for 48 h.		Mollah A. H., Allen D. G., 1999, <i>Biodegradation and Detoxification of Wood Leachate from Pentachlorophenol-Treated Poles</i> , The Canadian Journal of Chemical Engineering 77.
Blocks wood of <i>P. sylvestris</i> , <i>P. radiata</i> , <i>F. sylvatica</i> , <i>F. excelsior</i> , Southern yellow pine tretated with furfurylation process. Reference with chromated copper arsenate (CCA).	Demineralized water	After treatment the boards were cut into samples with dimensions 0,15x0,25x0,50cm for EN84 and Internal leaching procedure and 0,15x0,70x1,5cm for OECD procedure.	EN84: 10 water changes during 14 d in demineralized water with a water-over-wood volume equaling 5. Leaching water was collected after 1, 7, and 14 d. Internal leaching procedure: The specimens were submersed in demineralized water for 48 h with water changes and collection of leaching water after 0.5, 1, 6, 12, 24, and 48 h.  OECD Guideline 313: recommended ratio (0.4 cm <sup>2</sup> /ml) between the surface of the wood and the amount of water. The submersion test was continued for 32 d with demineralized water, during which the water was replaced eight times at set intervals. The leachates were collected after 0.25, 1, 2, 4, 6, 8, 15, 22, and 32d..	pH values below 7.5 were corrected by adding NaOH 0.5M aliquots	Pilgard A., Treu A., Zeeland V., Gosselink J. A., Westin M., 2010, <i>Toxic hazard and chemical analysis of leachates from furfurylated wood</i> , Environmental Toxicology and Chemistry 29, 1918–1924.

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Blocks wood of <i>Pinus radiata</i> , <i>Pinus sylvestris</i> treated with three different furfurylation process. Untreated <i>P. sylvestris</i> and <i>P. radiata</i> sapwood were used as reference material.	Demineralized water	Test specimens were larger than the standard recommendations and therefore have been cut at 0,15x0,70x1,5cm . The <i>P. radiata</i> material was planed prior to treatment, because the wood surface was suspected to have been treated with biocides (antisap-stain agents).	OECD Guideline 313: recommended ratio (0.4 cm <sup>2</sup> /ml) between the surface of the wood and the amount of water. The submersion test was continued for 32 d with demineralized water, during which the water was replaced eight times at set intervals. The leachates were collected after 0.25, 1, 2, 4, 6, 8, 15, 22, and 32d. The shower test: method was used to simulate rainfall. Every day, the specimens were sprayed with approximately 2 liters of freshwater over a time span of 1 h. This test was run for 2 weeks, and leachates were collected every day over a period of 10 d.	pH values below 7.5 were corrected by adding NaOH 0.1M aliquots	Pilgard A., Vetter L., Acker J.,z and Westin M., 2010b, <i>Toxic hazard of leachates from furfurylated wood: comparison between two different aquatic organisms</i> , Environmental Toxicology and Chemistry 29, 1067–1071.
Natural wood: <i>Populus tremuloides</i> , <i>Pinus contorta</i> , <i>Picea engelmannii</i> x <i>glauca</i> Parry, <i>Picea mariana</i> . As well as from two wood chipping sites using mixes of lodgepole pine and hybrid or black spruce.	1) Natural weather conditions 2) deionized water 3) deionized water		1) Tree species common to the Prince George Forest District mixed in two open 322-L containers at environmental condition for 23 month; 2)The static exposure consisted of placing 2 kg of wood chips in a polypropylene 1-cm opening mesh bag in 18 L of water for 28 days at room temperature and ambient light (9:1 water:wood). 3)A portable rainfall simulator was used to generate a heavy rainfall event of approximately 100 mm x h-1.		Rex J., Dubé S., Krauskopf P., Berch S., 2016, <i>Investigating Potential Toxicity of Leachate from Wood Chip Piles Generated by Roadside Biomass Operations</i> , Forests.
Natural wood: birch ( <i>Betula sp.</i> ) and spruce ( <i>Picea abies</i> ) woodchip, and their mixture with washed gravel (WG).	Tap water		Pure organic and mixed filling materials were used in one-stage batch leaching tests with a liquid to solid ratio of 10l·kg <sup>-1</sup> without size reduction. Using the calculated dry matter content of each material determined at the beginning, the equivalent mass of raw material (without any pre-treatment) was put into the 2l reagent bottles and filled with 1.6l of tap water. Reagent bottles were placed on the overhead shaker and mixed for 1–5 days at laboratory temperature with 5 RPM.	One sample of each material was decanted, centrifuged, and filtered using membrane paper (4 µm) on a daily basis.	Šereš Z., Došenović T., Šoronja-Simović D., Maravić N., Šaranović Z., Šereš L., Šćiban M., Djordjević M., 2019, <i>Modelling of cross-flow microfiltration coupled with bentonite treatment in sugar beet molasses purification</i> , The Canadian Journal of Chemical Engineering 97, 947-954.
Block of southern yellow pine treated with alkaline copper quaternary (ACQ), copper boron azole (CBA) and copper citrate (CC).	1) Toxicity Characteristic Leaching Procedure (TCLP) fluid 2) Synthetic Precipitation Leaching Procedure (SPLP) fluid, 3) deionized water, 4) synthetic seawater.	The samples used for the leaching analysis were cut into small blocks (2.5x5x10 cm) using a mill saw and then ground to particles less than 3 mm in diameter.	Treated wood samples were individually leached with four leaching fluids. Two liters of leaching fluids were combined with 100 g of ground wood (yielding a 1:20 solid/liquid ratio) in a glass container which was capped with a teflon-lined lid and placed on a rotary extractor for 18 ±2 hours.	The recovered leachates were filtered using a pressurized filtration apparatus with a 0,7 0 µm glass fiber filter.	Stook K., Dubey B., Ward M., Townsend T., Bitton G., Solo-Gabriele H., 2004, <i>Heavy Metal Toxicity of Pressure Treated Wood Leachates with MetPLATE™</i> , Bulletin Environmental Contamination and Toxicology 73, 987–994.

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Block of southern yellow pine treated with preservatives included chromated copper arsenate (CCA), alkaline copper quaternary (ACQ), copper boron azole (CBA), copper citrate (CC), and copper dimethyldithiocarbamate .	1) Toxicity Characteristic Leaching Procedure (TCLP) fluid 2) Synthetic Precipitation Leaching Procedure (SPLP) fluid, 3) deionized water, 4) synthetic seawater.	The samples used for the leaching analysis were cut into small blocks (2.5x5x10 cm) using a mill saw and then ground to particles less than 3 mm in diameter.	1)A 100-g, size-reduced, representative wood sample was placed into a 2.2-L Teflon-lined glass container. Two liters of the TCLP extraction fluid was added and the mixture was rotated for 18 (2 h). Other leaching tests were performed in the same manner as the TCLP, with the exception of the leaching fluid. The synthetic precipitation leaching procedure contains nitric and sulfuric acid at a 40/60 weight ratio. The synthetic seawater extraction solution was prepared by dissolving 0.5 cups of Instant Ocean in 3.8-L of deionized water; the resulting salinity of the solution was 3.2 psu.	The leachate was filtered through a 0.7-µm glass fiber filter	Stook K., Tolaymat T., Ward M., Dubey B., Townsend T., Solo-Gabriele H., Bitton G., 2005, <i>Relative Leaching and Aquatic Toxicity of Pressure-Treated Wood Products Using Batch Leaching Tests</i> , Environmental Science and Technology 39, 155-163.
Natural Sawdust and bark from: Pedunculate oak ( <i>Quercus robur</i> ), Scots pine ( <i>Pinus sylvestris</i> ), European larch ( <i>Larix decidua</i> ), Norway spruce ( <i>Picea abies</i> ) and European beech ( <i>Fagus sylvatica</i> )	Distilled water	The sawdust and the bark were obtained from sawmills located in Nybro, southern Sweden.	The leachate was produced according to the batch leaching Standard Method SS-EN 12457-2, Due to the very high water absorbing capacity of sawdust, which makes very difficult to run a leaching test with the ratio 10:1, a liquid to solid (L/S) ratio (40:1) four times the one recommended (10:1) was used. Leachate from bark of oak, pine and spruce where produced according to the same batch test method mentioned above. However, in these cases, the recommended L/S ratio of 10:1 was used.		Svensson H., Svensson B., Hogland W., Marques M., 2012, <i>Acute Toxic Effects Caused by Leachate from Five Different Tree Species on Artemia Salina and Vibrio Fischeri</i> , Journal of Biobased Materials and Bioenergy 6, 214–220.
1) water from pond that receives oak wood leachate generated due to log irrigation; 2) water from ditch that receives leachate generated due to rainfall contact with oak wood chip piles; 3) leachate generated via leaching test with oak sawdust under laboratory conditions.	1) irrigation water; 2) rainfall; 3) Distilled water	Vacuum filtration: filter paper grade 005 (20 µm), Munktell 00R (10 µm), and Watchman GF/C (1.2 µm); Next, with Millipore stirred Cell Model 8400 ultra-filtration discs Millipore 100 kDa (approximately 13 nm), 10 kDa (approximately 5 nm) and 1 kDa (approximately 2 nm). After each filtration step, 50 mL of water was removed and used for chemical analyses.	The leachate was produced according to the batch leaching Standard Method SS-EN 12457-2, Due to the very high water absorbing capacity of sawdust, which makes very difficult to run a leaching test with the ratio 10:1, a liquid to solid (L/S) ratio (40:1) four times the one recommended (10:1) was used. Leachate from bark of oak, pine and spruce where produced according to the same batch test method mentioned above. However, in these cases, the recommended L/S ratio of 10:1 was used.		Svensson H., Jani Y., Hogland W., Marques M., 2014, <i>Particle size characterization of oak wood leachate: chemical oxygen demand and toxicity distribution within different fractions</i> , Water Science & Technology 70.3
Natural sawdust obtained from oak ( <i>Quercus robur</i> ), maple ( <i>Acer platanoides</i> ), pine ( <i>Pinus sylvestris</i> ), beech ( <i>Fagus sylvatica</i> ) and wood chips from oak and pine.	Distilled water	Sawdust is wood debris with particles size 4 mm. Wood chips are larger than 4 mm and produced using a chipper that grinds wood to sizes up to 10 cm in diameter. 9 mg of sieved sawdust (particles 4 mm) were used to estimate the moisture content in the wood residues. The sawdust was dried in an oven for 24 h at 105°C.	The sawdust used in the leaching test was not dried in the oven, but the moisture content previously measured was taking into account for calculations purposes, as described in the Standard Method SS-EN 12457-2. Because of the very high absorption capacity of sawdust, the solid/liquid (S/L) ratio of 1 : 10 recommended by the Standard Method SS-EN 12457-2 after a preliminary trial was considered inappropriate, and S/L ratios of 1 : 20 (9 mg for 180 mL) and 1 : 40 (9 mg for 360 mL) were used instead in the batch tests.	The water phase was filtrated with a GF/C Whatman 0,45 µm microfiber filter	Svensson H., Marques M., Kaczala F., Hogland W., 2014, <i>Leaching patterns from wood of different tree species and environmental implications related to wood storage areas</i> , Water and Environment Journal.

Matrix	Type of leaching fluid	Wood treatment before leaching procedure	Description of leaching procedure	Post leaching procedure	Reference
Natural chips wood of aspen ( <i>Populus tremuloides</i> )	Dechlorinated tap water	Five young healthy trees, ranging 28 to 64 years in age and 9.7 to 15.7 cm butt diameter (mean 13.6 cm), were cut at ground level with a chain saw. Trees were topped, limbed, and cut into 2.5-m (8-ft) lengths in the field and then reduced to chips (0.5 to 10 cm in length) with a commercial chipper.	50 kg fresh weight (about 28 kg dry weight) of aspen chips in 250 L of water in a 280-L vat lined with polyethylene. The ratio of aspen to water was thus 8.9:1 by dry mass at room temperature. Leaching continued for 35 d at room temperature and until chemical and physical characteristics ceased to show rapid changes.	0.45 mm microfiber filter	Taylor B. R., Goudey J. S., Carmichael N. B., 1996, <i>Toxicity of aspen wood leachate to aquatic life: laboratory studies</i> , Environmental Toxicology and Chemistry 15, 150–159.
Leachate from natural woodpiles of trembling aspen ( <i>Populus tremuloides</i> )	Rainwater or snowmelt	The logs were transported to the field site, where they were stacked in two ranks, making a triangular logpile 5.5 m long and 1.5 m high. The logpile comprised 174 aspen logs with a total volume of 17.8 m <sup>3</sup> .	A polyethylene tarpaulin beneath the logpile, supported around the perimeter by more logs, created a basin (depth, 40 cm) that captured any runoff from the logpile. Part of the bottom row of logs could be half-submerged when the catchbasin was full.	Temperature of the air and leachate were measured and the specific conductance, pH, and dissolved oxygen tension of the leachate.	Taylor B. R., Carmichael N. B., 2003, <i>Toxicity and chemistry of aspen wood leachate to aquatic life: field study</i> , Environmental Toxicology and Chemistry 22, 2048–2056.
Natural wood with bark chipped from a mixture of conifer tree species including <i>Abies concolor</i> , <i>Pinus lambertiana</i> , <i>Calocedrus decurrens</i> , <i>Pinus jeffreyi</i> , <i>Abies magnifica</i> , and <i>Pinus contorta</i> .	Deionized water		Separate batches of wood-only chips and bark-only chips of the same Sierra Nevada mixed conifer tree species were soaked in water to create wood and bark leachates. The wood and bark were oven dried and 1.75 kg each were separately submerged in 14 L DI water for 6 days at 20°C.	The leachate liquids were poured through a 0.152-mm screen to remove solids prior to use as phytometer treatments.	Wolf E. C., Rejmánková E., Cooper D. J., 2019, Wood chip soil amendments in restored wetlands affect plant growth by reducing compaction and increasing dissolved phenolics, Restoration Ecology 27, 1128–1136.

**Allegato 2. Analisi bibliografica relativa alla caratterizzazione ecotossicologica e chimico-fisica dei lisciviati. / Attachment 2. Bibliographic analysis relating to the ecotoxicological and chemical-physical characterization of the leachates.**

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
Oak wood treated with commercial copper-based antifouling paint.	Total Cu, dissolved Cu, nanosized Cu, bulk Cu.		Cu <sub>2</sub> O particles in commercial antifouling paints may be released into natural waters during use. These released particles may include those that are nanoparticulate in size.		Adeleye A. S., Oranu E. A., Tao M., Keller A. A., 2016, <i>Release and detection of nanosized copper from a commercial antifouling paint</i> , Water Research, 102, 374-382.
Leachate from natural wood shavings from sawmill	COD, BOD, dissolved oxygen (DO), conductivity, chloride, sulphate, nitrate, pH, temperature, lignin and tannin, heavy metals: Pb, Cd, As, Cr Cu.	<i>C. gariepinus</i> , Sub-Lethal Concentrations and Micronucleus Analysis.	The concentrations of some of the SWL constituents were above permissible NESREA and USEPA limits	Leachate induced a time dependent acute toxicity and concentration-dependent significant increase in the frequencies of micronucleated peripheral erythrocytes and nuclear abnormalities in <i>C. gariepinus</i> .	Bakare A. A., Alimba C. G., Hassan T., Ajayi E. O., Sowunmi A. A., 2017, <i>Leachates biological treatment, toxicological characteristics and potential impacts on the environment</i> , Environmental Reserch Advances, 4.
Blocks wood treated with Chromated Copper Arsenate (CCA)	Metal Analysis, ecotoxicological test	<i>Ceriodaphnia dubia</i> , MetPLATE™, <i>Selenastrum capricornutum</i>	Arsenic has the highest concentration compared to the other two metals in all three types of soil. It appears, however, to have greater mobility in the sandy soil.	Despite the presence of copper, chromium and arsenic in elutriates collected from all three soil types, no toxicity was observed in any of the fractions collected from these columns.	Dagan R., Bitton G., Townsend T. G., 2006, <i>Metal Transport and Bioavailability in Soil Contaminated with CCA-Treated Wood Leachates</i> , Soil & Sediment Contamination, 15:1, 61-72.
Blocks of southern yellow pine (SYP) treated with alkaline copper quaternary (ACQ) and copper boron azole (CBA), along with chromated copper arsenate (CCA)- treated wood	pH, total dissolved solids (TDS), alkalinity, hardness, chloride, total organic carbon (TOC), chemical oxygen demand (COD), turbidity (in NTU). Cu, As, Cr, B concentrations, labile Cu.	MetPLATE	Results show that ACQ- and CBA-treated wood leach approximately 10 and 20 times more total Cu relative to CCA-treated wood and that the presence of organic and inorganic ligands in natural waters lowered the labile fraction of Cu relative to that from laboratory generated leaching solutions.	Lower toxicity was observed with <b>seawater</b> samples for each wood type compared with the other leachates. In general, among the other nine leachates for each wood treatment, toxicity followed the pattern CBA >ACQ>CCA. For all three treated wood leachates, the highest toxicity was observed from the DI leachate. This coincides with the relatively high labile copper concentrations associated with DI water.	Dubey B., Townsend T., Solo-Gabriele H., Bitton G., 2007, <i>Impact of Surface Water Conditions on Preservative Leaching and Aquatic Toxicity from Treated Wood Products</i> , Environmental Science Technology, 41, 3781-3786.
Leachate/runoff from wood recycling facilities	pH, BOD, COD, TSS, settleable solids, total Kjeldahl nitrogen (TKN), total phosphate-phosphorus (TP), tannin/lignin/phenolic (T/L/P) compounds, fecal coliforms, ecotoxicological test.	<i>D. rerio</i>	BOD, COD, and TSS concentrations were comparable to those of untreated domestic wastewater (raw sewage), although both lower and higher individual values were observed. TKN, TP and fecal coliform values were usually slightly lower than is typical for untreated domestic wastewater. BOD, COD, TSS, TKN, and TP median values were all much higher than for typical urban stormwater runoff, while fecal coliform levels were lower.	The developmental effects observed were yolk sac edema, abnormal spine curvature, kink tail, and delay in development. Only delay in development regularly occurred at lower concentrations. little acute toxicity other than the one case attributable to low pH was observed in the mulch leachate/runoff samples, and no correlation was found between delayed development and T/L/P.	Kannepalli S., Strom P. F., Krogmann U., Subroy V., Gimenez D., Miskewitz R., 2016, <i>Characterization of wood mulch and leachate/runoff from three wood recycling facilities</i> , Journal of Environmental Management 182, 421-428

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
Wood spruce ( <i>Picea</i> sp.) treated with chromated copper arsenate (CCA) and alkaline copper quaternary (ACQ)	Metal concentration with ICP-OES system, ecotoxicological test	<i>E. fetida</i>		We found that earthworm tissue concentrations of 80 mg As kg <sup>-1</sup> , 89 mg Cr kg <sup>-1</sup> and 90 mg Cu kg <sup>-1</sup> were not lethal to <i>E. fetida</i> . There was Cu bioaccumulation in <i>E. fetida</i> exposed to soil contaminated with a single metal (Cu from ACQ-treated wood), but Cu was less bioavailable in alkaline than neutral soil conditions. Exposure to Cu alone did not affect <i>E. fetida</i> growth or reproduction, but exposure to multiple metals (As, Cr and Cu from CCA-treated wood) produced soil-specific growth and reproduction outcomes.	Leduc F., Whalena J. K., Sunaharab G. I., 2008, <i>Growth and reproduction of the earthworm Eisenia fetida after exposure to leachate from wood preservatives</i> , Ecotoxicology and Environmental Safety 69, 219–226
Natural wood: <i>Pouteria guianensis</i> , <i>Minuartia guianensis</i> , <i>Eschweilera</i> spp, <i>Quercus</i> spp, <i>Picea abies</i> .	pH values 3, 24 and 72 h, Dissolved oxygen (DO), chemical oxygen demand (COD), ecotoxicological test.	<i>Artemia franciscana</i> , <i>Crassostrea gigas</i>		Leachate toxicities showed that AB<MM≈RO<AC<PA at 24 h and AB≈AC≈MM≈RO< PA at 72 h. From 24 to 72 h, toxic effects were reduced by more than 50% for all samples except for PA.	Libralato G., Losso C., Ghirardini A. V., 2007, <i>Toxicity of untreated wood leachates towards two saltwater organisms (Crassostrea gigas and Artemia franciscana)</i> , Journal of Hazardous Materials 144, 590–593
Wood chips of <i>Pinus resinosa</i> treated with pentachlorophenol;	PCP concentration, chloride ion	<i>Daphnia magna</i>	Complete degradation of PCP was also achieved in the cases for 120 mg PCP/L and a 12 h cycle time or 50 mg PCPiL and a 6 h cycle time	<i>Daphnia magna</i> toxicity tests showed that the presence of PCP in leachate caused toxicity, and the biodegradation of PCP resulted in significantly reducing the effluent toxicity to a value similar to that of PCP-free nutrient solution.	Mollah A. H., Allen D. G., 1999, <i>Biodegradation and Detoxification of Wood Leachate from Pentachlorophenol-Treated Poles</i> , The Canadian Journal of Chemical Engineering 77.
Blocks wood of <i>P. sylvestris</i> , <i>P. radiata</i> , <i>F. sylvatica</i> , <i>F. excelsior</i> , Southern yellow pine treated with furfurylation process. Reference with chromated copper arsenate (CCA).	Furan compound analysis, Maleic acid analysis, Ecotoxicological test	<i>Daphnia magna</i> , <i>Vibrio fischeri</i> (Microtox®)	The present study shows that the difference in toxicity most likely cannot be attributed to maleic acid, furan, furfural, furfuryl alcohol, or 2-furoic acid. However, the difference could be caused by the two substances 5-hydroxymethylfurfural and 2,5-furandimethanol.	The leachates from furfurylated wood have low toxicity. Earlier studies have shown that leachates from wood treated with furfuryl alcohol prepolymers have higher toxicity to <i>Vibrio fischeri</i> than leachates from wood treated with furfuryl alcohol monomers.	Pilgard A., Vetter L., Acker J.,z and Westin M., 2010b, <i>Toxic hazard of leachates from furfurylated wood: comparison between two different aquatic organisms</i> , Environmental Toxicology and Chemistry 29, 1067–1071.
Blocks wood of <i>Pinus radiata</i> , <i>Pinus sylvestris</i> treated with three different furfurylation process. Untreated <i>P. sylvestris</i> and <i>P. radiata</i> sapwood were used as reference material.	Ecotoxicological test	<i>Daphnia magna</i> , <i>Vibrio fischeri</i> (Microtox®)		The results from the toxicity tests show that the bacteria were more sensitive than <i>D. magna</i> to furfurylated wood leachates. These results indicate that, with general furfurylated wood, leachates are not more toxic than untreated wood leachates, the only exception being <i>P. radiata</i> treated with treatment A.	Pilgard A., Vetter L., Acker J.,z and Westin M., 2010b, <i>Toxic hazard of leachates from furfurylated wood: comparison between two different aquatic organisms</i> , Environmental Toxicology and Chemistry 29, 1067–1071.

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
Natural wood: <i>Populus tremuloides</i> , <i>Pinus contorta</i> , <i>Picea engelmannii</i> x <i>glauca</i> Parry, <i>Picea mariana</i> . As well as from two wood chipping sites using mixes of lodgepole pine and hybrid or black spruce.	pH, True Color, Total Organic Carbon, Chemical Oxygen Demand, Ammonium, Resin Acids, Phenols, Microtox.	<i>Vibrio fischeri</i> (Microtox®)	Aspen chips produced the most acidic leachate with higher organic, phenolic, and ammonia concentrations compared to the coniferous and mixed samples. Coniferous samples showed some subtle differences with the spruce samples being more similar to each other than they were to pine.	All samples collected during the 580 days of exposure produced a toxic response within the 15 minute test period; all leachate samples exhibited a consistent toxicity response over the 4-week period, with each toxic sample at concentrations below 10% by volume. There was no significant difference in leachate toxicity between dry and wet exposure within tree species or between tree species. The dry chip Muldowan 18 leachate samples required a higher concentration to induce toxicity than the wet sample and all other samples.	Rex J., Dubé S., Krauskopf P., Berch S., 2016, <i>Investigating Potential Toxicity of Leachate from Wood Chip Piles Generated by Roadside Biomass Operations</i> , Forests.
Natural wood: birch ( <i>Betula sp</i> ) and spruce ( <i>Picea abies</i> ) woodchip, and their mixture with washed gravel (WG).	total organic carbon, dissolved organic carbon, phenol index.	<i>Daphnia magna</i> , <i>D. subspicatus</i> , <i>Lemna minor</i> , <i>Vibrio fischeri</i> (Microtox®)	Statistical analysis of the data showed no significant influence of the leaching period on the quality of effluent represented by concentrations of FNI, TOC, DOC and an inhibitory effect on aquatic organisms. The type of filling medium resulted in significantly different levels of the leachate quality, in terms of TOC, DOC and FNI. The pure organic material (BWCH and SWCH) resulted in a significantly higher concentration of FNI and organic carbon as well as higher inhibition of the tested aquatic organisms.		Šereš Z., Došenović T., Šoronja-Simović D., Maravić N., Šaranović Z., Šereš L., Šćiban M., Djordjević M., 2019, <i>Modelling of cross-flow microfiltration coupled with bentonite treatment in sugar beet molasses purification</i> , The Canadian Journal of Chemical Engineering 97, 947-954.
Block of southern yellow pine treated with alkaline copper quaternary, copper boron azole and copper citrate.		MetPLATE		Leachate from untreated southern yellow pine were found to be no-toxic to the MetPLATE test bacteria. The toxicity was greatest for CCA-treated wood. Higher EC50 values were calculated in the second part of the experience using larger wooden blocks.	Stook K., Dubey B., Ward M., Townsend T., Bitton G., Solo-Gabriele H., 2004, <i>Heavy Metal Toxicity of Pressure Treated Wood Leachates with MetPLATE™</i> , Bulletin Environmental Contamination and Toxicology 73, 987-994.
Block of southern yellow pine treated with preservatives included chromated copper arsenate, alkaline copper quaternary, copper boron azole, copper citrate, and copper dimethyldithiocarbamate.	Arsenic, chromium, and copper	<i>Ceriodaphnia dubia</i> , <i>Raphidocelis subcapitata</i> , <i>Vibrio fischeri</i> (Microtox®)	CCA-treated wood leached greater concentrations of arsenic and copper relative to chromium, with copper leaching more with the TCLP and synthetic seawater. Copper leached at greater concentrations from the arsenic-free preservatives relative to CCA.	All of the arsenic free alternatives displayed a greater degree of aquatic toxicity compared to CCA. Invertebrate and algal assays were more sensitive than Microtox.	Stook K., Tolaymat T., Ward M., Dubey B., Townsend T., Solo-Gabriele H., Bitton G., 2005, <i>Relative Leaching and Aquatic Toxicity of Pressure-Treated Wood Products Using Batch Leaching Tests</i> , Environmental Science and Technology 39, 155-163.
Natural Sawdust and bark from: Pedunculate oak ( <i>Quercus robur</i> ), Scots pine ( <i>Pinus sylvestris</i> ), European larch ( <i>Larix decidua</i> ), Norway spruce ( <i>Picea abies</i> ) and European beech ( <i>Fagus sylvatica</i> )	pH, TOC, phenolic compounds (reported as total poly-phenols)	<i>Artemia salina</i> , <i>Vibrio fischeri</i> (Microtox®)	The pH varied from very acid (for oak sawdust leachate pH 4.0) to neutral (for spruce sawdust leachate pH 7.2). Similar to TOC, within the same species, the phenols content was also significantly higher (p < 005) in bark than in sawdust regardless the tree species tested (oak, pine and spruce).	Microtox® test is more sensitive to all tested waters than the crustacean <i>A. salina</i> . Regarding <i>V. fischeri</i> , two of the tested sawdust leachates (pine and oak) and all three tested bark leachates (spruce, pine and bark) were highly toxic while minor toxic effects was observed for the leachate from other species (spruce, beech and larch sawdust).	Svensson H., Svensson B., Hogland W., Marques M., 2012, <i>Acute Toxic Effects Caused by Leachate from Five Different Tree Species on Artemia Salina and Vibrio Fischeri</i> , Journal of Biobased Materials and Bioenergy 6, 214-220.



Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
1) water from pond that receives oak wood leachate generated due to log irrigation; 2) water from ditch that receives leachate generated due to rainfall contact with oak wood chip piles; 3) leachate generated via leaching test with oak sawdust under laboratory conditions.	pH, COD, total content of polyphenols (PP), ecotoxicological test	<i>Artemia salina</i> , <i>L. sativa</i> , <i>Vibrio fischeri</i> (Microtox®)	50% of the COD found in the samples collected in the field is in the 100 kDa fraction while only 10% of the COD is removed from the same water fraction after filtration of the laboratory leachate. The 100 kDa filter removes the largest amount of PP found in the field samples, while the amount of PP in the laboratory leachate is more evenly distributed along the fractions.	Only FSP 2 and LAB-L were toxic to <i>A. salina</i> and <i>V. fischeri</i> . No sample had a toxic effect on <i>L. sativa</i> germination. The toxic effect drops significantly for wood chips after the 100 kDa (over to) filter in both Microtox and Artemia tests, whereas the drop is not that significant for leachate water.	Svensson H., Jani Y., Hogland W., Marques M., 2014, <i>Particle size characterization of oak wood leachate: chemical oxygen demand and toxicity distribution within different fractions</i> , Water Science & Technology 70.3
Natural sawdust obtained from oak ( <i>Quercus robur</i> ), maple ( <i>Acer platanoides</i> ), pine ( <i>Pinus sylvestris</i> ), beech ( <i>Fagus sylvatica</i> ) and wood chips from oak and pine.	DOC, pH, conductivity, colour, phenols, tannins and lignin, biochemical oxygen demand (BOD).		The oak chips batch test released DOC up to 312 h of contact time when equilibrium was apparently reached. Based on sawdust leaching tests obtained from maple, oak, pine and beech, the organic compounds measured as DOC were mainly released during the first 24 h and the additional DOC release remains for up to 216 h of contact time. For pine sawdust, the DOC released was six times higher than that released from pine wood chips. For oak sawdust, the DOC released was 16 times higher than for oak wood chips. The pH range ranged from 4.1 (oak) to 6.8 (maple). Oak leachate color was found to be 8.7 and 15.2 times the color measured for pine, respectively. BOD7 / DOC ranged from 12 to 28% of the COD. Concentrations of phenol range from 12 to 77 mg / L. Unlike the total leaching of DOC per unit of dry matter, the DOC concentration (mg / L) in the aqueous phase is influenced by the S / L ratio. The DOC concentration in the leachate after a batch test decreased to approximately 40% after the second wash compared to unwashed sawdust.		Svensson H., Marques M., Kaczala F., Hogland W., 2014, <i>Leaching patterns from wood of different tree species and environmental implications related to wood storage areas</i> , Water and Environment Journal.
	Microtox, conductance, pH, DO, and absorbance (465 nm), 5-day BOD, COD, color, resin and fatty acids, nitrogen, total phenols, total organic carbon	<i>Daphnia magna</i> , <i>Oncorhynchus mykiss</i> , <i>Vibrio fischeri</i> (Microtox®)	The analyzes revealed an amber color, low pH (4.0), extremely high BOD (0.2.600 mg / L) and high conductivity (1140 mS / cm). The leachate was rich in phenols (30 mg / l), organic carbon (2,480 mg / l) and organic nitrogen (13 mg / l).	Median acutely toxic concentrations of leachate were consistently 1 to 2% of full strength for trout and <i>Daphnia</i> . Inhibition of bacterial metabolism began at concentrations below 0.3%. Leachate was less toxic to plant life but inhibited algal growth at concentrations of 12 to 16%.	Taylor B. R., Goudey J. S., Carmichael N. B., 1996, <i>Toxicity of aspen wood leachate to aquatic life: laboratory studies</i> , Environmental Toxicology and Chemistry 15, 150–159.
Leachate from natural woodpiles of trembling aspen ( <i>Populus tremuloides</i> )	pH, conductivity Color, BOD, COD, Phenols, TOC, Toxicity index	<i>Daphnia magna</i> , <i>Oncorhynchus mykiss</i> , <i>Vibrio fischeri</i> (Microtox®)	Aspen leachate was characterized by dark color, acid pH (5.0–6.5), high conductivity (200–500 mS / cm), high to very high BOD (500–5,000 mg / L) and concentrations of TOC (500–2,000 mg / L), varying levels of phenolic compounds (2–27 mg / L) and low dissolved oxygen voltages (.2 mg / L).	The leachate varied from weakly toxic (mean EC50 or LC50 . 10%) to very toxic (mean EC50 or LC50 , 1%). Leachate was produced in every season for two years and was chemically and toxicologically similar to that produced from wood chips in the laboratory (vedi Taylor, 1996)	Taylor B. R., Carmichael N. B., 2003, <i>Toxicity and chemistry of aspen wood leachate to aquatic life: field study</i> , Environmental Toxicology and Chemistry 22, 2048–2056.

Matrix	Type of test	Test species	Result Chemical test	Result Ecotox test	Reference
Natural wood with bark chipped from a mixture of conifer tree species including <i>Abies concolor</i> , <i>Pinus lambertiana</i> , <i>Calocedrus decurrens</i> , <i>Pinus jeffreyi</i> , <i>Abies magnifica</i> , and <i>Pinus contorta</i> .	Growth test, phenolics concentration	<i>Scirpus microcarpus</i>		Leachate of mixed conifer bark contains approximately 20 times higher phenolics concentration than similarly prepared wood-only leachate. High levels of phenolics (211 mg/L) inhibited <i>S. microcarpus</i> growth; this growth inhibition was more pronounced in nutrient-limited conditions and in smaller individuals.	Wolf E. C., Rejmánková E., Cooper D. J., 2019, Wood chip soil amendments in restored wetlands affect plant growth by reducing compaction and increasing dissolved phenolics, <i>Restoration Ecology</i> 27, 1128–1136.

### Allegato 3: Analisi chimiche dei campioni di lisciviato. / Attachment 3: Chemical analyses of leachate samples.

Di seguito vengono riportate le analisi chimiche effettuate da Agri.Te.Co, sono riportate solo i valori relativi al rame in quanto per le altre sostanze analizzate (polifenoli e terpeni) non risulta essere presente una quantità rilevante. / Below are the chemical analyzes carried out by Agri.Te.Co, only the values relating to copper are reported as for the other substances analyzed (polyphenols and terpenes) there is not a significant quantity present.

Sample		Parameter	MU	Value
UTW	t0	Copper	µg/L Cu	30,32
	t1	Copper	µg/L Cu	23,7
	t2	Copper	µg/L Cu	23,5
TM	t0	Copper	µg/L Cu	7,89
	t1	Copper	µg/L Cu	34,1
	t2	Copper	µg/L Cu	47,5
UC3	t0	Copper	µg/L Cu	2780
	t1	Copper	µg/L Cu	883
	t2	Copper	µg/L Cu	630