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13/11/2019

Dear Prof Zeng and Prof Sonne

Through the Editorial system (EVISE), I have sent you our manuscript entitled "Microplastic ingestion by commercial bivalves from genus *Meretrix*" by Cher Wei Yuan with Hoy. H. Sherilyn and Sarah P. Nelson. We would be grateful if you would consider it for publication as a full research paper in *Environmental Pollution*.

Evidence of microplastic (MP)-associated toxicity in humans garnered scientific interest in examining the susceptibility of popular commercial species to microplastics ingestion. Under this current scientific climate, we find it surprising that there are no studies on *Meretrix* spp., which are locally consumed in Southeast Asia and China. Here, we present the first evidence of MP uptake by *M. lusoria* and *M. meretrix*. Although most of our samples resisted MP uptake, the remaining exhibited MP ingestion rates affected by both size and species. We accounted for these effects in our statistical model which informs the shell length at which a particular *Meretrix* sp. is less likely to harbour MP and is thus safer for human consumption.

Our research adds to the repository of recent publications in *Environmental Pollution* elucidating MP uptake by commercial bivalves, namely "Microplastics in mussels along the coastal waters of China" by Li et al. (2016) and "Microplastics in mussels sampled from coastal waters and supermarkets in the United Kingdom" by Green et al. (2018). While they showed presence of MPs in field samples, we highlight laboratory experiments as a means to show the conditions at which the bivalves uptake less MPs. We hope our work translates to guidelines for shellfish farming (e.g. harvesting at a time when bivalves contain less MP) other food-safety policies that potentially affect millions in Asia, where clams serve as an affordable and common protein source.

The contents of this paper are original, have not been published and are not being considered for publication elsewhere. The authors declare that we have no conflict of interest and that all applicable guidelines for the care and use of animals were followed. All authors have approved the manuscript and agree with its submission to *Environmental Pollution*.

Yours sincerely,

Cher Wei Yuan

Undergraduate

Potential reviewers

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Microplastic ingestion by commercial bivalves from genus *Meretrix*

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A R T I C L E I N F O

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A B S T R A C T

Microplastics (MP) are ubiquitous in the ocean. Marine vertebrates and invertebrates, including bivalves, were shown to ingest MPs. As emerging evidence suggests toxicity associated with human consumption of MPs, there is an impetus to understand the propensity of commercial bivalves to ingest and retain MPs. Here, we subject the commercially valuable *Meretrix meretrix* and *Meretrix lusoria* to laboratory MP exposure experiments. A large number of replicates (n = 30 out of 48) did not show MP ingestion. Our multiple regression model indicated the interaction between shell length and species as a significant predictor explaining 11% of the variance. Taken together, *Meretrix* spp. generally resist MP ingestion but if the ingestion occurred, the rates were dependent on size and species. These results can guide health authorities in future directions for food safety.

1. Introduction

As a material, plastic has provided tremendous improvements in the quality of everyday life. The popularity of plastic use is reflected in polymer resin and synthetic fibre production, which soared from 380 million tonnes (Mt) from the 1950s to 2 Mt in 2015 (Geyer et al., 2017). After use, plastics were disposed of in landfills or the natural environment (Geyer et al., 2017). Physical abrasion or UV exposure can fragment them and produce MPs (Barnes et al., 2009), which are particles of size < 5 mm (Arthur et al., 2009). Besides fragmentation of large plastic pieces, MPs can also be produced directly, such as the MPs in cosmetics (Napper et al., 2015). A conservative estimate of 65 million MPs were discharged into the receiving water daily (Wright and Kelly, 2017). Unsurprisingly, MPs thus permeated all the major ocean and many freshwater environments (Rochman et al., 2015a).

MP ingestions were documented in many marine organisms (see Lusher et al., 2015; Moore et al., 2001; Murray and Cowie, 2011; Rochman et al., 2015b; Romeo et al., 2015). Concerns for human health were heightened when MPs were found in seafood (Smith et al., 2018). Together with emerging evidence suggesting consumption of MPs in seafood can be toxic to humans (extensively reviewed in Smith et al. (2018) and Wright and Kelly (2017)), the proclivity of commercial species to ingest MPs garnered scientific attention. Bivalves, as filter feeders that pump large volumes of water and retain particles on their gills, are recognized as potential food safety hazards (Wright and Kelly, 2017). Concerns on shellfish food safety thus led to field studies to assess MP ingestion by commercial bivalves (Li et al., 2016; Cauwenberghe and Janssen, 2014).

Currently, there is a lack of information on MP uptake by mangrove clams that are locally consumed in Asia. In China (Wang et al., 2013), Malaysia (Hamli et al., 2012) and Singapore, two commercial species, *Meretrix meretrix* and *Meretrix lusoria*, were not studied in relation to MPs. Here, we aim to examine the predisposition of these bivalves to MP intake. Together with future field studies, our work informs state-level public health agencies on future directions.

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2. Material and methods

We examined a total of 240 bivalves of two *Meretrix* spp. They were marketed as 'white venus' (N = 145) and 'la-la' (N = 95) clams but were identified as *M. lusoria* and *M. meretrix* with a species key (Hamli et al., 2016, see Supplementary Figure 1). Our experiments were conducted between 4 - 23 October 2019. We constructed a set-up (Figure 1) with aquarium air pumps (Super Beetle 12000 Air Pump and BOYU S-4000B) to circulate MPs in 1.5 L coke-light bottles containing the bivalves during the experiment. MPs denser than water were made by grinding PVC pipes using a hand-held orbital sander. By filtering the MPs through filters with known pore size, we know the MPs were roughly 25 microns. Each bottle contained five bivalves and 200 mg of MPs in approximately 850 mL of water. The amount of MPs used here were about 13-100 times higher than surface waters in coasts and reservoirs (Paul-Pont et al., 2018) to ensure MPs were not a limiting factor on consumption rates.

Previous records showed bivalves filtering 300 mL to 3L of water per hour (Marescaux et al., 2016; Vijayavel et al., 2007). Thus, we exposed the bivalves to circulating MPs for an hour to clear the entire water column (850 mL). For morphometrics, we followed López-Rocha et al. (2018) for measurement of shell length and height with Vernier calipers. Subsequently, the bivalves were storage at -20 ºC overnight prior to weight measurement and dissection. Next, using coffee filters (pore size of 20 microns), we isolated the MPs. We chose coffee filters because they do not capture tiny airborne particles that contaminate MP experiments (Yang et al., 2015). We later dried the filter paper in an oven (Shel Lab, model no. 1500E-2) in covered Petri dishes overnight. Then, the particles were isolated on a tray and weighed.

All statistical analysis we were performed in RStudio (Core RStudio Team, 2019; R Core Team, 2018). Prior to our experiments, we conducted power analyses using the pwr package (Champely et al., 2018). We aimed for a sample size of 30 for 80% power when the effect size is large, where Cohen's *f2* = 0.35 (Cohen, 1988). Nonetheless, we collected 48 replicates, which gave us 63.7% for medium and 95% power for large effect sizes. After the experiments, we calculated the mass of MP ingested with the following formula:

$$MP\ ingestion\ (mg) = 200 - mass\ of\ microplastic\ left\ in\ filter - mean\ MP\ loss\ in\ negative\ control$$

Four independent controls (with MPs but not bivalves) were used to estimate the loss of MPs during the experimental procedure. The mean MP loss in these negative controls was 47 mg (σ = 7.8 mg). Negative mass for MP ingestion after deduction was rounded to 0. We also had controls with bivalves but no MPs to account for bivalve excretions. Nevertheless, the weight obtained was too negligible to be picked up by the weighing scale.

A multiple regression was constructed to predict the mass of MP ingested based on height, length, weight and species. However, the morphometrics and weight were collinear (checked with vif package (Lin, 2011); since frozen weight was confounded by water content and shell length was strongly correlated with total body weight (r = 0.955, Zhang et al., 2018), only shell length was included in the model. We compared all possible linear models by AIC using MuMIn (Package and Inference, 2019). Graphics were plotted with ggplot (Wickham et al., 2019). The data and R scripts were uploaded to GitHub (https://github.com/CherWeiYuan/SP3203---Bivalve-Filtration).

3. Results

Our results show that *M. lusoria* (white venus, n = 19) and *M. meretrix* (la-la, n = 29) can ingest MPs of size 20 microns within an hour (mean of MP ingested = 7.7 ± 14.6 mg). The MP ingested were observed in our dissected bivalves. However, a majority of the bivalve replicates did not show any detectable MP ingestion (white venus: n = 17; la-la: n = 14). We constructed a multiple regression model to examine MP ingestion based on species and shell length (Table 1). The model diagnostic plots indicated heteroscedasticity which cannot be resolved by log transformation or the addition of variance structures in GLS. The maximal model was marginally behind the model with only species (difference of AIC = 0.1, or delta = 0.07); we picked the maximal model because its interaction term explains species-length trends we observed during exploratory data analysis. Indeed, the interaction term explained a significant amount of variance (10.96%, Table 2). Also, all explanatory variables were significantly different from zero (Table 1). We plotted a regression line for the model (Figure 2) and detected two trends: firstly, except for two replicates, the remaining *M. lusoria* consumed more MPs than all the smaller-sized *M. meretrix*. Secondly, there was a decrease of 1.05 mg of MP for every mm increase in shell length for *M. meretrix*. In contrast, for every mm increase in shell length for *M. lusoria*, there was an increase of 0.27 mg of MP uptake (Table 1).

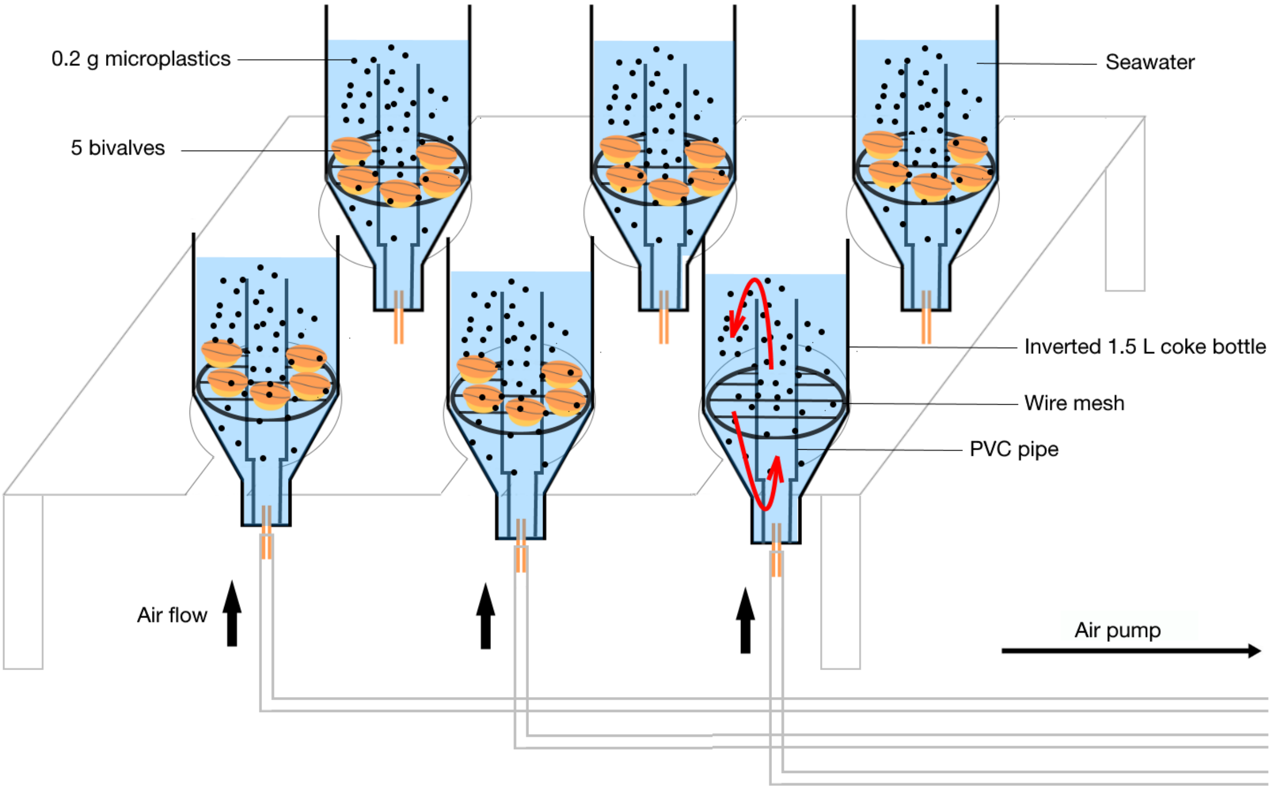

Figure 1. Set-up to circulate MPs. A photo of the actual set-up is provided in Supplementary Figure 2.

Table 1. Coefficient and intercept values for the multiple regression: MP ingested = $\beta_1 \times$ length + $\beta_2 \times$ species + $\beta_3 \times$ species $*$ length. The multiple R^2 value is 0.12.

	Estimate	Std. Error	t value	p
β_1	-1.05	0.50	-2.10	0.04 *
β_2 (*M. meretrix*)	175.69	80.95	2.17	0.03 *
β_2 (*M. lusoria*)	-41.42	94.3	-2.30	0.02 *
β_3	1.32	0.56	-2.30	0.02 *

Table 2. ANOVA table for multiple regression model.

	Sum Sq	Mean Sq	F value	p	Percentage variance explained (%)
Length	52.1	52.1	0.26	0.61	0.52
Species	49.3	49.3	0.24	0.62	0.49
Length: Species	1091.7	1091.7	5.48	0.02 *	10.96
Residuals	8764.7	199.2	N.A.	N.A.	88.01

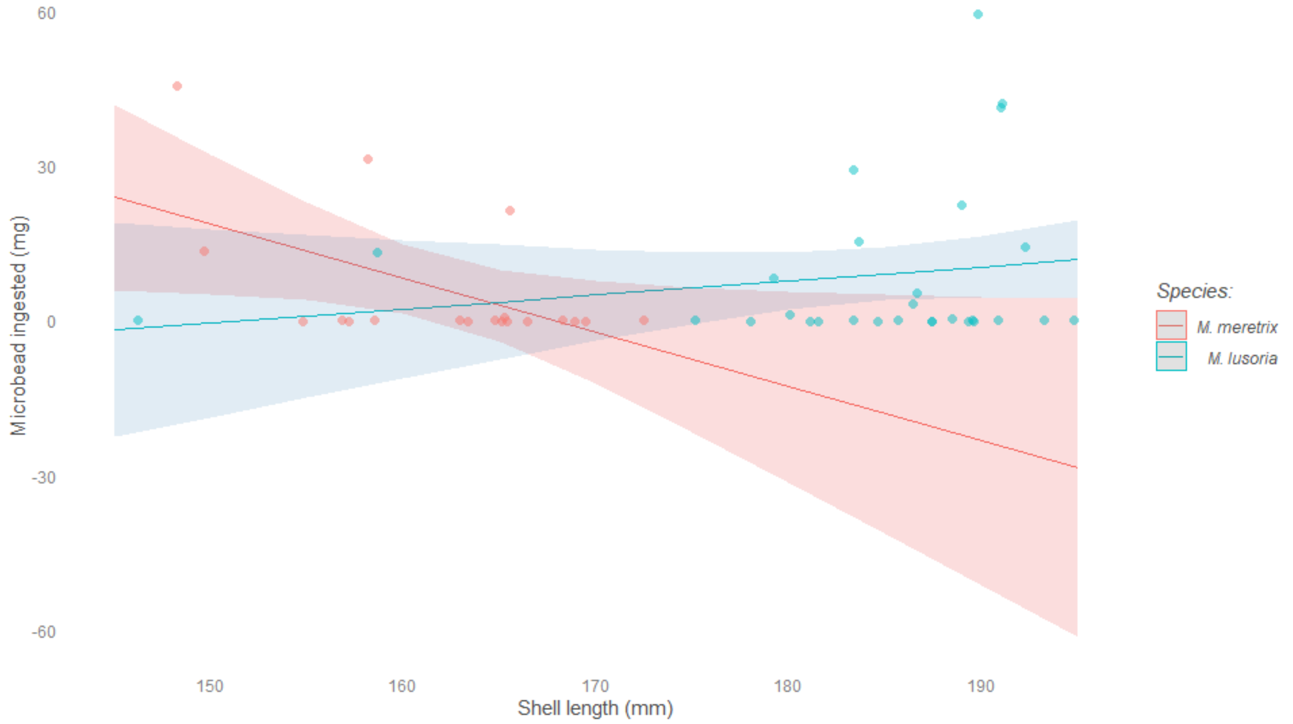

Figure 2. Multiple regression model showing the relationship between MP ingested (mg), bivalve height (mm) and species. The standard error is shown in coloured bands along the regression line.

4. Discussion

In this study, we exposed *M. lusoria* and *M. meretrix*, two commercially important bivalves in Malaysia (Hamli et al., 2012) and Singapore, to MPs in an experimental setting and confirmed they ingest MP. We dissected a bivalve from each set-up and found, especially for the replicates with high MP ingestions, MPs on the outer surface (e.g. mantle and foot) and also within the digestive tract. This supports the view that MP intake can occur via the gastrointestinal tract or by adherence to tissues (Kolandhasamy et al., 2018).

However, both species showed resistance to MP uptake. We observed 65% of our replicates did not have more MP loss than the negative control. This may be explained by MP expulsion from gills or digestive glands as observed in *Mytilus edulis* (Woods et al., 2018). Alternatively, *Meretrix* spp. might behave similarly to *Mytilus galloprovincialis*, which preferentially uptake MP fibres but not MP granules (Ding et al., 2018). Hence, it is possible that the MP ingestion rates can be more detectable if microfibres, instead of granules, were used. Notably, the bivalves we bought from the market were kept for extended periods without food. Consequently, they were in an unhealthy state, which may also explain their reduced MP intake. Future studies may consider maintaining healthy bivalves by rearing them at optimal conditions prior to the experiment.

Our data showed species-specific ingestion patterns. For *M. lusoria*, a larger shell length predicts higher MP ingestion (Figure 2). This trend is mirrored in various bivalves, where larger size provides greater surface area for tissue adherence (Kolandhasamy et al., 2018) and also correlates with higher filtration rates (Metaxatos and Ignatiades, 2011). In contrast, the larger *M. meretrix* uptake less MPs, a phenomenon observed but not explained by any biophysical mechanics yet (Rosa et al., 2018). The species-specific trends here can guide policies mandating the harvest of bivalves at particular sizes to minimise their MP content prior to human consumption.

Heteroscedasticity was detected in our model (increasing variance of residuals with increase fitted values) but could not be resolved by fitting variance structures or log transformation. The consequence is possibly an erroneous calculation of the standard error, which suggests that the beta values in the model may actually not be significant. Since we fitted a maximal model, the non-normality of residuals could mean we missed an important variable, resulting in impure heteroscedasticity. We suspect the missing variable is siphon size as larger siphons enable the bivalves to take in a greater range of MP, which explains larger variability at higher fitted values. Thus, we suggest future studies to measure siphon size after dissection.

A limitation of our study is the use of one MP type. An ideal experiment should mimic the natural environment, which may require a diverse range of MPs (type, size, quantity and colour) in different conditions (weathering status and organic/ inorganic coating) (Paul-Pont et al., 2018; Phuong et al., 2016). Future work should consider surveying farm sites of *Meretrix* spp. to determine the types of model MP suitable for experimental manipulation. Also, the MPs can be weathered in the farm prior to the experiments.

5. Conclusion

M. lusoria and *M. meretrix* are commercially important yet understudied with respect to MP ingestion. Our results confirmed that both bivalve species uptake MP. We suggest the use of our modelling results to inform the sizes at which the bivalves can be consumed more safely due to their lower MP content.

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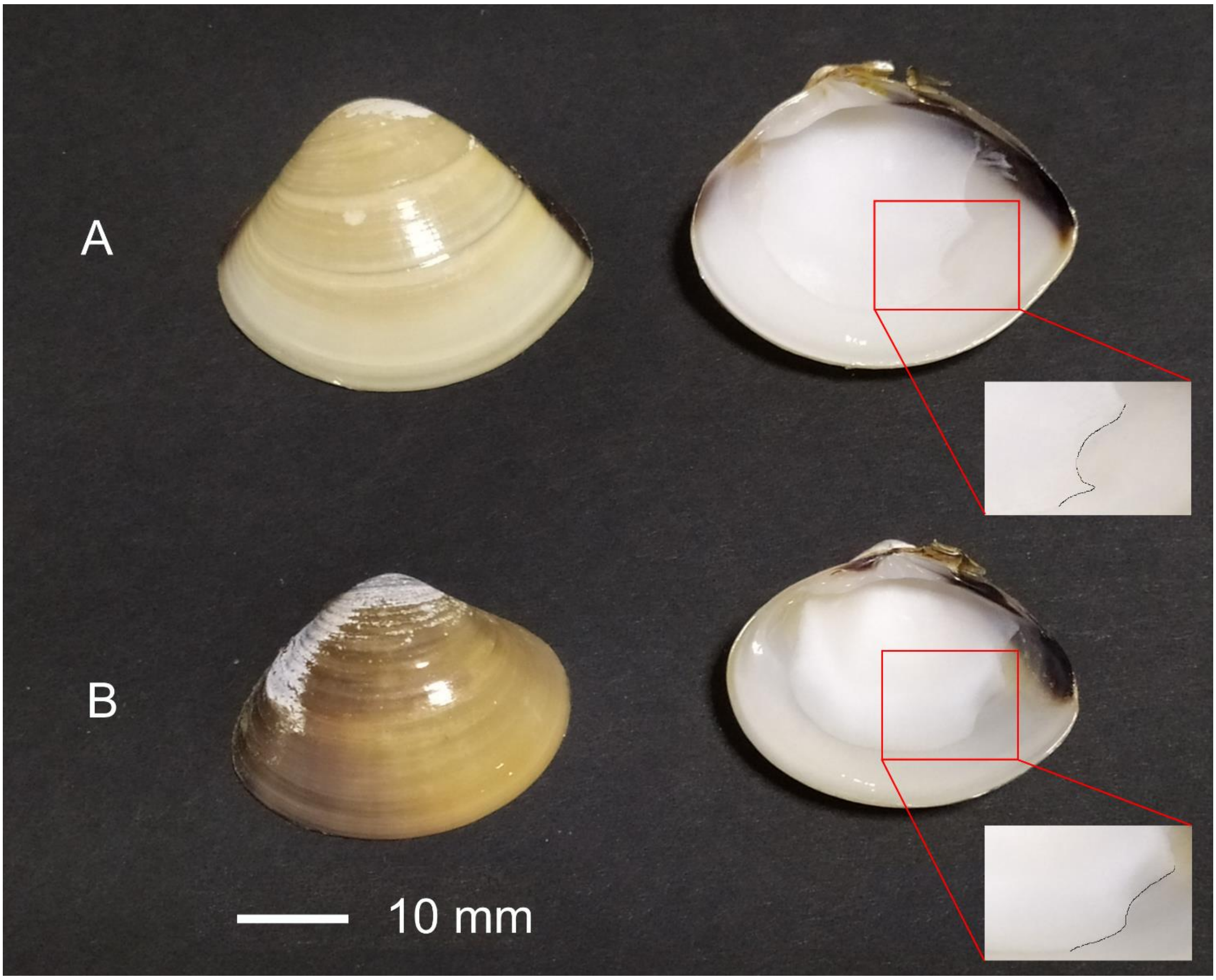
SUPPLEMENTARY DATA



Supplementary Figure 1. Identification of bivalve species using key for *Meretrix* spp. in Southeast Asia from Hamli et al. (2016). The enlarged areas shows the pallial sinus scar, which is traced using a black line. Both the (A) white venus and (B) la-la clams have smooth shells without undulate patterns, hence they are unlikely *Meretrix lyrata*. (A) The white venus has a white shell and a distinct L-shaped pallial sinus scar pattern, which fits the description of the *Meretrix lusoria*. (B) La-la clams have brownish shells without the distinct 'L' pattern which helped us identify it as *Meretrix meretrix*.

Supplementary Figure 2. A photo of the set-up as illustrated in Figure 1.