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TITLE:

Rapid Testing of Resistance of Timber to Biodegradation by Marine Wood-Boring Crustaceans

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KEYWORDS:

Limnoria, gribble, faecal pellets, feeding rate, wood preservation, EN275, marine biodegradation

SUMMARY:

This protocol presents a method for assessing the feeding rate of the wood-boring crustacean, *Limnoria*, by measuring faecal pellet production. This method is designed for use in non-specialist labs and has potential for incorporation into standard testing protocols, to evaluate enhanced wood durability under marine conditions.

ABSTRACT:

Wood-boring invertebrates rapidly destroy marine timbers and wooden coastal infrastructure, causing billions of dollars of damage around the globe every year. As treatments of wood with broad spectrum biocides, such as creosote and chromated copper arsenate (CCA), are now restricted in marine use by legislation, naturally durable timber species and novel preservation methods of wood are required. These methods undergo testing in order to meet regulatory standards, such as the European standard for testing wood preservatives against marine borers, EN 275. Initial investigation of durable timbers species or wood preservative treatments can be achieved quickly and inexpensively through laboratory testing, which offers many advantages over marine field trials that are typically costly, long-term endeavours. Many species of *Limnoria* (gribble) are marine wood-boring crustaceans. *Limnoria* are ideal for use in laboratory testing of biodegradation of wood by marine wood-borers, due to the practicality of rearing them in aquaria and the ease of measuring their feeding rates on wood. Herein, we outline a standardizable laboratory test for assessing wood biodegradation using gribble.

INTRODUCTION:

Wood-borers can cause extensive damage to marine wooden structures, such as sea

defences, piers, and aquaculture structures; the replacement or restoration of which costs billions of dollars per annum worldwide[1-3]. In order to protect these structures, timber is often treated to reduce biodegradation. However, due to the restriction of use of broad-spectrum biocides in the US, UK, Australia and EU, in the marine environment, new modification techniques and species of wood that are naturally durable to borers are sought after[4-7]. Novel techniques for the preservation of wood in the marine environment require thorough testing in order to meet regulatory standards and limit environmental impacts from hazards such as leaching of any chemical preservative. For example, the European standard, EN 275, which is the current European standard from 1992, used to evaluate wood preservation treatments against marine wood-borer damage[8,9]. This standard, along with other legislations against the use of biocidal compounds, such as CCA[4-7] and creosote[10], necessitates sustainable, non-toxic methods of wood protection and the use of naturally durable timber species to replace biocidal treatments[11,12]. Marine trials, such as those specified in EN 275, require long exposure periods and are thus expensive and slow to yield meaningful results. Laboratory tests, however, provide a much quicker alternative to test methods of preserving timber products against marine wood-borer attack, allowing rapid evaluation of adjustments to treatment schedules[13]. Results from this rapid laboratory experiment are designed to inform novel modification processes of wood and to identify timber species with natural durability to borer damage. A low feeding rate and vitality can indicate increased resistance in potential products and this information can then be fed back to industry partners to allow them to improve designs. Our method allows a nimble and rapid response, that is desirable in industry, and once promising products have been identified, results can be supplemented with those from marine trials.

Gribbles *(Limnoria)* are a genus of isopod crustacean in the family Limnoriidae. There are over 60 species of *Limnoria* worldwide[13-15], with three common species found in the UK, *Limnoria lignorum, Limnoria tripunctata* and *Limnoria quadripunctata*[16]. They bore tunnels on the surface of wood that is submerged in seawater, often causing economically significant damage. Gribbles are highly abundant in coastal UK waters and are easy to maintain under laboratory conditions, making them ideal organisms for the study of wood biodegradation by marine wood-boring invertebrates. Evaluating the feeding rates and vitality of gribbles on different timber species and wood preservation methods can determine the efficacy of their resistance to biodegradation. The following protocol sets out a standard method for measuring gribble feeding rates, developed from that described by Borges and colleagues[12,17], in addition to streamlining the introduction of image analysis to make the process operable in non-specialist labs. Image analysis is also used to reduce the practical limitations of manually counting large number of samples. Durability in long-term marine testing, according to the British Standard EN350-1:1994, are graded in reference to *Pinus sylvestris* sapwood[18]. In the short-term laboratory testing presented here, we use Scots pine (*Pinus sylvestris* L) sapwood as a control to testing heartwood of the species ekki (*Lophira alata* Banks ex C.F Gaertn), beech (*Fagus sylvatica* L), sweet chestnut (*Castanea sativa* Mill) and turpentine (*Syncarpia glomulifera* (Sm.) Nied). Average faecal pellet production and vitality among eight replicates per wood species was used as an indicator of durability. We provide illustrative data collected from a typical evaluation, using the gribble species *Limnoria quadripunctata* and a range of naturally durable timber species. *Limnoria quadripunctata*, identified by the keys provided by Menzies (1951), was selected as the optimal species for biodegradation trials due to the fact that it is the most well-studied member of the family and is well-established as a

model species for use in biodegradation trials. This protocol is also applicable for testing woods of different treatments although the control used should be untreated replications of the same species.

PROTOCOL:

1. Preparing Test Sticks

1.1. After any treatment processes are complete, cut dry wood into test sticks to size 2 mm x 4 mm x 20 mm (**Figure 1**). Air dry sticks to a constant weight, under laboratory conditions. Use at least 5 replicates of each wood being tested.

[Place **Figure 1** here]

1.2. Vacuum Impregnation

1.2.1. Post wood preparation (i.e., cutting and treatment, if applicable), place sticks under a mesh in a food-safe plastic container, inside the vacuum desiccator and replace lid ensuring there is a tight seal, facilitated by a coating of vacuum grease (**Figure 2**).

1.2.2. Attach a three-way valve between the tubing connecting the desiccator and pump, with a third tube leading to open air (**Figure 2**). Ensure that the three-way valve is closed off to the air and run the pump to achieve a vacuum of between -0.75 to -1.0 bar within the vacuum desiccator and hold this vacuum for 45 minutes – 1 hour.

1.2.3. Submerge the open end of the third tube into a container of seawater. Switch the pump off and close the valve leading to the pump, then slowly open the valve until seawater is drawn by the vacuum into the desiccator. Allow the water to flow until it fills the plastic container, above the level of the mesh.

1.2.4. Then withdraw the tube from the seawater in the container, allowing air to enter, until the desiccator returns to atmospheric pressure. Keep the sticks submerged under the mesh until they sink to the bottom of the plastic container.

[Place **Figure 2** here]

1.3. Leaching Wood

1.3.1. Submerge seawater-saturated test sticks in seawater contained in 50 mL tubes (**Figure 3**). Replace water regularly for a period of 20 days.

NOTE: The leaching process applies to any experimental wood under test, including treated or natural woods.

[Place **Figure 3** here]

2. Extracting Gribble

2.1. Extract individual specimens of gribble from an infested wood block. Use a pair of fine forceps and a thin (size 000/0.4 mm or smaller) paintbrush. Carefully peel back any wood that is covering the gribble burrow with the forceps

NOTE: Burrows are found on the surface of wood and can be identified by small holes (**Figure 4**).

2.2. Once gribble have been exposed, use a paintbrush to gently pick up individuals from underneath and deposit in a petri dish filled with seawater. Check gribble under a microscope to identify species and to ensure no damage was caused while extracting.

NOTE: Beating pleopods are a sign of vitality.

2.2.1. Discard any females brooding eggs as gravid females have a reduced feeding capacity.

[Place **Figure 4** here]

2.3. Identifying *Limnoria quadripunctata*

2.3.1. Identify *Limnoria quadripunctata* under a stereomicroscope by the four distinct tubercles, arranged in a square pattern, on the animal's pleotelson in addition to an X-shaped carina on the fifth pleonite[19] (**Figure 5**).

[Place **Figure 5** here]

3. Preparing Well Plates

3.1. In multi-well plates with wells of diameter 20 mm, place one test stick and 5 mL of unfiltered seawater, between 32-35 PSU, per well (**Figure 6**).

3.2. Place treatments/species of wood systematically throughout the well plate so that each type of wood is represented at least once per plate. Add one gribble per well.

NOTE: Temperature should be kept stable in an incubator at 20 °C ± 2 °C for the species *L. quadripunctata,* other species of *Limnoria* can be used with adjustments to the temperature made to suit the specific species.

3.3. Keep plates in constant dark conditions as the photoperiod does not have an effect on gribble feeding rate[15].

[Place **Figure 6** here]

4. Collecting and Counting Faecal Pellets and Assessing Vitality.

4.1. Twice per week, remove the test stick and each gribble (one per well) from the well plate and place into a freshly pre-prepared well plate (containing 5 mL of seawater per well

[32-35 PSU, 18-22 °C]).

4.2. Use a paintbrush to gently brush off any faecal pellets from the stick before transferring and retain the faecal pellets within the original well.

NOTE: Prior to transferring the gribble to a fresh well plate, vitality can be assessed on a scale of 1-5; 1= dead, 2 = passive, not on the wood, 3 = actively swimming or beating pleopods, not on the wood, 4 = crawling on the surface of the wood, 5 = burrowed into the wood.

4.3. Image Processing

4.3.1. Use a fine paintbrush to separate any clumps so that individual pellets are visible and brush pellets away from the very edges of the well. Take a detailed, photograph under a stereo microscope, at magnification x4 and upload to a computer (**Figure 7**).

NOTE: Ensure the pellets are in focus and the background is uniform, with no shadows or light reflections on the surface of the water.

[Place **Figure 7** here]

4.3.2. Process to Generate Faecal Pellet Count Using Image J.

4.3.2.1. Download ImageJ (latest version as of 03/08/21, 1.8.0_172) from https://imagej.nih.gov/ij/download.html or run from the computer's browser.

4.3.2.2. Upload a stack of images by dragging and dropping or by selecting **File | Import | Image sequence | Browse**. Do not change any parameters then select **Okay**.

4.3.2.3. Next, use the circle tool to select the bottom section of the well containing the faecal pellets. Remove the well edges, select **Edit | Clear outside**. Make the image binary, select **Process | Make binary**.

4.3.2.4. Calibrate by selecting **Analyse | Set scale** and choose the number of pixels per millimeter for the image (for example 10 pixels = 1 mm). Count the pellets, select **Analyse | Analyse particles**.

4.3.2.5. In the box next to **Size (unit^2)**, select a lower threshold that is the same as the smallest size pellet, using the unit scale set earlier (for example, if 10 pixels = 1 mm and the smallest pellet is 0.5 mm, choose 5-infinity).

4.3.2.6. In the **Show** drop down box, select **Outlines** and then tick **Summarise** and press **Okay** (**Figure 8**).

NOTE: Further information can be found at https://imagej.nih.gov/ij/docs/guide/index.html

[Place **Figure 8** here]

4.4. Data Analysis

4.4.1. Convert pellet counts to pellets per day, which gives and indirect measure of feeding rate. Discard data from any moulting individuals on days that moulting occurred (**Figure 9**).

NOTE: Moulting occurs over 1-3 days and can be identified when a full moult of the exoskeleton can be seen.

[Place **Figure 9** here]

REPRESENTATIVE RESULTS:

A feeding experiment of *L. quadripunctata* was conducted over 20 days, using five different wood types (Scots pine (*Pinus sylvestris* L) sapwood, and heartwood of beech (*Fagus sylvatica* L), ekki (*Lophira alata* Banks ex C. F Gaertn), sweet chestnut (*Castanea sativa* Mil), and turpentine (*Syncarpia glomulifera* (Sm.) Neid)) (See **Table of Materials**), in November 2020. Eight replicate sticks were used per wood species and one specimen of *Limnoria quadripunctata* was fed per stick. All gribble were acquired from stocks that are maintained in aquaria at the Institute of Marine Sciences, University of Portsmouth, UK. Stocks are regularly supplemented with wild collections from the south coast of England. Animals are well acclimatized to the stable and consistent culture conditions prior to the experiment. Wood sticks (20 mm x 4 mm x 2 mm) were leached in seawater for two weeks prior to the feeding trial. One gribble, one test stick and 5 mL of seawater were placed per well in a 12 multi-well plate and kept in an incubator at stable conditions of 20 °C (± 0.2 °C) and in constant dark conditions. Faecal pellets were counted and collected every 2 to 5 days, with full water changes at each collection. Eight replicates of each wood species were used, giving a total of forty sticks with one individual gribble each. Seawater used for leaching wood and used throughout the experiment was obtained directly from the aquarium used to rear specimens. Seawater conditions are stable in the aquarium and stable in the incubator. Evaporation from the small volume of water used per well is minimised by the lid design of the well plates and full water changes occurring every 2-5 days.

Pellets were counted automatically using Image J (version 1.8.0_112).

Gribble feeding on Scots pine sapwood wood as a control, produced the most faecal pellets per day consistently, apart from at Day 20 where pellet production was overtaken by beech. Ekki produced the lowest faecal pellets per day of all the wood species tested. The second highest faecal pellet production was seen on beech, followed by sweet chestnut and turpentine. There was an increase in faecal pellet production in all species from Day 5 to Day 7. Pellet production dropped in all species, other than ekki, between Day 7 and Day 12, possibly due to the increased time between water changes. After this, faecal pellet production remained fairly consistent among each of the wood species. From Day 14, Scots pine decreased in daily faecal pellet production, while beech increased (**Figure 10**).

[Place **Figure 10** here]

The highest vitality of 5 was seen in most individuals feeding on Scots pine wood, other than the one dead individual. 5 indicates animals that have burrowed into the wood and this was

seen only on Scots pine sapwood and beech heartwood. By Day 12 for Scots pine and Day 20 for beech, all living individuals had burrowed into the wood. Sweet chestnut had the highest percent mortality but did not increase over time. The remainder of living individuals stayed at a vitality of 4 (crawling on the wood surface), apart from at Day 14 where two individuals were off the wood (vitality of 3). Ekki and turpentine also had the majority of individuals at a vitality of 4 over the duration of the experiment, apart from at Day 14 and Day 5 for turpentine. Mortality did not show an increase over time across any of the wood species. Only burrowing was seen to increase on Scots pine and beech while the other three wood species mostly remained at a vitality of 4 (**Figure 11**).

[Place **Figure 11** here]

Results from this testing method can be used to identify wood types or treatments that have an increased resistance to marine wood-borer damage. Then, marine field trials, as described in the European Standard EN 275, can be conducted and durability can be graded (0= 'no attack', 1= 'slight attack', 2= 'moderate attack', 3= 'severe attack', 4='failure' [20]) in addition to comparison to non-durable control wood.

Figure 1: Test sticks used in short-term laboratory testing to assess gribble feeding rates.
Test wood sticks sized 2 mm x 4 mm x 20 mm. From left to right: ekki, turpentine, sweet chestnut and beech heartwood and Scots pine sapwood. Scale bar 4 mm.

Figure 2: Equipment used to vacuum impregnate wood sticks with seawater, in preparation for feeding to gribbles during a laboratory feeding assay.
A) Vacuum desiccator; B) Pump; C) Pressure gauge for the vacuum desiccator; D) The three-way valve leading to the vacuum desiccator, pump and to open air or seawater (orange tube).

Figure 3: Leachate from wood sticks for preparation for feeding to gribbles during a laboratory feeding assay.
Wood that was fully submerged in seawater contained in a 50 ml Falcon tube, with regular water change (1-3 days), produced distinctly coloured leachate. From left to right leachate from heartwood of; sweet chestnut, turpentine, ekki, and beech and Scots pine sapwood.

Figure 4: Image of a gribble burrow with two typical ventilation holes.
L. quadripunctata burrow on a stick of Radiata pine wood, sized 2 mm x 4 mm x 20 mm. Two smaller ventilation holes can be seen next to the burrow entrance. Scale bar 2 mm.

Figure 5: *Limnoria quadripunctata* identifying features.
Image of dorsal surface *Limnoria quadripunctata,* taken on a stereomicroscope at X20 magnification. Identifying features shown by red arrow - indicates the X- shaped carina and blue arrow – indicates four tubercles on pleotelson. Scale bar 1 mm.

Figure 6: Experimental set up for gribble feeding assay.
An example of a 12 multi-well plate used in the laboratory testing of gribble feeding rate. Each well contains 5 ml seawater and one test stick (20 mm x 4 mm x 2 mm) of different wood species; Scots Pine sapwood and ekki, beech, sweet chestnut, and turpentine heartwood. Scale bar 20 mm.

Figure 7: Image of gribble faecal pellets.
L. quadripunctata faecal pellets (small, cylindrical, brown pellets) from feeding on Radiata pine wood in one well of a multi-well plate. Taken at X4 magnification. Images prior to manipulation for image analysis (see Figure 7). A) Example of a suitable image to be used for automated counting in Image J. Pellets are sufficiently spread out and away from the edges of the well. The well is centred and there are no obstructions or reflections. B) An example of an image that is unsuitable for image analysis. The well is off-centre, cutting off the bottom half. Blue (dotted) circle shows light reflection off the surface of the water. Orange (solid) circle shows pellets that are clumped too closely together and too near the edge of the well. Red (dashed) circle shows a wood chip that was not removed. Scale bar 5 mm.

Figure 8: A flow diagram of the process used in ImageJ to count faecal pellets.
A) Importing an image sequence in the File tab of ImageJ. B) The browse button in the 'Import Image Sequence' dialog box to import a sequence of images from a local device. C) Using the circle tool to select area containing faecal pellets D) Clear outside button in the edit tab area to remove outside of selected area. E) Make binary button in the process tab. F) Set scale button in the Analyse tab. Distance in pixels is equivalent to the number of pixels to one unit of measurement (mm). G) Analyse particles button in the Analyse tab. Size (unit^2) set to the lower threshold of faecal pellet size, in pixels, to infinity. Show 'outlines' and 'summarise' are selected.

Figure 9: Example of a gribble moult.
Gribble (*L. quadripunctata)* moulting, on a Radiata pine wood test stick sized 20 mm x 4 mm x 2 mm. Moults are indicated by red circles. Scale bar 2 mm.

Figure 10: Number of faecal pellets per day (n=40) (mean ± SE) produced by *L. quadripunctata* using different wood species, over 20 days. Turpentine, sweet chestnut, beech and ekki heartwood tested, with Scots pine sapwood used as a control.

Figure 11: Vitality of individuals over time, as a percentage of replicates, feeding on different wood species.
Turpentine, sweet chestnut, beech and ekki heartwood tested, with Scots pine sapwood used as a control. Of eight replicates per wood species, the percentage at different vitalities were plotted over the 20-day experimental period. Dark blue represents a vitality of 5 (burrowing), light blue a vitality of 4 (on wood), grey a vitality of 3 (off wood but active), purple a vitality of 2 (off wood and passive) and black shows a vitality of 1 or dead individuals.

DISCUSSION:
Prior to selecting gribble specimens to be used in the feeding experiment, individuals should be screened to assess their suitability. There can be some variation in feeding rate between individuals due to size so only specimens that have achieved full adult should be used. No significant difference between feeding rate of individuals between 1.5 mm and 3 mm length was detected by[17]. Female *Limnoria* brood their eggs, during which time have a reduced feeding rate. Therefore, any brooding females should be checked for and discarded while selecting specimens. Similarly, moulting individuals will also have a reduced feeding rate[21]. Therefore, faecal pellet counts on days when individuals are moulting should be discarded[17].

As moulting occurs for more than a day, moults are counted when a full exoskeleton moult can be seen on pellet collection days. *Limnoria,* when creating their burrows, have an increased faecal pellet production and will also produce more frass (personal observation); fine wood waste that is not incorporated into the faecal pellets[22]. The high levels of frass can interfere with identification of faecal pellets but can carefully be removed under stereomicroscope observation, using a pipette or fine paintbrush, prior to image capture for automatic counting. Alternatively, pellets can be counted manually.

The software ImageJ requires quality in-focus images for image processing. To this end, images should be captured in which faecal pellets are not obstructed by the well walls and a paintbrush should be used to separate individual faecal pellets. The background of the image must be uniform with no areas of light or shadow. Which would interfere when the image is transformed to binary for processing in ImageJ. There is no need to adjust contrast or light prior to image processing. When importing a stack of images, all photographs must be taken in the same plane so no errors occur while processing.

Vacuum impregnating wood with seawater causes the wood to sink and become readily accessible to the gribble. Leaching wood prior to exposing it to gribbles will remove any water-soluble extractives that may impact their feeding rate or cause mortality[12]. Mortality due to extractives in the water is not representative of mortality to be expected in the sea, where extractives will become rapidly diluted. Well plates should be kept at a constant temperature that is the optimal for the gribble species being tested. The common southern British species, *L. quadripunctata*, feeds well between 15 and 25 °C and has an optimum feeding rate at 20 °C[17] so well plates can be conveniently kept in an incubator at a constant 20 °C ± 0.5 °C.

Assessing the vitality of the feeding gribble detects sublethal or pre-lethal effects of wood treatments or naturally durable timbers. A high vitality of 5 indicates that the gribble is demonstrating natural behaviour by burrowing into the wood and suffers no adverse effect from contact with it. A vitality of 4 shows that while not having burrowed into the wood, the gribble is still comfortable to crawl along its surface. A score of 3 is given to gribble that are not on the wood, but instead actively swimming in the water or are stationary but with rapidly beating legs and pleopods. A low vitality of 2 means that the gribble is exposed and/or has little energy. This may come about from a prolonged period of low feeding rate or from extractives either leaching into the water or becoming accessible during feeding. If high mortality is seen after 7-8 weeks, this may be due to starvation, as starved gribbles (kept in wells with just 5 ml of seawater and no wood) can survive for this long (personal observation).

The benefits of using a short-term laboratory assay as opposed to longer-term marine field trials, is that novel treatments and wood products can be rapidly tested to identify their potential to be used commercially. Furthermore, such assays can facilitate rapid optimization of treatment processes. If a significantly lower faecal pellet production is seen compared to a control wood, then testing can be supplemented by marine trials. Slevin *et al*., 2015[23] and Westin *et al*., 2016[24] demonstrate a good correlation between laboratory and field assessments through testing the same wood in two different settings, indicating a capable predictive ability of the former. A short-term assay can be run for several weeks. Starved gribbles can survive for 7-8 weeks when kept in well aerated water without wood (personal observations) which may provide additional comparison if investigating the mortality

response to different types of woods. However, through recent, unpublished observations, there is no significant fluctuation in faecal pellet production over a time period longer than 20 days, other than when mortality begins to occur. In addition, previous methods, such as that used by Borges *et al*., 2008 and 2009, runs for 15 days. Therefore, 20 days is a sufficient time for a rapid laboratory-based test to provide indication of wood durability.

While this method is suitable for short-term trials, findings should be complemented by long-term marine field experiments. Laboratory conditions cannot replicate the variety of biotic and abiotic factors that may affect wood in the marine environment. Biofouling organisms, along with other species of marine wood-borers (such as shipworms) may still be present and cause damage to the wood[25,26]. In addition, abrasion from wave-thrown shingle or sand can wear wood down, which may then become accessible for gribbles[27]. However, a standard laboratory method can provide an initial screening of new products which show promise for marine applications. By assessing the faecal pellet production and vitality, woods that are better at reducing gribble feeding rate can be identified.

Due to the regulations and restrictions of wood preservatives, such as CCA and creosote, it is important to find novel products to replace these treatments. Timber is subject to high levels of biodegradation in the marine environment but is still one of the most renewable construction materials available and retains its strength and structure well in seawater[27,28]. Not only will timber that is resistant to biodegradation reduce costs but will also be more environmentally friendly than using alternative materials such as concrete or steel, which require high energy input during manufacture[29,30], or broad-spectrum biocide preservatives that may leach out and affect the surrounding ecosystem[31-37].

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DISCLOSURES:

The authors have no conflicts of interest related to this study.

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Figure 1

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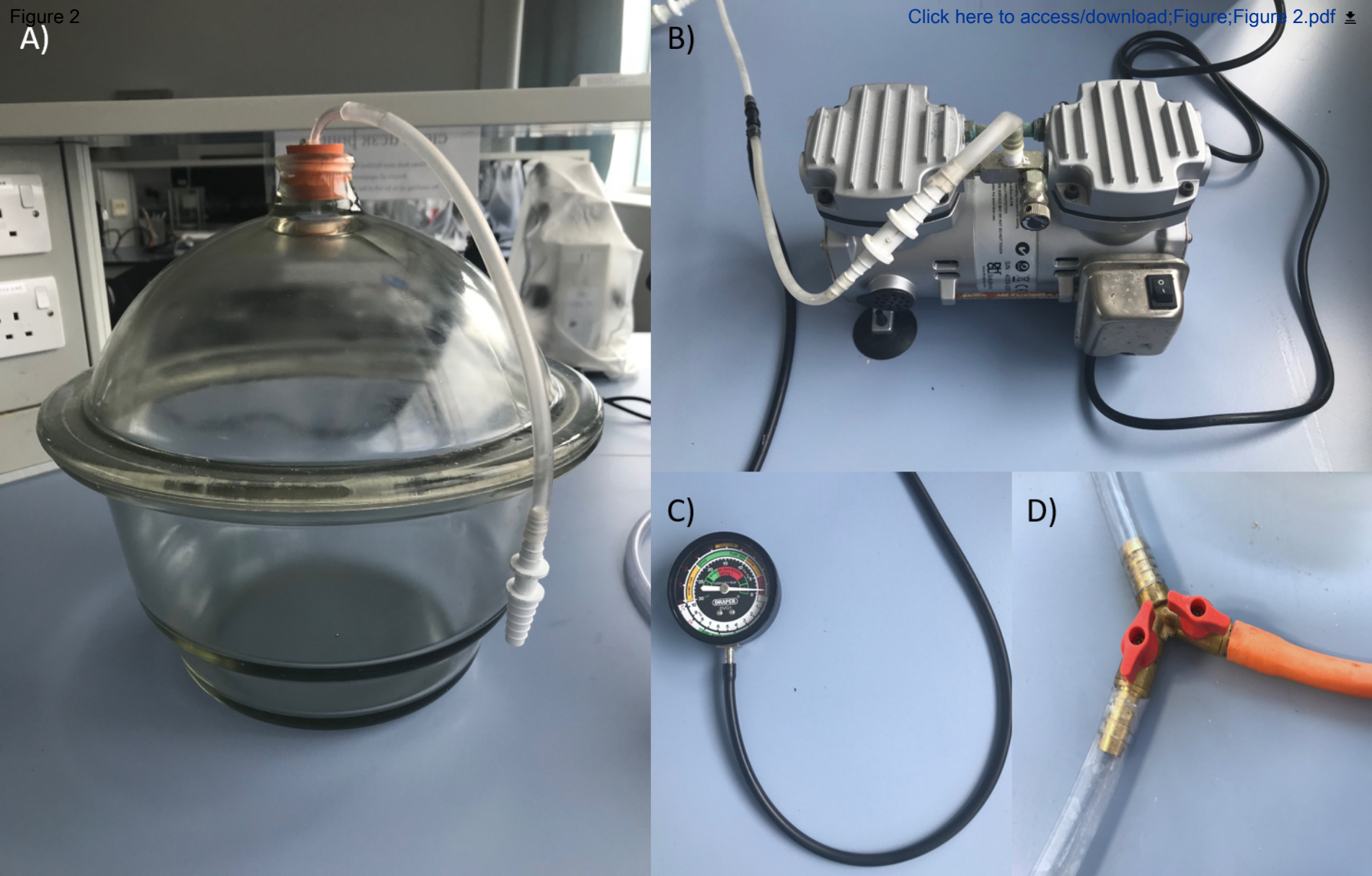
Figure 2



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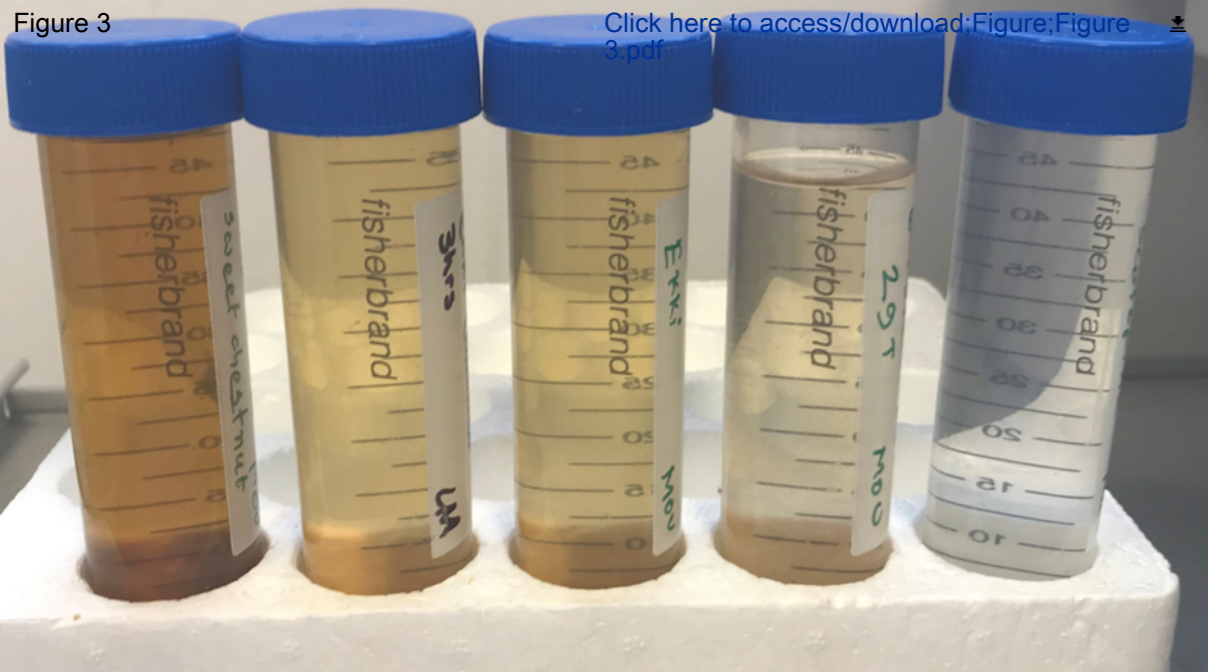

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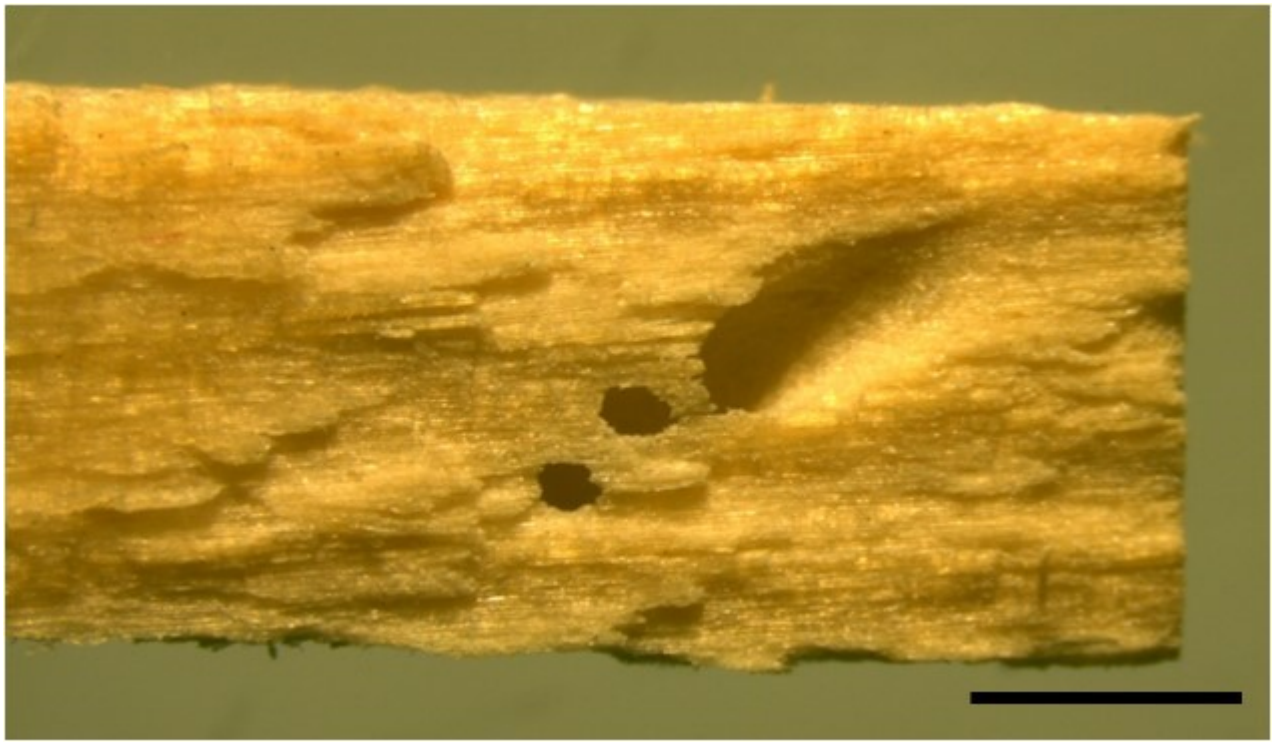

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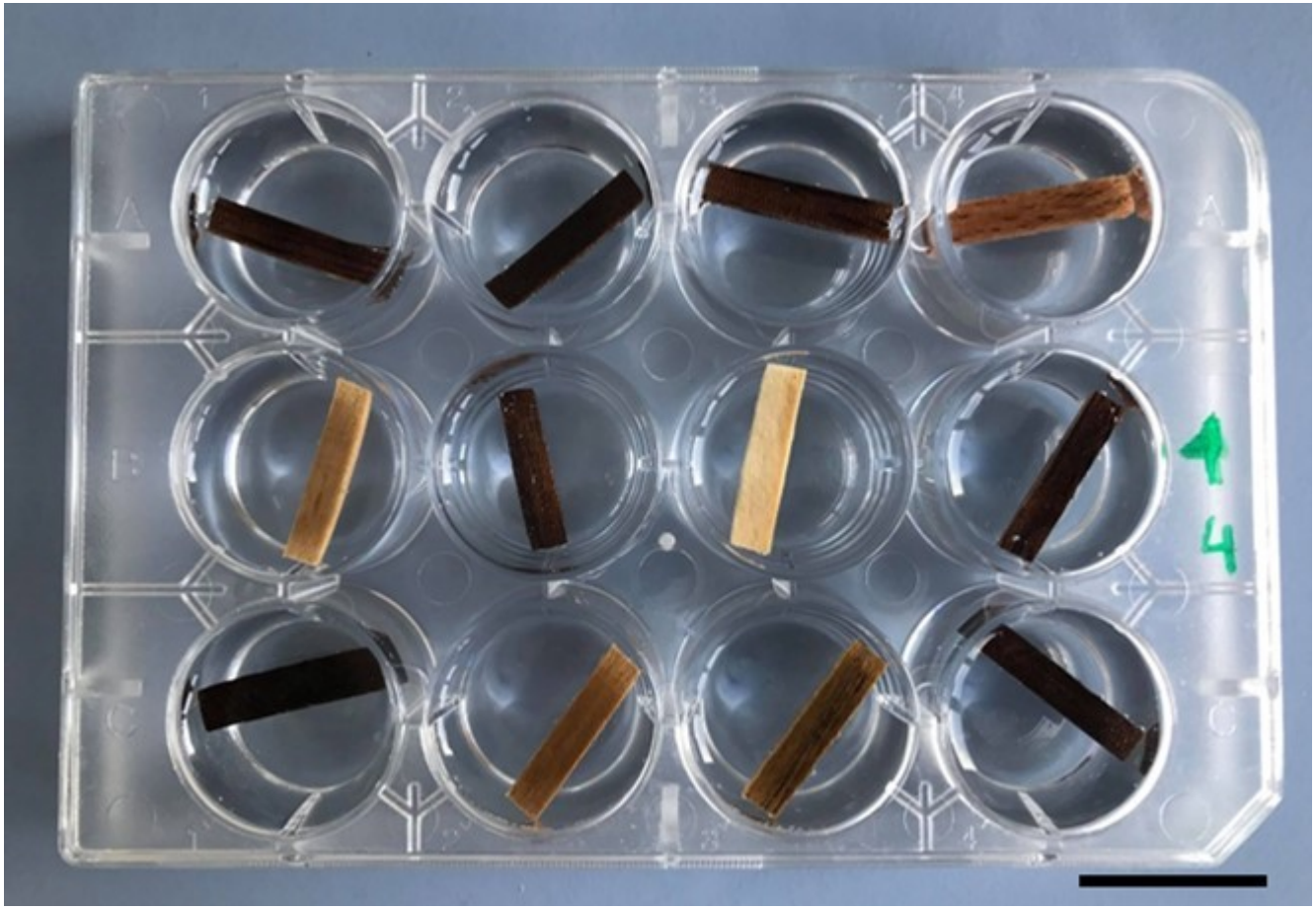

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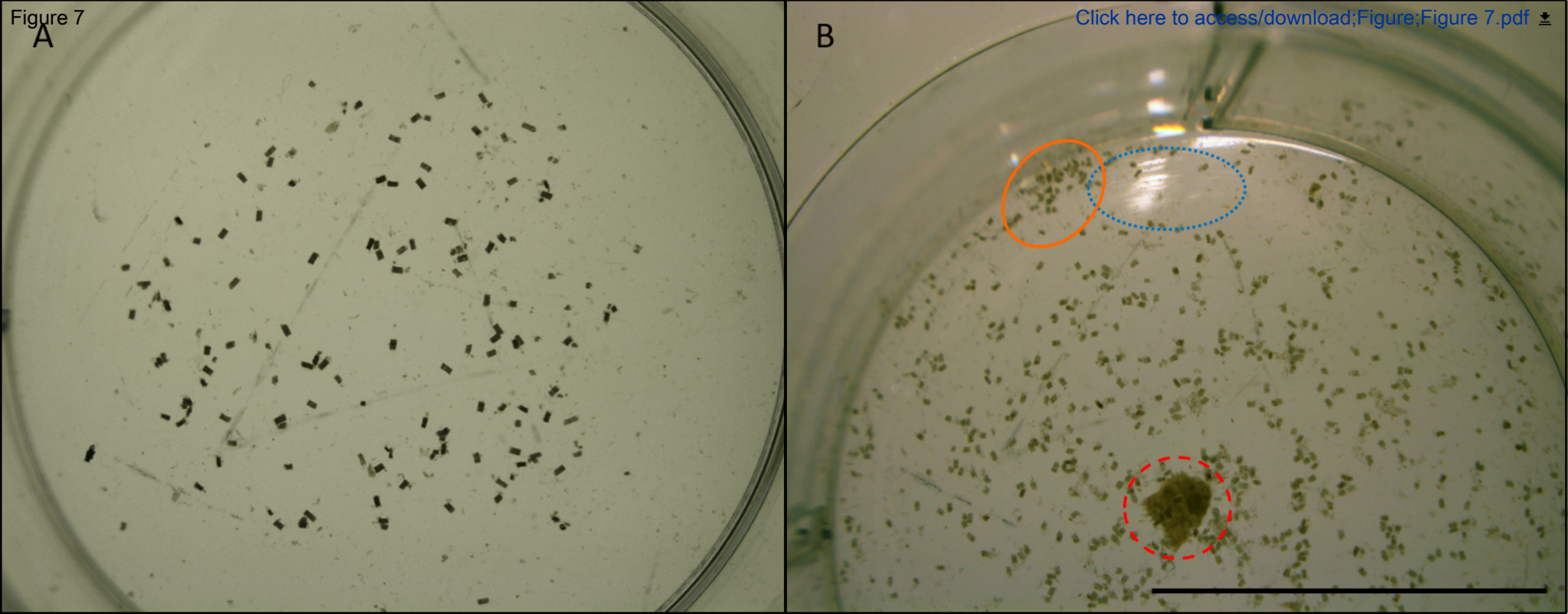

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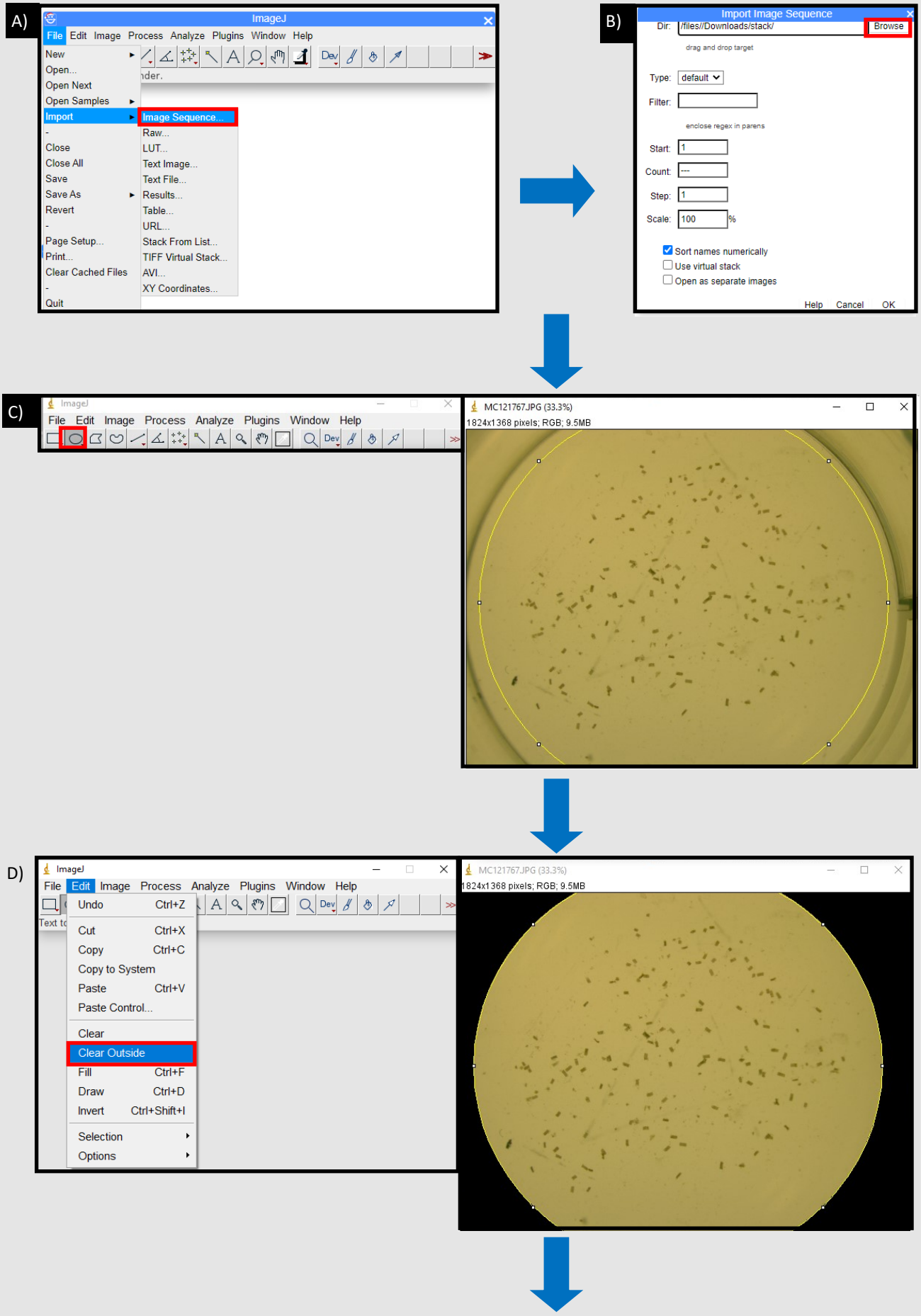

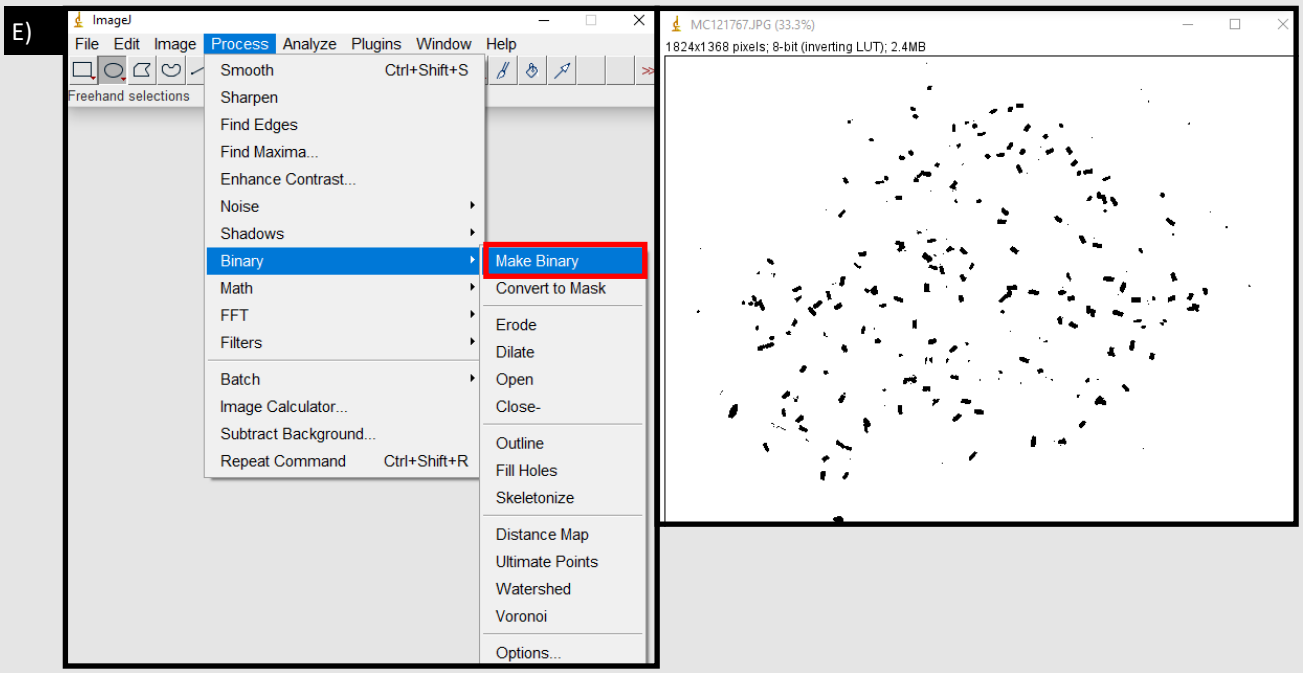
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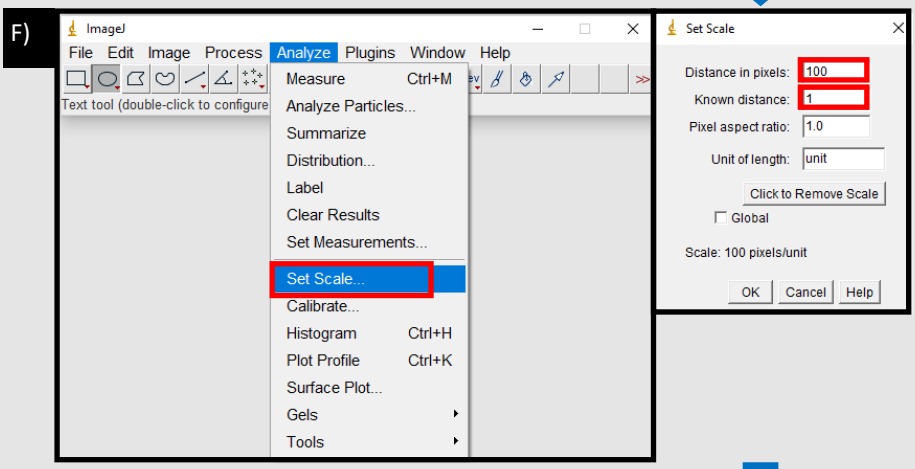
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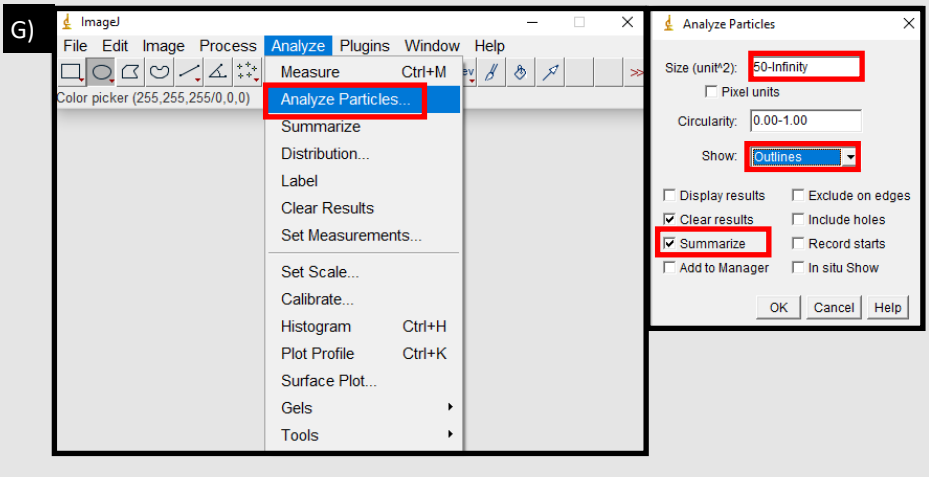
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Figure 9

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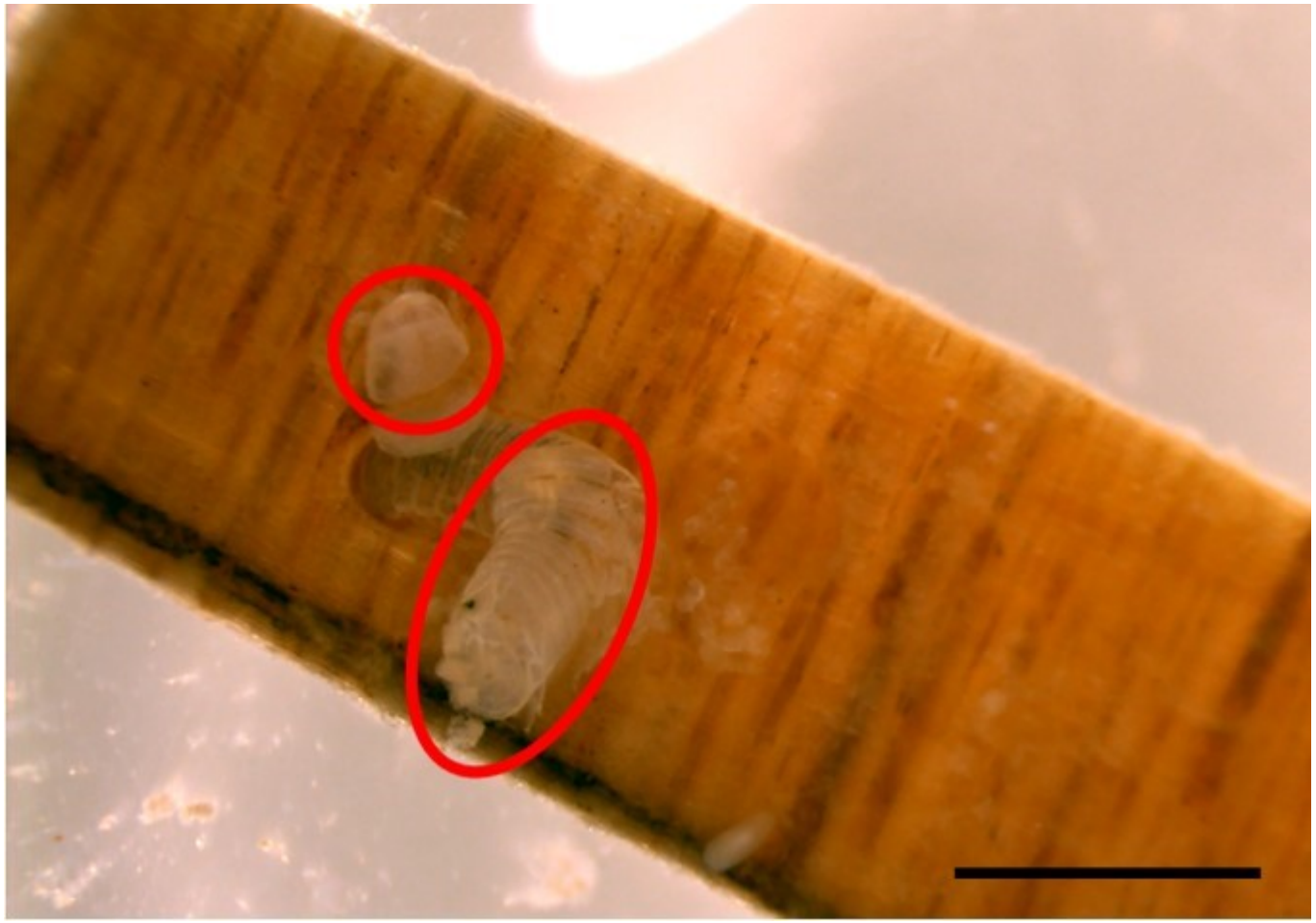

Figure 10

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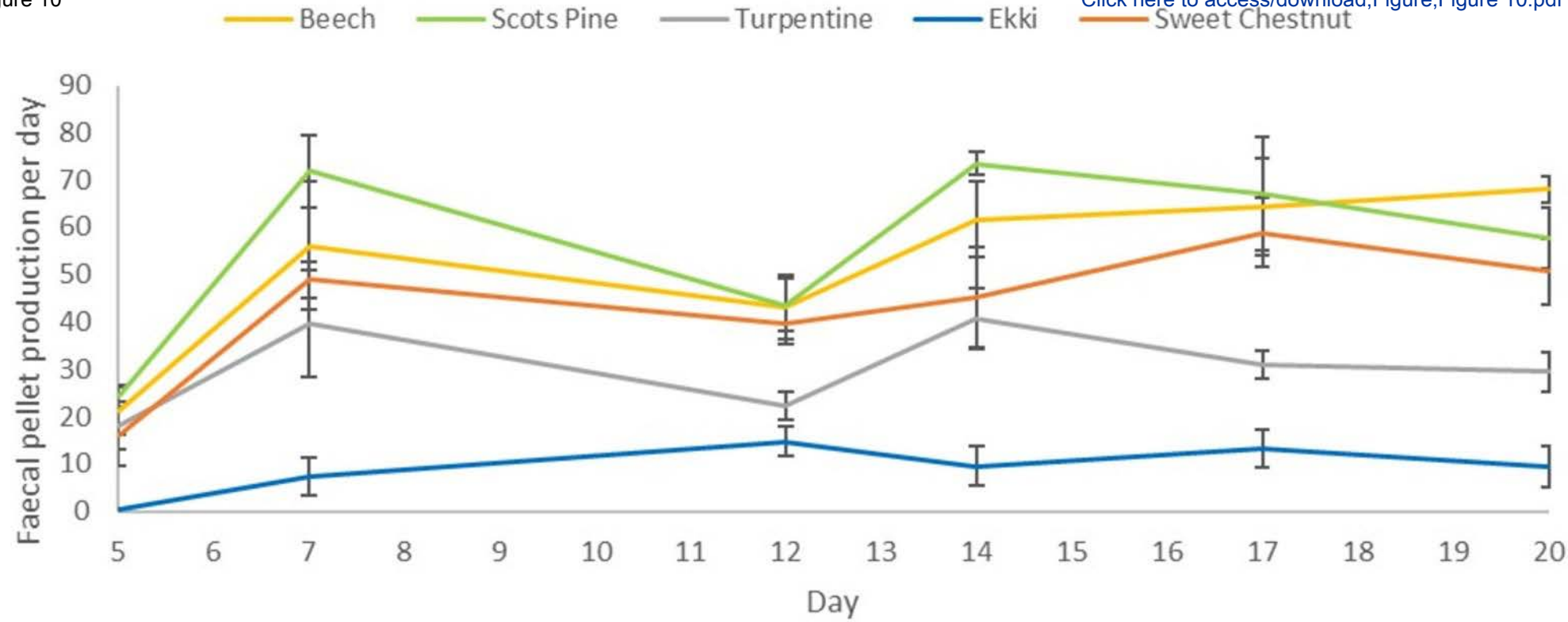

Figure 11

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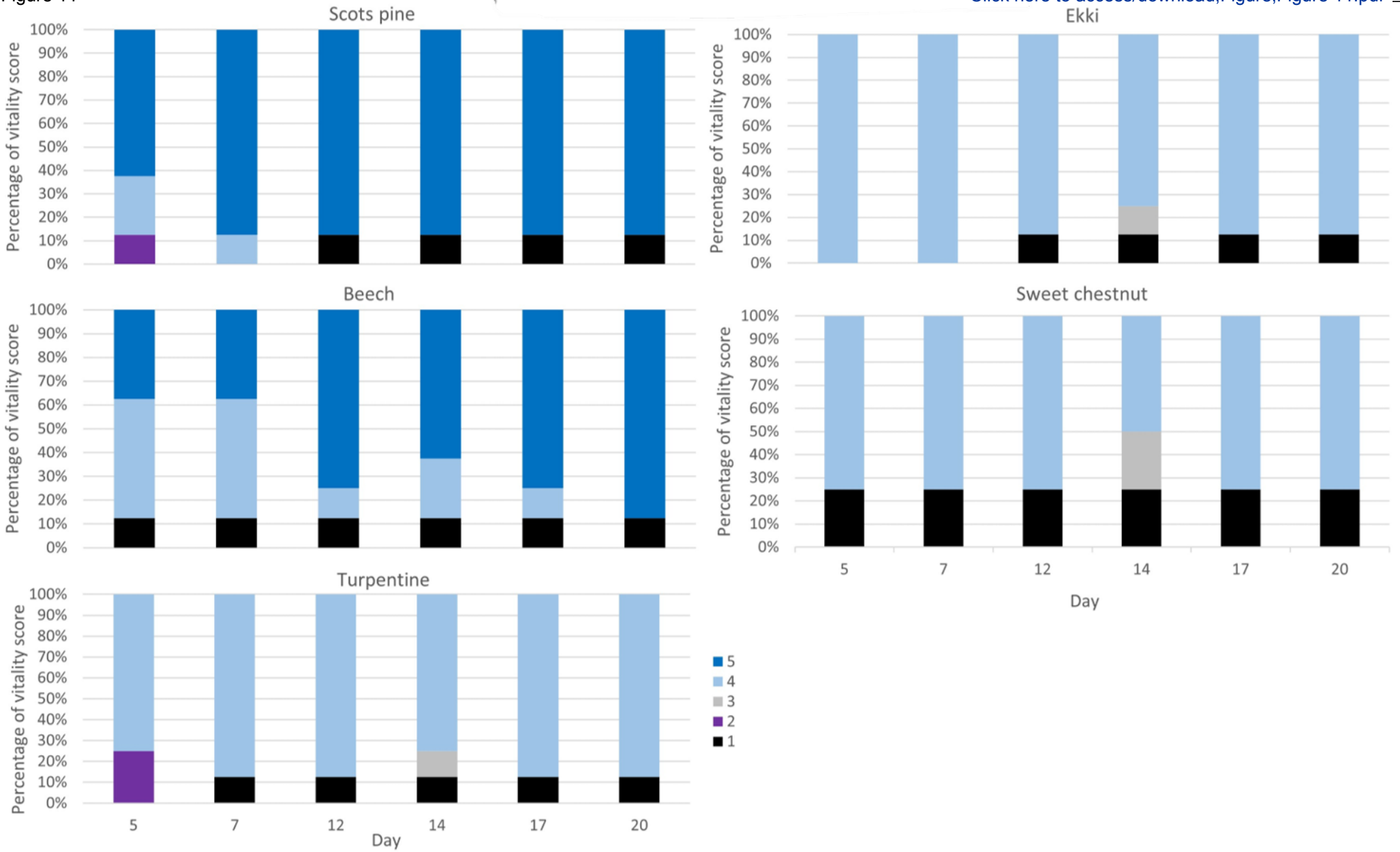

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Table of Materials

Table of materials.xlsx

Response to Reviewers: Rapid testing of resistance of timber to biodegradation by marine wood-boring crustaceans

31st July 2021

Dear Dr. Bajaj

Thank you for your comments and suggestions, and those of the reviewers, for the manuscript titled 'A laboratory method for rapid testing of resistance of timber to biodegradation by marine wood-boring crustaceans' (JoVE62776R1). After careful consideration, we have made several changes to the manuscript, outlined below. We feel that the suggestions of the reviewers have greatly improved the quality of this manuscript. We hope you will find the updated manuscript adequately addresses the reviewer's suggestions and we look forward seeing the final publication.

Kind regards

Dr Reuben Shipway

Editorial and production comments:

1 Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

E1.1 Checked

2. Please check with your funding source that you are allowed to publish standard access.

E1.2 Checked

3. Please make the title concise: Remove the phrase "a laboratory method"

E1.3 Title changed as follows “Rapid testing of resistance of timber to biodegradation by marine wood-boring crustaceans”.

4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “This protocol presents…”

E1.4 Done.

5. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

E1.5 Done.

6. Do you need an approval from IACUC for working with crustaceans? If yes, please include an ethics statement to show that the study was approved by your animal research committee.

E1.6 This group of animals are not protected under the Animal Scientific Procedures Act 1986, so a Home Office licence is not required. However, all work is approved by the University of Portsmouth’s Animal Welfare Ethical Review Board prior to commencing.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

E1.7 Done.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

E1.8 Steps in the protocol have been changed to direct actions and additional information has been removed or changed to “Note”.

9. The Protocol should contain only action items that direct the reader to do something.

E1.9 Changed (see E1.8).

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

E1.10 Done

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step

E1.11 Done

12. Please ensure the results are described in the context of the presented technique e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

E1.12 Done

13. Please include all the Figure Legends together at the end of the Representative Results and before the Discussion section in the manuscript text.

E1.13 Figure legends have been moved to page 6.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager

account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

E1.14 Figures that have been in previous publication (Martin *et al*. (2021), Validating a short-term laboratory method to assess the resistance of timber to biodegradation by marine wood-borers, IRG/WP 21-10975) have been changed.

15. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

E1.15 Done

16. For the reference section, please ensure that they are numbered in the order of citation in the text. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

E1.16 Done.

17. Please sort the materials table in alphabetical order.

E1.17 Done

2 Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written

manuscript and vice versa. The video and the written manuscript should be reflections of each other.

E2.1 Done. All figures (bar Figure 2 – Equipment used to vacuum impregnate wood and Figure 8 – Flow chart for using Image J) have been added to the video.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

E2.2 Done. Narration has been updated to match the protocol from the manuscript.

3. Please include protocol chapter title cards reflecting the subsections presented in the text.

E2.3 Title cards have been added.

4. Please ensure that the narration is equalized.

E2.4 Done.

5. Title Cards:

• Please capitalize the first letter of every important word in your title.

• 10:53 Please capitalize the first letter of every important word in your chapter title.

E2.5 Done.

6. Audio Editing and Pacing:

• Audio levels are too high. Please ensure to balance the audio level peaks average around -9 dB.

E2.6 Done.

Once done please ensure that the video is no more than 15 min in length. Please upload high resolution video at

https://www.dropbox.com/request/T5iK0ecwvY69bDULuoB7?oref=e

Reviewer #1:

Manuscript Summary:

R1.1: The purpose of the study remains clear. The abstract implies that the article aims at understanding how attack by marine borer, so that the results allow to select appropriate wood materials.

Author (R1.1): We thank the reviewer for their comments.

Reviewer #2:

General comments

R2.1: In this paper, the authors present a very interesting experiment, with a good description of the methodological apparatus and a captivating visual representation, which is well suited to this journal. The approach is smart and could well complement many of the standard tests used for evaluating the durability of woods. It uses a simple and low-tech lab equipment and is therefore suitable for being offered even in countries with reduced economic possibilities. For these reasons the proposed method has a strong potential to become a standardized method and therefore deserves publication. Nonetheless some questions and considerations arise.

Author (R2.1): We thank the reviewer for their comments.

R2.2: The title would suggest that an already solid method is presented, which instead can be such only after a long series of experiments and related statistical analyzes aimed at evaluating the replicability of the method and its stability depending on the wood species, species of Limnoria used. seasonal physiological responses, origin of the test animals.

Author (R2.2): Previous studies by Borges *et al*. (2008/2009), Cragg *et al*. (2007/2017), Janus *et al.* (2018), Bowen *et al.* (2017), Klüppel *et al*. (2015),

Sivrikaya *et al*. (2009), Malyon (2011), Green-Extabe (2013), Papadopoulos *et al*. (2008) and Slevin *et al.* (2015) (see end for references) have demonstrated the efficacy of this method for the testing wood biodegradation in the marine against the borers *Chelura* and *Limnoria*. These methods vary in leaching time, experimental time and type of wood tested, but clearly demonstrate that the use of equipment and general methodology is very effective for a rapid laboratory examination of feeding by marine wood-borers. We aim to consolidate these procedures to create a standard laboratory testing method that is under consideration for incorporation into the revision of European Standard EN275 – Wood Preservatives: Determination of the protective effectiveness against marine borers. This method can be adapted for a variety of different species or treatments of wood being tested and is applicable to any xylotrophic species of *Limnoria,* with the only adjustment required being the temperature well plates are incubated in.

R2.3: Have you tested the protocol with L. quadripunctata only? In theory, all species of Limnoria can be used, but are you expecting similar results? Or what do you think are the experimental parameters to be evaluated for the different species? Are the animals taken from laboratory strains or from the environment? If they are raised in the laboratory, can you refer to breeding protocols? Will you present (or have you already presented) the breeding techniques in another paper? If they are taken from the environment, do you think all sites give similar results? And in this case, how do you take into account the seasonality and stress associated with the transfer from the environment to the laboratory, conditions that can be extremely different?

Author (R2.3): The protocol has been tested predominantly with *Limnoria quadripunctata* as this species is the most well-studied in the family. Further, this species is common around the British Isles and is most accessible for collection and culturing in our aquarium.

It is potentially possible to use all species of *Limnoria* that specialise in xylotrophy (as opposed to the several species that live and feed on kelp or seagrasses) for assessing rates of biodegradation in timber, and we note that the biology of wood digestion is likely to be broadly similar throughout the family.

The animals were acquired from the research aquarium at the Institute of Marine Sciences, University of Portsmouth, which is regularly supplemented from wild collections along the south coast of the UK. All specimens utilised in this study were well acclimated to the stable and consistent culture conditions in the aquarium prior to use in degradation trials. We have updated the methodology section to make this point clear in the manuscript (page 5).

Indeed, our protocol on the field collections, breeding, and culturing of gribble is the subject of a current an ongoing manuscript that we hope to publish in the future and a separate subject from this current body of work.

R2.4: Have you tried the test with other species of Limnoria (and perhaps also with Chelura)? If so, with what results?

Author (R2.4): We have conducted pilot studies with *L. lignorum* and *L. tripunctata*, and have found broadly similar results to trials with *L. quadripunctata*. However, these results are preliminary and ongoing. A similar

test has also been conducted with *Chelura terebrans* although the scope of the study was to investigate feeding rate in the presence or absence of *L. quadripunctata* and not to look at testing the wood substrate (Green-Extabe, 2013).

R2.5: It is not clear how the results describe a durability scale or if they should be put as a ratio to reference against Pinus sylvestris.

Author (R2.5):

We thank the reviewer for this useful suggestion and we shall indeed be providing durability scales in future work that incorporates long-term trials of biodegradation in both laboratory and field settings. However, the purpose of this current work is to rapidly identify promising timbers or timber modification techniques that can then proceed to these long-term trials where the durability scale is a more relevant metric. In rigorous tests the comparators are 1) borer vitality control - Scots pine sapwood (no natural resistance to borer activity) and beech if a denser non-durable timber is needed; 2) commercially viable borer suppression comparator - ekki (we have experience of how to adequately leach this timber to avoid unrealistic effects of leachate – see discussion in R2.6).

R2.6: Leaching is very important and should be considered carefully as it could also greatly affect the results, especially considering that you operate in very small environments. You must clarify whether the test is aimed at natural woods or even those treated with impregnating agents, in this case the issue of leaching is even more delicate. In my opinion, there is a need to standardize the leaching method more, you use a static type of washing (which facilitates the spread of the method) but a dynamic washing would be much more effective even with a simple stirrer. The volumetric wood / water ratios must be carefully evaluated

and it is good to refer to leaching methods used in ecotoxicology. The timing must be justified, it is not enough for the water to be "clear" to the eye. There are prompt release woods and longer release woods. Consider whether or not to oxygenate the water in a closed falcon (I think at 20 ° C, but it is not specified) in case of hypoxia anoxic conditions could occur with the development of toxic substances. Should the pH be adjusted / tamonated during leaching and during the experiment? do you measure it? In case of untreated woods it is clear that the toxic effect of the extractives can be sensibly lowered by leaching, simulating the permanence in the aquatic environment, but will the materials treated with preservatives have the same behavior? For example, in the case of impregnation with metals, the ionic composition of the water can have an importan role in metallic ion mobilization, as well as the redox. A leacheate toxicity test could give indications on residual effects in wood as well as indicate possible environmental effects.

Author (R2.6):

These comments are very helpful for our ongoing work. We recognise the need for both a standardised leaching protocol and a suitably simple toxicity protocol specific to wood products. As shown in papers cited below there has been a significant amount of investigation of the effects of leachate from CCA treated wood.

Borges *et al.* (2008) shows how to sort out toxicity from insoluble extractives retained in wood from toxicity of leachates. We aim to provide sufficient leaching to avoid effects of water-soluble extractives or biocides.

The addition of vitality measurements into this procedure provides evidence of sublethal effects of leachates on borer activity.

R2.7: Does the proposed protocol have similarities with some toxicity tests: did the authors look for any parallels that could be borrowed (eg leaching techniques, expression of results ...)?

Author (R2.7):

See comments below for R2.10

R2.8: The paper seems to me written in a sober and fluent style although, not being a mother tongue I am not able to adequately judge this aspect.

Author (R2.8): We thank the reviewer for their comment.

R2.9: The figures seem clear to me and the bibliographical references sufficient although something more could be added regarding the durability methods in use.

Author (R2.9): We refer the reviewer to comment R2.5 in which we address issues with durability.

Here are some minor detailed comments. Since the pages and lines are not numbered in my copy of the manuscript, I have to insert quoted references.

R2.10:

Page 1:

"Novel techniques for the preservation of wood in the marine environment require thorough testing in order to meet regulatory standards and limit environmental impacts."

Add references for this statement, what are the regulatory standards and environmental impacts you refer to? How does this test helps to meet standards and limit environmental impacts?

Author (R2.10): We are not aware of any timber-specific standards for assessing the toxicity of leachate from timber products in seawater. However, research on the topic of the effect of leachate from timber treated with CCA (copper chromium and arsenic compounds) on marine biota has been evaluated (Brown *et al*., 2003; Lebow *et al*., 1999), as has the effect of creosote pilings used in aquaculture (Smith, 2008). Methods for measuring leachate from CCA treated wood have been tested, though seawater was not used (Mercer & Frostick, 2012). The vitality observations are used to indicate sublethal and potentially lethal effects of leachate. We have a paper in press which shows that animals can maintain feeding rates on Scots pine over many weeks in the experimental setup described in this paper.

Our protocol assesses readily measured behavioural attributes of animals exposed to wood. Where tunnelling does not take place, further studies are required to establish why certain timbers are resistant to tunnelling despite the animals being able to feed on them. Hardness is an issue, but does not provide a complete explanation (Cragg *et al*., 2007). Avoidance of wood demonstrates potential antifeedant properties, while death during the leaching period.

R2.11:

Page 2:

"Gribbles (Limnoria) are a genus of isopod crustacean in the family Limnoriidae"

It would be helpful to say how many species of Limnoria there are in the world (which is in favor of the fact that the test can be easily exported to many places)

Author (R2.11): There are over 60 species of Limnoria distributed globally, although not all are wood borers (e.g. some are specialised to feed on kelp or seagrasses). The three species that can be found in UK waters are *Limnoria quadripunctata*, *Limnoria tripunctata* and *Limnoria lignorum*. Additional

information has been included in the text (page 2). We have also added a section in the protocol specific to identifying *L. quadripunctata* (page 3) (also see R3.3).

R2.12:

"species ekki, beech, sweet chestnut and turpentine heartwood was used"

Although reported in the table of materials, the first time you mention a wood species it would be correct to put the scientific name and the author.

Author (R2.12): We thank you for the comment. The relevant information has been added in the text (page 2 -"In the short-term laboratory testing presented here, we use Scots pine (*Pinus sylvestris* L) sapwood as a control to testing heartwood of the species ekki (*Lophira alata* Banks ex C.F Gaertn), beech (*Fagus sylvatica* L), sweet chestnut (*Castanea sativa* Mill) and turpentine (*Syncarpia glomulifera* (Sm.) Nied)").

R2.13:

"Preparing test sticks"

Should the wood samples have particular characteristics as in EN275 (for example number of rings per cm etc.)?

Author (R2.13): Ideally yes as this correlates with density. We use Scots pine from southern sources as they are faster grown and thus less dense.

R2.14:

"Vacuum impregnation."

Is this chapter valid also in case of wood impregnted with biocides?

Author (R2.14): Yes, this method is applicable for treated woods as well as testing natural resistance in different wood species. This technique has also been applied to wood subject to modification or biocidal treatments.

R2.15:

"under a mesh in a plastic container,"

It would be useful to specify the kind of plastic (e.g HDPE? Acrilic? Atoxic? For food use?)

Author (R2.15): Thank you for the comment, the plastic used was food safe and this detail has been added into the manuscript (page 3).

R2.16:

Page 3

"as evidenced by the water remaining clear"

It is a weak end point, maybe it is better to propose a given period of time.

Author (R2.16): Agreed and thank you for the suggestion, this has now been updated to 20 days. Clear water is reassuring, but previous experiments where animal mortality and vitality is examined guide this period.

R2.17:

"Extracting gribble."

It could make a lot of difference whether the animals are taken from the environment or come from a laboratory culture. If taken from the environment they need a period of acclimatization, have you tried to see if there are different physiological responses in different periods of the year?

Author (R2.17): As per author comment R2.3 above, we have now updated the methodology section to address issues with environmental acclimatisation of gribble prior to trials.

This is normalised by comparing Scots pine with experimental wood, using animals from the same culture, so the method compensates for any annual

variations. However, the cultures have been maintained in the lab over a long period of time, so they have limited access to seasonal cues. Also, lab cultures are kept at constant temperature. We also have data on effects of temperature on feeding rate (Borges *et al*., 2009).

R2.18:

"Preparing well plates"

What kind of sea water is used? Filtered, sterilized? Salinity? Is the pH buffered?

What photoperiod is used?

"different wood species"

from the left to the right?

Author (R2.18): In order to maintain a simple and straightforward test method, and to be comparable to environmental data, we did not use treated seawater. Unfiltered seawater, consistent with that used to maintain the aquarium cultures, was used and animals were acclimatised to this over a long period prior to the experiment. The seawater salinity ranged between 32PSU and 35PSU depending on evaporation within the well plates, but this was minimised with the lid design of the plates and due to the fact that water was changed fully every three to five days. Seawater is a good pH buffer itself and was therefore not modified. Seawater was not sterilised as microbes associated with these animals are part of the maintenance of vitality. In addition, water changes would avoid any build-up of microbes. Gribble also carry a copious amount of microbes on their exoskeleton which would be transferred with them into the wells. No photoperiod was used as Borges *et al.* (2009) found that this did not affect feeding rate. during the experimental period, gribble were kept in constant dark conditions inside the incubator.

Different treatments or species of wood should be placed systematically throughout the well plate so that each type of wood is represented at least once per plate. Indeed, the wood species shown in Figure 5 (now Figure 6) shows different species from left to right.

R2.19:

Page 4

Specify if you use a single animal for each well

"Image processing"

Do you adjust contrast and brightness?

Author (R2.19): A single specimen of *L. quadripunctata* was indeed used per well. This was previously stated in the manuscript, and has now been reiterated at further points in the protocol (page 4 '**3 Preparing well plates**' and '**4 Collecting and counting faecal pellets and assessing vitality**' and page 5 '**Representative results'**). Brightness and contrast were not adjusted for image processing as the images were converted to binary for counting.

R2.20:

Page 5

why 20 days? How did you decide the experiment (test) duration?

Author (R2.20): A period of 20 days was chosen based on previous experiments using this method - Borges *et al.* (2008/2009) and Papadopoulos *et al*. (2008), who used 15 and 21 days respectively. Additional preliminary trials, one for a period of 4 weeks and one for a period of 8 weeks, corroborate our experimental design. The longer 8-week assay demonstrated no increased faecal pellet production beyond the shorter assay. Therefore, 20 days is sufficient time to quickly and effectively gain enough information to determine a wood's

resistance against marine borers. This allows rapid feedback of potential wood products to industry and can then be progressed into the second phase of testing, which is to use longer term marine trials as outlined in EN 275.

R2.21:

"A longer assay would be able to identify if these trends were to continue over time or if they are part of a normal fluctuation in daily faecal pellet production."
Why didn't you run the experiment longer?

Author (R2.21): We address this question in R2.20. To reiterate, a longer (8-week) preliminary trial provided no additional relevant data beyond the shorter 4-week trial. As such, we have now removed this sentence from the manuscript.

R2.22:

Figure 9
give in te caption also he number of gribbles n= xx although reported in the text

Author (R2.22): Thank you, this information has been added.

R2.23:

Page 6
"high resolution"
Could you give a measure of resolution suggested?

Author (R2.23): 'High resolution' was removed as a suggestion for image capturing.

R2.24:

"Vacuum impregnating wood with seawater causes the wood to sink and become readily accessible to the gribble. In the absence of vacuum impregnation"

Propose one choice, did you test the difefrence between vacuum impregnated and "drowned" specimens?

Author (R2.24): The option for 'drowning' wood using weights has been removed as both vacuum impregnation and leaching cause the wood to sink. Weights have been used in past preliminary trials of this experiment and were not seen to adversely affect gribble feeding.

R2.25:

Page 7

" If high mortality is seen after 7-8 weeks, this may be due to starvation, as starved gribbles"

"Therefore, conducting this laboratory experiment for eight weeks or longer can compare mortality from wood products to starved individuals."

The experiment reported here lasts 3 weeks (20 days), so do you suggest continuing it? You should consider the length of the tets you propose.

Author (R2.25): Starved gribbles can survive for 7-8 weeks which can allow comparisons when looking at the mortality rates of different preservative treatments for example. However, as this protocol primarily focuses on faecal pellet production and vitality as a means to rapidly test responses to different types of wood (including both naturally durable and treated woods) this has now been altered in the text (page 8). Recent, unpublished experiments running for 8 weeks show that there is no fluctuation in faecal pellet production over a time period longer than 20 days (See R2.20 and R2.21). In addition, previous methods, such as that used by Borges *et al*. (2008/2009), runs for 15 days.

Therefore, 20 days is a sufficient time for a rapid laboratory-based test to provide indication of wood durability.

R2.26:

"Not only will timber that is resistant to biodegradation reduce costs but will also be more environmentally friendly than using alternative materials such as concrete or steel, which require high energy input during manufacture, or broadspectrum biocide preservatives that may leach out and affect the surrounding ecosystem (Pernell, 2013;"

Could you add more references about the environmental effects of biocide leaching (by the way Pernell or Purnell?)

Author (R2.26): Thank you for the suggestion, additional references on impacts of biocidal wood preservative leaching have been added in the manuscript (see also R2.10). Also thank you for picking out this typo, Purnell is the correct author spelling.

R2.27:

References and figures

Figures 1, 4 and 8

Pictures seem to be out of focus, is it a pdf compression problem?

Author (R2.27): Yes, this appears to be a PDF compression issue. The images submitted were of high quality and were not out of focus.

R2.28:

Figure 9

Do you have an idea why 12-day readings reveal a generally lower number of fecal pellets? Are the differences in the production of fecal pellets statistically significant?

Author (R2.28): Faecal pellet production tends to be highest at around 3-7 days as the animals begin to create their burrows and therefore shift larger quantities of wood. Following this, on day 12 there was a reduction in vitality across species that saw a drop in faecal pellet production and a few individuals had died on Scots pine and ekki. Some mortality at the beginning of the experiment is expected due to the stress of collection and extraction (in addition to animals that die due to natural causes), although every effort is made to minimise this.

Reviewer #3:

Manuscript Summary:

R3.1: The method presented and proposed for standardization is highly interesting and well presented. The method has high potential to get implemented in wood durability research and thus in wood preservation.

Author (R3.1): We thank the reviewer for their comments. We are working towards this end with colleagues on the working party responsible for updating the relevant standard (EN275).

R3.2:

Major Concerns:

None.

Author (R3.2): N/A

Minor Concerns:

R3.3: Expert knowledge is needed to identify Limnoria species. Some guidance should be provided, at least suitable references shoud be provided.

Author (R3.3):

Limnoria quadripunctata, identified by the keys provided by Cookson (1991), Cookson and Vic (1997) and Menzies (1951), was selected as the optimal species for biodegradation trials because it is the most well-studied member of the family and is well-established as a model species for use in biodegradation trials (also see R2.3). Information on identifying *Limnoria quadripunctata* has been added to the protocol (page 3).

R3.4: What are the preferred Linoria species for test? L. quadripunctata?

Author (R3.4): Please see R3.3 above.

R3.5: Are there any further requirements for sampling the sea water (T, sampling depth etc.)? Do such factors affect the results?

Author (R3.5): Seawater was taken from the same source as that used in the aquarium cultures of Limnoria, from which we obtained our experimental animals (also see R2.18). This species does well in salinities 32-35ppt and temperature ranges between 15-25°C. Salinity was monitored and remained within this range through water changes and temperature was controlled at 20°C using an incubator.

R3.6: A major flaw of any screening test (and the presented method might be considered as a screening test) is its limited transferability to field conditions or real service conditions respectively. It would be helpful to briefly discuss the predictive power of the method with respect to the resistance of wood against gribble in the sea, and against other marine borers such shipworms etc.

Author (R3.6): We thank the reviewer for this suggestion and further information has now been added to the discussion (page 8). Yes, this method could be considered a screening test for novel products to be rapidly identified, for wood treatment methods to be optimised and for different sources of the same species of naturally durable wood to be compared. Results from testing with this method show a good correlation with results from marine testing. This is seen by comparing the findings of Slevin *et al*. (2015), who tested furfurylated wood in laboratory conditions, with those of Westin *et al*. (2016), who tested the same wood in marine field trials.

R3.7: Since living animals are involved in the experiment, I was wondering whether there are any legal limitations to be considered. Respective hints would be helpful for potential applicants.

Author (R3.7): This group of animals are not protected under the Animal Scientific Procedures Act 1986, so a Home Office licence is not required. However, all work is approved by the University of Portsmouth's Animal Welfare Ethical Review Board prior to commencing.

R3.8: The EN 275 protocol is the basis for assigning durability classes to wood-based materials. Would be beneficial to learn more about the interpretation of the test results and how they might be used for durability classification of wood.

Author (R3.8): Results from this rapid laboratory experiment are designed to inform novel modification processes of wood and to identify timber species with natural durability to borer damage. A low feeding rate and vitality can indicate increased resistance in potential products and this information can then be fed back to industry partners to allow them to improve designs. EN 275 currently

only assesses wood from long-term marine trials, rates test materials as '0= no attack', '1= slight attack', '2= moderate attack', '3= severe attack', '4=failure' (Palanti *et al.*, 2015). Our method allows a nimble and rapid response, that is desirable in industry, and once promising products have been identified, results can be supplemented with those from marine trials. – This has been added to the manuscript (page 2).

R3.9: Figure 9 and 10 are not trivial and their interpretation takes a while. Therefore they should be displayed for longer during the video.

Author (R3.9): We have now increased the duration of these images in the video

Reviewer #4:

R4.1: The manuscript describes a method for assessing the durability of timber in marine settings by testing its resistance to gribble in a laboratory set-up. This is relevant as marine testing using for example EN275 is expensive, requires expertise and specialised equipment, and may take years. The method demonstrated here can give indications within a few weeks using standard laboratory equipment, thus allowing for screening before engaging in marine testing.

Author (R4.1): We thanks the review for their comments.

The manuscript has some minor issues that need to be attended to before publication.

R4.2: CCA and Creosote being banned: Please be more specific about what region(s) of the world this applies to.

Author (R4.2): Information on creosote and CCA restrictions in the UK, EU, USA and Australia have been added (page 2).

R4.3: Please provide Latin species names within the text of the timbers included in the study (only mentioned in table of materials).

Author (R4.3): We thank you for the suggestion, this has been added within the manuscript (page 2 -"In the short-term laboratory testing presented here, we use Scots pine (*Pinus sylvestris* L) sapwood as a control to testing heartwood of the species ekki (*Lophira alata* Banks ex C.F Gaertn), beech (*Fagus sylvatica* L), sweet chestnut (*Castanea sativa* Mill) and turpentine (*Syncarpia glomulifera* (Sm.) Nied)")..

R4.4: Cutting of sticks and their dimensions: This is a bit unclear, as dimensions of wood depend on the moisture content. Are the sticks cut in a wet state, as the sticks should be air dried afterwards? Please be more specific - for which moisture state are the dimensions given?

Author (R4.4): All sticks were air dried prior to cutting and dimensions are given in an air-dried state. Step 1 of the methodology has been updated accordingly to clarify this point (page 2).

R4.5: Vacuum impregnation: The vacuum is stated to be 0.8-1.0 bar, but I suspect this can't be right as 1 bar roughly equals 1 atm. Should it perhaps be 0.8-1.0 mbar?

Author (R4.5): This is correct, the values should be negative and we have since changed the manuscript accordingly.

R4.6: Leaching: This step of the instruction is not operational enough. How is it determined when to stop the leaching? Ideally some UV-VIS measurement of the liquid and quantifiable comparison to clean, demineralised water could be

used. In absence of that, manual evaluation of the colour compared to pure water using standardised light conditions and a standardized white background could work. Or a fixed, standardized time.

Author (R4.6): It would indeed be ideal to test the water to ensure all leachate has been removed. However, as this method is designed to be useable in non-specialist labs, a simpler standardisation is required. We have updated the protocol to give a fixed time of 20 days for the leaching period (page 3). Previous work has demonstrated that a leaching period of one week is sufficient to allow trials to proceed (Borges *et al*., 2008/2009, Papadopoulos *et al*., 2008, Slevin *et al*., 2015) (see also R2.6).

R4.7: Leaching: 50 mk Falcon tubes -> 50 ml Falcon tubes

Author (R4.7): This is correct and we have since changed the manuscript accordingly.

R4.8: Feeding in multi-well plates: Please be more specific about how many replicates to use and how these should be placed in the well (random?). Are the plates placed in light or darkness (normally no light within incubators, right?), or does it not matter?

Author (R4.8): At least five replicates should be used for each type of wood being tested (EN275). Here, eight replicate sticks were used per species of wood with one gribble per stick. Samples in individual wells should be placed systematically so that each wood species or treatment is represented at least once in each plate. This is to avoid any potential external factors affecting a single well plate and impacting many replicates of the same treatment/species. Following work by Borges *et al.* (2009), that found photoperiod did not affect feeding rate, gribble were kept in the incubator in constant dark conditions.

R4.9: Taking photographs of faecal pellets: Please specify which magnification is used and whether a stereomicroscope or composite microscope is used. Please also give some hints for adjusting the light and image capture settings correctly (high/low intensity, contrast, time etc.). It would be useful to also give a few examples of "bad" images, where automatic image analysis does not work satisfactorily.

Author (R4.9): Thank you for the suggestions, the information on magnification and the type of microscope used has been added in the manuscript (page 4). We have also included an additional figure demonstrating poor quality images (Figure 7).

R4.10: ImageJ instructions: Please specify which version of ImageJ this procedure refers to - it might be outdated when/if ImageJ is updated.

Author (R4.10): The version used while writing this paper was 1.8.0_112. This how now been updated in the methodology.

R4.11: Moulting: Please be more specific here for readers not familiar with gribble biology. How long time does moulting take? One day? Should all animals be checked daily for this, or is the whole period leading up to a counting session discarded, if a moulting animals is encountered? Could moulting take place unnoticed in-between counting sessions?

Author (R4.11): Gribble moulting occurs for over a day with an intermoult period of around 32 days for the species *L. quadripunctata* (Delgery *et al*., 2006). A moult is detected and counted when an entire exoskeleton moult is seen within the well. Burrowed gribbles will remove the moult from the wood stick so they are very distinct when a moult has occurred. Delgery *et al*. (2006) found that the

pre-moult period also caused a reduction in feeding rate and feeding rate returned to normal within a few days after moulting. Therefore, the entire period between pellet counts is discarding once a moult is detected. If a moult occurs in between counting sessions, it is picked up at the next water change and the entire interval between the last viable count and when the moult was detected is discarded. For example, if a moult occurred on day 15 or 16, the count for day 17 (when the moult will have been detected) will be discarded, leaving the previously occurring count at day 14.

R4.12: Acknowledgements: Please also provide names/numbers of grants.

Author (R4.12): We have updated the acknowledgments with the grant names.

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