THE PHYSIOLOGICAL IMPACT OF MICROPLASTICS ON

Holothuria leucospilota

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BOGOR

2015
I hereby declare that the thesis entitled The physiological impact of microplastics on *Holothuria leucospilota* is my own work under the direction of an advisory committee. It has not yet been presented in any form to any Education Institution. The sources of information which is published and unpublished by other researchers have been mentioned and listed in the references of this thesis.

Bogor, September 2015

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C551130121
RINGKASAN

KHOIRUNNISA ASSIDQI. Dampak Fisiologis Mikroplastik Terhadap Holothuria leucospilota. Dibimbing oleh NEVIATY PUTRI ZAMANI dan HAWIS H. MADDUPPA.


Polyvinylchloride (PVC) dan fluoranthene merupakan representatif model plastik dan model polutan yang dipakai. Ukuran partikel PVC yang digunakan adalah 50-200 µm dan telah dicemari oleh fluoranthene terlebih dahulu. Kelompok teripang (n = 15) dibagi menjadi 6 skenario pencemaran yang berbeda (yaitu 0%; 0,03%; 0,3%; 1%; 3%; 3% yang tanpa dicemari dengan fluoranthene) penentuan berdasarkan presentase densitas mikroplastik pada berat sedimen. Selanjutnya, teripang yang telah terpapar mikroplastik yang dicemari dan yang tidak dicemari fluoranthene menghadapi kondisi hipoksia digunakan sebagai variabel respon untuk menyelidiki apakah H. leucospilota memiliki resistensi yang lebih rendah terhadap stres lingkungan akibat telah mencerna mikroplastik sebelumnya.

Hasil penelitian menunjukkan bahwa setelah 60 hari paparan mikroplastik partikel PVC yang dicemari dan yang tidak dicemari oleh fluoranthene tidak memiliki pengaruh negatif pada fisiologis (laju respirasi, produksi kotoran dan tingkat kelangsungan hidup) dari teripang, H. leucospilota. Penelitian ini menunjukkan bahwa H. leucospilota rentan terhadap penurunan konsentrasi oksigen. Kemungkinan, penelitian secara jangka panjang akan mengungkapkan dampak negatif yang tidak terdeteksi dalam penelitian ini. Penelitian ini juga mengumpulkan dan menemukan mikroplastik di seluruh sampel sedimen, kedalaman dan lokasi pengambilan sampel di pulau Rambut. Komposisi kelimpahan tipe mikroplastik yang ditemukan pada lokasi pengambilan sampel adalah fiber, partikel foam, film dan fragment. Penelitian lebih lanjut perlu dilakukan untuk mengungkapkan pengaruh kemungkinan kerentanan hewan laut terhadap biodiversitas laut terutama di pulau Rambut, salah satu pulau yang terdekat dengan Teluk Jakarta.

Kata kunci: Mikroplastik, PVC, fluoranthene, teripang, tingkah laku, kelangsungan hidup, Pulau Rambut
SUMMARY

KHOIRUNNISA ASSIDQI. The Physiological Impact of Microplastics on Holothuria leucospilota. Supervised by NEVIATY PUTRI ZAMANI and HAWIS H. MADDUPPA.

Nowadays plastic debris are found in oceans. Various processes lead to a transformation of macroplastics into microplastics (i.e. particles < 5mm). Sunlight and the action of the waves can degrade plastic and shred the material over time into smaller pieces. Furthermore, they can be found as ingredients of different products e.g. cosmetics and therefore enter the marine environment. Microplastics could have negative effects on marine organisms, especially for benthic organisms but up to now little is known about potential consequences. This study aims to investigate the physiological impact of microplastics on marine deposit-feeding invertebrates like sea cucumber Holothuria leucospilota.

Polyvinylchloride (PVC) and fluoranthene was selected as a representative model plastic and model pollutant. The size range of PVC particles were 50-200 µm and have been previously polluted with fluoranthene. In laboratory, groups of sea cucumbers (n=15) were exposed to 6 different pollution scenarios (i.e. 0%; 0.03%; 0.3%; 1%; 3% and 3% non polluted with fluoranthene) which differed regard to the microplastics density (% by weight of sediment). Furthermore, after exposed from PVC particles polluted and non polluted with fluoranthene, H. leucospilota would faced hypoxia condition used as a common response variable to investigate if H. leucospilota has a lower resistance to environmental stress due to previous microplastics ingestion. The study revealed that after 60 days exposure to microplastic PVC particles polluted and non polluted with fluoranthene is not given any negatively influence on physiological parameters (respiration rate, faeces production and survival rate) of black sea cucumber H. leucospilota. This study clearly indicates that H. leucospilota is susceptible to oxygen depletion. Probably, long-term experiments will reveal negative impacts that were not detected in the present study. Another aspects from this study, microplastics particles were collected and found in all sediment samples, depths and sampling locations at Rambut Island which also took H. leucospilota but in different site. The diverse composition of microplastics particularly on type at the sampling location Rambut Island were found fibers, foamed particles, films and fragments. Further, these findings revealed a possible threshold of marine organisms on the marine biodiversity especially in Rambut Island, one of closest island to Jakarta Bay.

Keywords: Microplastic, PVC, fluoranthene, holothurian, behavioral, survival, Rambut Island
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THE PHYSIOLOGICAL IMPACT OF MICROPLASTICS ON
Holothuria leucospilota

KHOIRUNNISA ASSIDQI

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1 INTRODUCTION

Background

The abundance of plastic debris is uncountable. It is unlikely that plastic production can be entirely stopped in near future, but everyone can do his best to reduce the use of plastic. Many scientists focusing on marine pollution have studied and reported about the composition, abundance, distribution and quantification of the types and amount of plastic debris (Ryan et al. 2009; Viehman et al. 2011; Law et al. 2014). Plastic debris is becoming an increasing problem for the world, not only in the land but also in the ocean. Huge quantities of plastic debris can be found in the ocean (UNEP 2014). Many marine organisms are harmed by plastic debris (Ryan 2008; Gregory et al. 2009; NOAA 2014; Bond et al. 2013). Uneputty and Evans (1997) found that plastic debris on shores of the Thousand islands originated from Jakarta and it could spread out 30-45 km from the mainland, which were carried and stayed in uninhabited island.

According to research by Willoughby et al. (1997) the amount of plastic debris has increased from 1985 to 1995 in the Thousand Islands, Indonesia, where plastic debris poses new problems for life in the marine environment. A lot of plastic debris from Jakarta Bay is carried by currents and thus enters the marine environment and settles along the shorelines of the Thousand Island. Jambeck et al. (2015) has been reported Indonesia is the second largest country that ranks in the amount of plastic debris.

The major part of plastic debris comes from the land floating in the rivers and drifting into the ocean environment. The plastic debris travels all over the ocean, being transported by currents (Zarfl and Matthies 2010) without any clear boundaries in the marine environment. The physical factors in the ocean can carry debris overall in the ocean. According to Cozar et al. (2014) distribution of plastic debris is worldwide, however, it will accumulate in the convergence zones of the five subtropical gyres. Even at conservation islands or protected islands, plastic debris can be found. But unfortunately many animals do not know how to distinguish between their food and plastics. This case makes a problem for them because they eat plastics (Mansui et al. 2014). UNEP (2014) also reported plastics become entanglement of marine mammals for example dolphins and large whales. So plastics is a hazard for marine organisms. The secondary problem is not only debris comes from land, but also from in the sea, such as fisherman losses there some part fishing net when they are fishing and do fisheries culture activities (Vieira et al. 2014).

Some of the scientists already investigated that sunlight and the action of the waves can breakdown plastics and shred the material over time into smaller pieces. Sunlight and UV radiation as we known photodegradation will make plastic losses their elasticity and hydrolysis because the reaction with seawater (Andrady 2011), these cases also determine how long plastic can degrade depending on their polymer types. Deraik (2002) said plastic debris in marine environmental will degradation into smaller fragments, some of which are microscopic in size and described as microplastics. Microplastics have been reported on beaches, subtidal sediments and surface waters (Eriksen et al. 2013
Furthermore, they can be found as ingredients of different products like cosmetics such as facial cleansers, toothpaste and clothes industries, it is called primary microplastics, because it is already produced by microscopic size (Fendall and Sewell 2009; Cole et al. 2011).

Thompson et al. (2004) have reported similar fibers were found in the sediment habitats, some fragments were granular, the size more less than 20 µm with brightly colored. Many of the studies already reported the sensitivity of benthic invertebrates with microplastics exposure. Microplastics could have negative effects on marine organisms, especially for benthic organisms, but up to now little is known about the potential consequences.

Microplastics from polyvinylchloride (PVC) and polyethylene (PE) have potential to absorb and desorb the persistent organic pollutants (POPs) (Teuten et al. 2007, 2009 and Bakir et al. 2014). Bakir et al. 2014 have observed the persistent organic pollutants (POPs) or plastic combination examined polyethylene (PE) with phenanthrene (Phe) gave the highest potential for transport to organisms. For example, microplastics ingestion in the marine worm, Besseling et al. 2012 have been observed microplastics have a reduction in feeding activity and weight loss in a polystyrene dose of 7.4% dry weight by Arenicola marina. Wright et al. 2013 also reported A. marina, was shown to significantly deplete energy reserves by up to 50% as a result of a combination of reduced feeding activity, longer gut residence times of ingested material and gut inflammation.

Von Moos et al. (2012) have observed that microplastic particles are taken up into digestive cells of Mytilus edulis L. Graham and Thompson (2009) have been investigated for Holothurians ingest nylon and PVC fragments along with sediment. Moreover, plastic ingested by benthic foragers, many of whom occupy a low trophic level, may be a means of reintroducing settled plastic debris to littoral, nektonic, and pelagic food webs (Graham and Thompson 2009).

The extreme condition in marine environment is not predictability by disturbances (Vilnäss et al. 2012). The internal factor because of global warming temperature can leads to reduce oxygen level in ocean. The external factor from anthropogenic activities always increasing annually, which can decrease drastically oxygen level. Gray et al. (2002) has been reviewed that marine life can not thrive and it will increasing mortality when the concentration dissolved oxygen in the water are below 2 mg O₂ L⁻¹ to 0.5 mg O₂ L⁻¹. The primary effect of the marine environment are pollutants and nutrients as stressor will decrease dissolved oxygen. Hypoxia also can build up in this condition.

Consequently, it would affects to loss biodiversity in the benthic ecosystem services, because of it, they could not survive in heavy sedimentation (Worm et al. 2006). Thus, it would be impair the performance of holothurian. Hypoxia phenomenon will impact to their survival in marine ecosystems. Some studies already reported that hypoxia became a stressor could gave a bad impact for the sustainability marine organisms such as physiological stress and the worst impact were reduced growth and reproduction (Vaquer-Sunyer and Duarte 2008; Loddington 2011).

Holothurian has a role in the coastal ecosystem, which according MacTavish (2012) had investigated holothurian can be used as biotubators, the
number of bacterial abundance and the exchange of nutrients and dissolved oxygen in water or marine coastal sediments. There is a connectivity between the physical and biological processes in the marine coastal environment. Holothurian is one of the deposit feeder respires with all tentacles around their skin and consume a lot of water inside their body and take dissolved oxygen from that. Holothurian could be a biological signal for marine coastal ecosystem because their responses from the physiological and behavioral will describe the ecological process and to immediate the ecological status (Riedel et al. 2014).

**Problem formulation**

Plastic debris can be found in large quantities at marine coasts as well as in the open sea. As the development of science, Cole et al. (2011) have found that plastics as we known, it is called macroplastic, due to the sunlight and the action waves can breakdown plastics and shred the material over time into smaller pieces, if the size is less than 5 mm and described as microplastic (Deraik 2002). Apart from that the effect of microplastics in marine organisms are not well understood. *Holothuria leucospilota* as a test organism, because it is a robust and quantitatively important deposit feeder at the base of the marine food web and also has a role in the coastal ecosystem. The uptake of plastics by sea cucumber has been described before by Graham and Thompson (2009).

Moreover, microplastics have recently been detected in sea cucumbers. In earlier studies that address the effects of microplastics on bioaccumulation of POPs to marine benthic organisms are negligible. Further, microplastics could accumulate and transport POPs such as polychlorinates biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) which uptake by low trophic level into higher food chain which is humans. Polyvinylchloride (PVC) and fluoranthene was selected as a representative model plastic and model pollutant, since PVC is one of the five main high production volume plastics that make up about 90% of the total plastic demand, and is therefore commonly found in the marine environment. Therefore, because the density of PVC is higher than that of seawater, it may settle faster than equally sized microplastics with a lower density, thus contributing to increased concentrations in sediment.

Fluoranthene is one of PAHs as model pollutant due to its toxicity, it has potential to carcinogenic and mutagenic for aquatic organisms and most abundant in the sediment (Zhou et al. 1998). Meador et al. (1995) and Browne et al. (2013) reported that sediment ingestion by marine invertebrates has bioaccumulation to fluoranthene and transferred into tissues. Microplastics is one of waste contributor in the marine environment. Accordingly, overenrichment and pollutants enter to the marine environment, this situation will potentially lead to sedimentation and causing oxygen depletion as well as environmental stressor. Therefore, hypoxia condition used as a common response variable to investigate if *Holothuria leucospilota* has a lower resistance to environmental stress due to previous microplastics ingestion. The relevant pathway was microplastics’ pollution may have implied to the physiological, behavioral and survival of *H. leucospilota* which assessed of hypoxia condition (Figure 1).
Objectives of study

1. To determine the effect of microplastic PVC particles (polluted and non-polluted with fluoranthene) in the sediment diet of *Holothuria leucospilota* on physiological parameters which are respiration rate, feces production and survival rate of *Holothuria leucospilota*.

2. To determine the effect of microplastic PVC particles (polluted and non-polluted with fluoranthene) on the behavioral and survival of *Holothuria leucospilota* to hypoxia stress.
Hypotheses

The following null-hypotheses were tested:

1. Ingestion of microplastic PVC particles non polluted with fluoranthene has no significant effect on the respiration rate, feces production and survival rate of *H. leucospilota* (H0₁).

2. Ingestion of microplastic PVC particles polluted with fluoranthene has no significant effect on the respiration rate, feces production and survival rate of *H. leucospilota* (H0₂).

3. Ingestion of microplastic PVC particles polluted and non polluted with fluoranthene has no significant effect on the behavioral and survival of *H. leucospilota* under hypoxia stress (H0₃).

2 METHODOLOGY

Time frame and study locations

Preparations for the experiment carried out on April 2014. The sampling of target organisms and experiments carried out on May 2014 and ran until the end of September 2014. The experimental organisms and the sediment were collected at Rambut Island (Thousand Islands) (Figure 2). Experimental work (pilot study and feeding experiments) was carried out in Marine Habitat Laboratorium facilities of Faculty of Fisheries and Marine Science Technology at Bogor Agricultural University (IPB).

Materials and Methods

Field microplastic monitoring and analysis

Sampling site

Sediment samples were taken at the southern tip of Rambut Island (5°58'41.9"S 106°41'40.2"E), which is located 1.8 km from Jakarta, Indonesia (Figure 2). Rambut Island belongs to the maritime region of the Thousands Islands and is the island of the archipelago that is closest to Jakarta Bay. Jakarta Bay, which borders with Tanjung Kerawang in the eastern part of Bekasi and Tanjung Pasir in the western part of Tangerang. The bay is part of the metropolitan region of Jabodetabek, which has an outstandingly high degree of urbanization, and therefore receives wastewaters from industrial, agricultural and domestic human activities.
Figure 2 Map of Rambut Island, dot represent the location of the microplastics monitoring and diamond represent sea cucumbers were sampled. The sites were different between microplastics monitoring and sea cucumbers due to the conditions in Rambut island, it had not possibility taken at the same site.

Sediment sampling

Sediment sampling was conducted on June 19, 2014. For this, two transect lines separated by a distance of 5 m were placed in parallel to the waterline at the southern tip of Rambut Island. One of these transects followed the high tide line, while the other marked the low tide line (Figure 3). Local low and high water levels were observed before the transects were laid. Per transect we placed three quadrats (replicates, 1m$^2$ each) at a distance of 10 m from each other and took 5 sediment cores (sub-replicates) with a diameter of 10 cm and a length of 10 cm from each quadrat. For this, a glass corer was chosen to prevent any unintended contamination of the samples with microplastics. For further analysis, each core was divided into a top layer (0-5 cm) and a bottom layer (5-10 cm) and all five top and bottom layers of a quadrat were then pooled into a single container.

Sediment processing

Sediment samples were dried in an electric oven for 24 hours at a temperature of 60°C. The dried sediment was then sieved two times with different mesh sizes: First with a mesh size of 4000 µm to exclude larger debris items and then again with a mesh size of 500 µm to retain all particles larger than this. To extract all low density plastic items from the resulting fraction, a
hypersaline solution with 350 g NaCl/L freshwater was added to the residue (Thompson et al., 2004, Browne et al., 2010). However, this solution was not suitable to extract high density polymers such as polyvinylchloride (PVC) that has a density of 1.14-1.56 g cm⁻³ or polyethylene terephtlate (PET) with 1.32-1.421 g cm⁻³ (Claessens et al. 2013).

The mixture of sediment (200 g) and hypersaline solution (600 ml) was incubated for 24 hours on a mechanical shaker to homogenize the suspension. After this, we let the sediment particles settle down for 3 hours and then the supernatant was decanted and filtered using a vacuum pump and a Whatman paper filter with a pore size of 1.5 µm and a diameter of 55 mm. Filters were then placed into petri dishes to facilitate the counting and classification of microplastic particles under a stereo microscope used 20 times magnification (Olympus SZ61). Our particle classification followed Van Cauwenberghe and Janssen (2014) and Laglbauer et al. (2015). According to them, particles were categorized by colour and type (i.e. fibers, films, foams, fragments, pellets and spheres). However, this method does not allow to identify the origin and the polymer type of the classified particles. Finally, the total number of microplastics particles per sample was recorded.

Figure 3 The sediment sampling procedures: samples were collected along the high and low tide lines using a glass core (diameter = 10 cm, high = 10 cm) in each quadrat transect (1m²).

Sea cucumbers collection

The test organisms of black sea cucumber *Holothurian leucospilota* were collected during low tide in Rambut Island (5°58’30.9"S 106°41’42.9"E) (Figure 2). *H. leucospilota* were found at their habitat characterized fine-grained sediments just below the low tide line. The sea cucumbers used for experiment ranged in length were from approximately 10 cm to 15 cm. *H. leucospilota* are deposit feeder which use their peltate tentacles to push sediment into their...
mouths, ingest and digest those. The sea cucumbers were kept in cooled insulation box with seawater. During transportation in the laboratory, every 2 hours always exchanging with new seawater to provide a dissolved oxygen inside the box. Upon return to the laboratory, every 5 sea cucumbers each were transferred into a glass aquarium with 20 l of seawater with constantly aeration. The seawater was always changed daily to prevent accumulation of metabolic wastes. After the acclimatisation with laboratory conditions for 10 days, in addition, the sea cucumbers were fed with sediment collected from the origin until the main experiment was applied.

Preparation of microplastic (PVC) polluted with fluoranthene

We used PVC (polyvinylchloride) particles in a size range 50-200 µm which purchased the material from PyroPowders (www.pyropowders.de) as model microplastic particles for our feeding experiment. There were white color, fluffy and irregular shape. The fluoranthene is hydrophobic and solid, it has to be dissolved in an organic solvent. The fluoranthene was dissolved in acetone then aliquots of a stock solution with 100 µg fluoranthene per 1 ml acetone were mixed with seawater. The PVC particles were mixed into the fluoranthene seawater solution. For contaminating the PVC particles, 100 g of PVC were mixed into 500 ml of seawater that contained fluoranthene in a concentration of 2 µg. It is important to mix the suspension well, thus the PVC particles distributed homogeneously in the water body. For a constant mixing, a small pump or stirring device installed in the tank. Every 4th day the fluoranthene seawater solution was renewed to maximize the loading of the PVC particles. During changing with a new fluoranthene seawater solution, first let the PVC particles deposited then removed as much water as possible without losing particles. The total incubation time was 3 weeks. Every week started a new incubation. After the 3 weeks, the solution removed without losing particles and the same amount of clean seawater were added. In this the particles washed for one hour to remove any fluoranthene that did not absorb to the plastic surface. The PVC particles used directly after the loading, it was stored in a small amount of seawater in a dark and cool place.

Experimental setup

For the study of the potential impact of microplastics on sea cucumbers, we carried out feeding experiments microparticles in the laboratory for 62 days. The experiment follows a 1-factorial ANOVA design, which sea cucumbers were exposed to several pollution scenarios that differed with regard to the amount of PVC particles by weight of sediment. Each treatment group consisted of 15 replicates (Table 1). The 3% PVC particles was chosen as referred from Carson et al. (2012) which closed to 3.2% of microplastics were found at Kamilo Beach, Hawaii. Beforehand, for initiate PVC particles polluted with fluoranthene exposed to sea cucumbers, 100 g of sandy sediment and PVC particles (at different treatment groups) was assessed into experimental containers. Seawater in the experimental containers was renewed every morning. Every 3 weeks was
added new sandy sediment and PVC particles polluted with fluoranthene in the same amount directly on the top after water exchanged.

Table 1  Treatment groups for microplastics polluted with fluoranthene. F indicates of microplastics polluted with fluoranthene

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Microplastics density (% by weight of sediment)</th>
<th>Fluoranthene</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>No</td>
<td>15 individuals</td>
</tr>
<tr>
<td>B</td>
<td>0.03F</td>
<td>Yes</td>
<td>15 individuals</td>
</tr>
<tr>
<td>C</td>
<td>0.3F</td>
<td>Yes</td>
<td>15 individuals</td>
</tr>
<tr>
<td>D</td>
<td>1F</td>
<td>Yes</td>
<td>15 individuals</td>
</tr>
<tr>
<td>E</td>
<td>3F</td>
<td>Yes</td>
<td>15 individuals</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>No</td>
<td>15 individuals</td>
</tr>
</tbody>
</table>

Response variables

Respiration rate

During exposure PVC particles contaminated with fluoranthene, per single individual was checked the respiration rate. Time to measure respiration rate per each individual was 1 hour. We performed the sea cucumber in a single container with 2 l of seawater completely filled and the container had a lid to prevent the oxygen outside flow through into the container. On the top of container, we installed an Oxygen sensor (CellOx 325 for WTW Oxical 3205) with a silicon rubber attached tidely to check the value of dissolved oxygen in the water (Figure 4). During the 1 hour measurement of respiration rate, the water stirred slowly with magnet stirrer below the container. We always maintained the value before beginning to measure and 1 hour after.

Figure 4  (a) Oxygen sensor was installed in the container; (b) One sea cucumber was measured per 1 hour
Feces production

The purpose of this test was for ensuring the animal had ingested the microplastics into their gut. With many differences concentration microplastics might caused the animal to react in a different ways and look the potential hazard of pollutant for *H. leucospilota*. Every 3 weeks took the feces pellets from the single container with the animal inside. Every container checked which they produced feces in the morning, then prepared a small tray to catch feces when suck out it from container (Figure 5). It was done carefully during suck out feces pellets to avoid other sediment was also sucked. After collecting feces, we dried the feces pellets into the oven at 60° C for 24 hours, and measured the dry weight.

![Figure 5 Design of feces pellets collection (Grossmann, 2014)](image)

The survival rate of sea cucumbers treated with microplastics

Survival rates were determined for all sea cucumbers at each treatment groups. Mortality was monitored daily before water exchanged by checking every single sea cucumber. They were dead if they did not show any movements and their body was smelly. Live individuals were continued to hypoxia stress test.

Hypoxia stress tolerance

For initiate hypoxia stress tolerance, prior to setup hypoxia circulation system. The system comprised for different dissolved oxygen (DO) concentrations. The DO concentrations was measured by a digital oxygen meter, the sensor type is CellOx 325 for WTW Oxical 3205, Weilheim, Germany. The hypoxic water collected in header tank to distribute into experimental containers. For connecting the system, The inflow connected to the seawater header tank which had one hole on the top for inflow the nitrogen. The concentration of dissolved oxygen in the seawater header tank will be reduced by bubbling...
nitrogen gas into it (Seitz et al. 2003; Long et al. 2008; Cheung et al. 2008), while the target DO concentrations should reached 2 mg O$_2$ L$^{-1}$, 3 mg O$_2$ L$^{-1}$ (for pilot study) (Table 2) and 0.5 – 1.0 mg O$_2$ L$^{-1}$ (for main hypoxia stress tolerance). The outflow will be distributed the seawater filled in experimental containers (Figure 6).

![Figure 6](image) Design of hypoxia circulation system with one bottle of nitrogen through in header tank and distribute to each experimental container

Each individual test animal placed in a separate container and the containers closed tightly with a lid to prevent re-oxygenation of the water. The seawater in containers each always changed daily. If the water gets re-oxygenized may need to fill in de-oxygenated water from the header tank again. During the water exchange, the containers and the top of the header tank have to be opened until the water body inside the containers has entirely been replaced with hypoxic water from the header tank. While, normoxia condition was applied > 6 mg O$_2$ L$^{-1}$ (only in pilot study) (Table 2) as control and provided with aeration. However, presumably for all dissolved oxygen concentrations did not feed the sea cucumbers during the hypoxia test (Cheung et al. 2008).
Table 2: Treatment groups for pilot study of hypoxia stress tolerance

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Concentration levels of dissolved oxygen</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>2 O₂ mg L⁻¹</td>
<td>5 individuals</td>
</tr>
<tr>
<td>Y</td>
<td>3 O₂ mg L⁻¹</td>
<td>5 individuals</td>
</tr>
<tr>
<td>Z</td>
<td>&gt; 6 O₂ mg L⁻¹</td>
<td>5 individuals</td>
</tr>
</tbody>
</table>

The aims of this study were to investigate the behavioral and the survival rates of *H. leucospilota* face in hypoxia stress tolerance with *ex situ* observations in the same DO concentration after exposed from microplastics polluted and non polluted with fluoranthene, while the target DO concentration for main hypoxia stress tolerance was 0.5 – 1.0 mg O₂ L⁻¹. DO concentrations < 2 mg O₂ L⁻¹ is one of the triggers of stress with the severe concentration in benthic community (Diaz and Rosenberg 1995; Long et al. 2008). We observed their behavioral during under hypoxia condition twice a day included the survival rates. Prior to analysis the ammonia concentrations in the seawater after death revealed to ensure it was not caused for mortality.

Determinant of fluoranthene concentrations in microplastic particles and sea cucumber tissue after the experiment

**Detection of fluoranthene bound to PVC particles**

For detection fluoranthene bound to PVC particles, in the first step was extracted with 6 mL hexane added to 1 gr PVC particles in a beaker and shook it gently for 1 minute. Afterwards transferred the solution without the particles only the supernatant into a glass test tube and then evaporated the solvent using gentle stream of nitrogen for approximately 15 – 20 minutes. The solvent was dissolved the residue with 500 µL acetonitrile and took with pipette 200 µL in an HPLC (High Performance Liquid Chromatography) sample vial. Only 20 µL for the final volume injected into the HPLC analysis (Ex-λ-460 nm, Em-λ-360 nm) (Martin and Appel 2014).

**Detection of fluoranthene in tissue**

For detection fluoranthene in the sea cucumbers tissue, we prepared 5 g per treatment groups. In the first step the tissue homogenized or mixed in a blender. Afterwards, the tissue was added into 5 mL centrifuge tube and carefully shaken with vortex for 1 minute. Directly was added again for 8 mL ACN, and once again shooked it for 1 minute. The next step was added Bond Elut QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) AOAC salt packet, for an extraction step, then shaken it for 1 minute afterwards directly put into centrifuge at 4000 rpm for 5 minutes. After these step took 200 µL of supernatant and added Bond Elut QuEChERS SPE (a dispersive solid-phase extraction) into 15 mL tube. Shaked and centrifuged it again at the same time as before. The last step was filtered through a 0.45 um PVDF syringe filter. This
step was for clean the samples from particles left when took the supernatant. Then transferred 0.2 mL extract to an autosampler vial. Finally, the samples were ready for HPLC analysis (Martin and Appel 2014).

Data analysis

Prior to further analysis, the microplastics monitoring data were analyzed by the total amount of microplastic particles in the sediment and by their color, shape and type (Hidalgo-Ruz et al. 2012). Statistical analyses was applied two way ANOVA test for multiple comparison microplastics particles in sediments at different transect line and depth layer. If this test indicated significant differences continued with pairwise comparisons used t-test.

One way analysis of variance (ANOVA) was used to analyse the effects of microplastics and fluoranthene exposure in different densities on the respiration rate and feces production rate. Homogenous variances and normality of the data were tested with Fligner Killen test and Shapiro test (Field et al. 2012). Contrast analysis were used for comparing the differences between the groups during exposure with microplastics and fluoranthene (Field et al. 2012). Data were displayed as a box plot which showed the distribution of quantitative data.

The behavioral and the survival rates of *H. leucospilota* were used Cox proportional hazard (Coxph) test for censored data if the risk of behavior and the risk of death phase experimental groups differed between the treatments (Bewick et al. 2004; Goel et al. 2010). Data were presented as the Kaplan Meier curves which showed the probability for an individual survival and behavioral. All statistical tests, curves and graphics were calculated and produced using the free R-statistic software (version 3.1.2 (2014-10-31) “Pumpkin Helmet”) (R Core Team, 2014). The significance result was assumed if p-value lower than 0.05.

3 RESULTS

Microplastic monitoring

Quantities of microplastic particles differed between high and low tide line as well as between the two sediment layers. At both transects, particle densities were consistently higher in the deeper sediment layers. Fibers were the most common and also, with regard to size and colour, most diverse particle type at both transect lines, while their abundance was higher at the low tide/bottom (77%) than at high tide line/bottom (56%). Films, on the contrary, were more common at the high tide/bottom (40%) than at the low tide/bottom (18%). Finally, foamed particles (50% and 4%) were found only at the high tide line (top and bottom), while fragments (5%) only occurred at low tide/bottom (Figure 7).

Figure 7 Composition of microplastics particles (500 – 4000 µm) collected at the high and low tide line at the southern tip of Rambut Island, Indonesia.

Accordingly, the highest number of particles was found in the bottom layer (5-10 cm) at the low tide line/bottom layer with 7.34 particles per kg dry weight sediment, while the lowest density was observed at the high tide line/top layer with 1.55 particles per kg dry weight sediment (Table 3).

Table 3 Quantities of microplastic particles (500 – 4000 µm) collected at the high and the low tide line at the southern tip of Rambut Island, Indonesia

<table>
<thead>
<tr>
<th>Transect/Layer</th>
<th>Number of particles</th>
<th>Sample sediment dry weight (kg)</th>
<th>Number of particles/ kg dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>high/top</td>
<td>12</td>
<td>7.74</td>
<td>1.55</td>
</tr>
<tr>
<td>high/bottom</td>
<td>25</td>
<td>7.75</td>
<td>3.23</td>
</tr>
<tr>
<td>low/top</td>
<td>34</td>
<td>5.59</td>
<td>6.07</td>
</tr>
<tr>
<td>low/bottom</td>
<td>62</td>
<td>8.44</td>
<td>7.34</td>
</tr>
</tbody>
</table>
When comparing particles densities statistically, we found insignificant interaction between transect line and sediment layer ($p = 0.083$, $F = 3.31$, $R^2 = 0.29$) (Figure 8). Furthermore, particle density was only significantly higher at the low than at high tide line (pairwise t-test $p = 0.031$, $F = 3.31$, $R^2 = 0.29$), but densities did not differ between sediment layers (Figure 8).

![Figure 8 Density of microplastic particles at the two transects and in the two sediment layers sampled at the southern tip of Rambut Island. Boxes indicate inner quartiles, the line is the median and the whiskers show outer quartiles.](image_url)

In both sampling sites and sediment layers about 76% of fibers were found light blue colored and 24% which found fiber with black colored (Figure 9a). Foamed particles were found in an oval shape with white color and films were light blue with an irregular shape. Both were found in the upper sediment layer at the high tide line (Figure 9b), however, a blue colored film with irregular shape was also detected in another sample (Figure 9c). Fragments only had a share of 3% of the particles in the bottom layer (5-10 cm) at the low tide line. They were irregular in shape of dark brown color with hard material (Figure 9d).
Figure 9 Microplastic particles extracted from sediment samples taken at Rambut Island. (a) Fibers with light blue colored (b) Foams with an oval shape and white colored and associated with one film blue colored (c) Films with blue colored (d) Fragments with dark brown colored

Respiration rate

After 60 days of exposure to microplastics, respiration rates in *Holothuria leucospilota* were found not significantly different between treatment groups (Figure 10). ANOVA test was accepted H$_{01}$ and H$_{02}$ which showed (p = 0.7478). The Shapiro test (p = 0.08086) and the Fligner Killen test (p = 0.783). The comparison between 0% (control group) and all treatment groups were showed microplastics not affected respiration rate. Neither the presence of the lowest microplastics density polluted with fluoranthene and the highest microplastics density non polluted with fluoranthene. For all treatment groups with microplastics showed no clear pattern about the impact of microplastics to the respiration rate in *H. leucospilota*. 
Figure 10  Respiration rates of *H. leucospilota* after 60 days of exposure to different microplastics density. The box indicates the inner quartile 25-75% of the data, black horizontal line indicates the median and black vertical line (whisker plot) indicates the outer quartile 0-25% and 75-100% of the data. Black dot indicate outliers (n=5)

**Feces production**

During the 60 days of the experiment, we observed the production of feces under microscope stereo to ensure *H. leucospilota* ingested microplastics to their gut system (Figure 11).

Figure 11  (a) Sea cucumber sandy sediment without artificially added microplastic PVC particles (b) Ingestion of microplastic PVC particles by sea cucumber. The size of microplastics is smaller than the sand which directed by arrows
Feces production in *H. leucospilota* revealed that moderate significantly different between the treatment groups. ANOVA test was accepted H$_{01}$ and H$_{02}$ which showed (p = 0.07131). The Shapiro test (p = 0.2607) and the Fligner Killen test (p = 0.9139). The 0% (control group) produced more feces than all treatment groups (Figure 12). It was proved by Contrast analysis the 0% microplastics was differ with all treatment groups with microplastics. It was strongly significant result (p = 0.00475**). The feces production (g dry weight (dw)) was standardized by wet weight (ww) of sea cucumbers because we could not measured the dry weight after the microplastics exposure.

![Box plot showing feces production](image)

**Figure 12**

Feces production of *H. leucospilota* after 60 days of exposure to different microplastics density. The box indicates the inner quartile 25-75% of the data, black horizontal line indicates the median and black vertical line (whisker plot) indicates the outer quartile 0-25% and 75-100% of the data. Black dot indicate outliers, dw is dry weight and ww is wet weight (n=5).

Survival rate

The suprising result that all of organisms used for experiment and exposed to the microplastics polluted and non polluted with fluoranthene for 60 days, they were still alive 100% survived. It was meant that microplastics not showed any affected to their mortality. None of them died because of microplastics densities neither the fluoranthene itself. See in (Figure 13) its...
showed no any pattern only straight line for all treatment levels and also for the survival test said could not calculate.

Figure 13 The proportion of survivor during microplastic exposure in *H. leucospilota* for 60 days. F indicates that microplastics concentration polluted with fluoranthene. The straight line indicate no sea cucumbers were dead (n=15)

**Hypoxia pilot study**

*Holothuria leucospilota* have been exposed to hypoxia stress tolerance for 22 days, behavior as well as survivor were found to low percentages with oxygen depletion (Figure 14 & 15). The high percentages was seen by normoxia condition, due to holothurians did not emit their cuvierian tubules (Figure 14 & 15). The different oxygen concentrations had a significant influence to behavior of *H. leucospilota* with ejected their cuvierian tubules (Figure 15). Its proved by Cox-ph test: Chisq = 8.1249, df = 2, p = 0.01721*. The relationship between hypoxia and holothurians behavior were strongly affected by oxygen depletion concentrations.

Figure 14 The proportion of behavior of *H. leucospilota* showed by ejection of cuvierian tubules during hypoxia stress tolerance with different oxygen concentrations for 22 days (n=5)
The survival rates of *H. leucospilota* even showed a decrease by 18% and 60% at 2 mg O$_2$ L$^{-1}$ and 3 mg O$_2$ L$^{-1}$ in comparison to the > 6 mg O$_2$ L$^{-1}$ group by 100% survived (Figure 15). The line drop of proportion of survivors in all oxygen concentrations indicated the death phase of holothurians. This study revealed that hypoxia compared to normoxia condition had significant interaction (Figure 14 Coxph-test: Chisq = 7.1477, df = 2, p = 0.02805*). The lowest concentrations of dissolved oxygen (2 mg O$_2$ L$^{-1}$) faster dead compared with the higher concentrations (3 mg O$_2$ L$^{-1}$) and normal concentration (> 6 mg O$_2$ L$^{-1}$). In holothurians that indicated more susceptible to 2 mg O$_2$ L$^{-1}$ concentration. The lowest concentration (2 mg O$_2$ L$^{-1}$) occurred shortly before death in ca. 20% of the animals and during the check when death was confirmed in the rest.

![Graph](image)

**Figure 15** The proportion of survivor of *H. leucospilota* showed by declining curves during hypoxia stress tolerance with different oxygen concentrations for 22 days (n=5)

When they became inactive condition *H. leucospilota* were slow reaction touched while big open their mouth which hanging in the lid of container (Figure 16a), the tentacles being white some part (Figure 16b) and in long term white for overall and followed with chronic condition looked weaks and ejection of their cuvierian tubules (Figure 16c). In normoxia condition *H. leucospilota* was always moved from one part to another part and looked healthier than in hypoxia condition (Figure 16d). There was no differences behavior between under 2 mg O$_2$ L$^{-1}$ and 3 mg O$_2$ L$^{-1}$, but as indicated only faster appeared and followed with mortality. We tested the ammonia concentrations, the result none of them had lower than 0.05 mg L$^{-1}$, it was proved that ammonia not caused their mortality. In order to ensure their mortality, we touched the body, if did not any movement or reaction its occured dead. Generally things, holothurians always ejected parts of their cuvierian tubules when under pressure or under stress following to chronic condition then died.
Figure 16  The difference appearances between under hypoxia and normoxia condition (a) The anterior body hanging on the top and big open mouth (b) The tentacles transform from black to white (c) *H. leucospilota* ejected part of cuvierian tubules (d) *H. leucospilota* in normoxia condition not showing in understress

**Hypoxia after PVC particles and fluoranthene exposure**

The proportion of survivors of all sea cucumbers were heavily suppressed under hypoxia (Coxph-test: Chisq = 13.55, df = 5, p = 0.01866*) (rejected H0) (Figure 17). The median survival that were differ between the 0% (day 5) compared to 0.03% (day 3) and 1% (day 3) polluted with fluoranthene treatment groups, but the control group was no differ between 0.3% (day 5) polluted with fluoranthene, 3% (day 5) polluted with fluoranthene and 3% (day 5) non polluted. There was significantly interaction between survival and hypoxia stress test. But neither interaction between microplastics polluted nor non polluted with fluoranthene and hypoxia stress test. Microplastic and fluoranthene did not affect sea cucumbers death. This study shows that sea cucumbers was more susceptible under hypoxia stress test. The ammonium concentration was checked in each container per treatments after a 24 h cycle and the value was < 0.05 mg L⁻¹ for all treatment groups. It has been proved that the ammonium not affected their mortality.
The proportion of survivor of *H. leucospilota* showed by declining curves during hypoxia stress tolerance with different microplastics concentrations polluted and non polluted with fluoranthene for 6 days (n=15). The DO concentration from 1st day to 3rd day was 0.5 mg O$_2$ L$^{-1}$ then increased until 1 mg O$_2$ L$^{-1}$ due to the survival already more and less than 50%. Only in 1%F no one individuals left. F indicates microplastic polluted with fluoranthene.

Based on the pilot study we choosed 0.5 mg O$_2$ L$^{-1}$ oxygen target concentration in the main hypoxia stress test as it induced mortality within a suitable time frame. But unfortunately the average for all survivals were more and less than 50% of individuals during 3 days tested. Afterwards we increased again the concentration until 1 mg O$_2$ L$^{-1}$. Every morning we monitored their condition before started to exchange the water and every 6 hours after exchanged the water. The difficulties cases we left the sea cucumber in a noted position until the next check in the morning and we found several of them already died.

During hypoxia conditions many of behavior types did by the seacucumbers. In the first day under 0.5 mg O$_2$ L$^{-1}$ the mouth and the tentacles looked open so big followed the body part hanging in the container wall or lid while the rest was still attached (Figure 18a & 18b) In the second day during the day they began laying down, falling off the wall and the mouth and the tentacles open so big. In the third day several individuals began dead we proved it when it was no longer responding to touch, their skin had some lessions or bloated, they ejected parts of their gut (cuvierian tubules) (Figure 18c), they started to smell rotten (Figure 18e).
Figure 18  (a) The body part hanging in the container wall (b) The mouth and tentacles open so big (c) The ejection parts of their guts (cuverian tubules) (d) The ejection cuverian tubules and bloated (e) The seacucumbers severely bloated, the skin had some lessions and started to smell rotten (f) The skin already dissolution into water and the smell really rotten

Another cases if their body already bloated, ejected cuvierian tubules and when measured the oxygen level dropped drastically but they were still responding to touch (Figure 18d) usually these followed moderate behaviour types and would be temporary to chronic. The next day we found left of them dead and became worst not seems like before the smell were really rotten followed the skin dissolution into the water (Figure 18f). Because several individulas looked so weak and started dying, we increased the concentration up to 1 mg O₂ L⁻¹ in the third day. They could lived until day 5 and day 6 even in
two of groups already dead all of them (group 0.03%F and 1%F). We could not recorded the wet weight and dry weight after they dead because their body already dissolution into water, so it would less accurately for the measurement.

When the sea cucumbers enter the death phase, some of them occurred worst behavior types i.e. the formation of lessions and the dissolution of skin then the water would to smell rotten. The hypoxia stress test was terminated after 6 days due to the animals. Only 20% of animals were survived in the control group and all other animals that had not died were frozen and processed for fluoranthene detection in their tissues.

Fluoranthene detection in microplastics and sample tissues by HPLC

Microplastic PVC particles incubated with fluoranthene, unfortunately, it was negative from fluoranthene concentrations when tested by an HPLC, what indicates that the fluoranthene was not successful absorp onto the microplastic. The surprising and confusing result we detected fluoranthene in sea cucumber tissue samples. But not in all treatment groups we found fluoranthene in the tissues only in 0%; 3%F; 3% (Figure 19). In 0% group found fluoranthene concentration value was 0.33 ng g\(^{-1}\) ww (wet weight). In 3% microplastics group non polluted with fluoranthene was 0.31 ng g\(^{-1}\) ww. For 3% group microplastics polluted with fluoranthene was 0.48 ng g\(^{-1}\) ww.

Figure 19 Accumulation of fluoranthene in sea cucumber tissue samples after exposed to microplastic PVC particles polluted and non polluted with fluoranthene. F indicates fluoranthene

4 DISCUSSION

Microplastics monitoring in marine environment

The analysis of the microplastic types shows that fibers, as the only type of particles, occur at all sampled transect lines. Similar results were reported by Mathalon and Hill (2014) who detected 20 to 80 of fibers per 10 g sediment at the high and the low tide line on one exposed and two protected beaches in the
Eastern Passage of Nova Scotia, Canada. We assessed fibers more diverse at the low tide line, due to their lower density in comparison to other particle types. Fibers are commonly made from polyester and polyamides which has low density than another polymer types (Browne et al. 2011). Furthermore, they might easily be washed away and drag in by wave actions and carried to the low tide line by tides (Mathalon and Hill 2014). The most common sources for fibers are fishing nets and ropes (Claessens et al. 2011 and Hidalgo-Ruz et al. 2012). These are commonly used for different activities in Untung Jawa Island, which is close to Rambut Island, including fishing, aquaculture and harbour activities such as loading and unloading of goods on board. When fishing nets and ropes float on sea surface or get washed to a beach, they are continuously exposed to UV radiation, what enhances their fragmentation into pieces that can spread out in the marine environment (Claessens et al. 2011).

In addition to fishing gear, the shore line of Rambut Island is polluted by plastic bags and food packages, what presumably explains the occurrence of the second most abundant particle type (films). Foamed particles were also found at the high tide line, which may originate from food packaging made of Styrofoam (Free et al. 2014; Nuelle et al. 2014). Fragments were only found in low amounts at the low tide line. This could be due to the long time it takes for rigid plastic items, such as buckets, bottles, bottle caps, lighters and others, to break down into particles of microscopic size which are commonly made from PVC (Andrady 2011; Free et al. 2014; Nilsen et al. 2014).

According to Browne et al. (2010) the spatial distribution of microplastics in the marine environment is probably influenced by several factors such as wind, currents, waves and most importantly, the density of the microplastic material itself. During low tide, the microplastics observed in this island could be already deposited in deeper layers because long process of degradation have been relegated before and occurs continuously then new sediment covered the last sediment in upper layer. Therefore, tides and wave actions may have transported microplastics to the low tide line, what promoted their accumulation in deeper layers and could have caused the divergence in the composition of microplastics between high and low tide line. The differences in the abundance of microplastics between sites and between layers in our study could also have been influenced by the sampling time.

The current study was observed microplastics particles in all sediment samples at all transect lines and depths. This may pose a problem for marine organisms living there which is several studies findings microplastics could bound pollutants ingested by them and causes negative performance even mortality. Rambut Island is located very close to Untung Jawa Island, which is a touristic place with recreational activities and permanent inhabitants. Due to the local circulation currents from the east to the west prevailing in Thousand islands connecting the two islands, Rambut Island could possibly be impacted by the human activities (urban waste), industries activities (industrial waste), fish culture activities and harbor activities (shipping waste) (Willoughby 1997).

Plastic debris at Rambut Island presumably comes along with the river run-off from three estuaries from different areas included Jakarta Bay, Tangerang and Bekasi. It is also carried to the northern part of the bay by seasonal currents, winds and accumulates along the islands shorelines.
Willoughby et al. (1997) reported that the plastic debris, which they collected at the shores of 23 of the Thousand Islands close to Jakarta Bay, was carried there by currents or originated from tourism on the islands. The authors counted nearly 34,000 items in 11 categories. They explained that the total amount of plastic debris had increased drastically from 1985 to 1995. Estimations speak of nearly 1 kg of debris that is generated per day per capita in Jakarta, which adds up to a total amount of about 5 million tons of debris per year. Although most of this goes into landfills, still a significant share enters the marine environment via rivers (Willoughby et al. 1997).

Microplastics can have a negative impact on marine ecosystems, food webs and organisms (Cole et al. 2011; Farrel and Nelson 2013; Fossi et al. 2014; Chua et al. 2014). For instance benthic organisms e.g. sea cucumbers, deposit feeders, and mussels, filter feeders, can ingest microplastics passively with their food. They take up microplastics in the sediment into their gut and this could lead to inflammations in the digestive tract (Graham and Thompson 2009). Rochman et al. (2013) found that fish, which were fed with virgin polyethylene fragments with chemical pollutants absorbed to their surface, showed signs of the symptoms of tumor in hepatic organ. Graham and Thompson (2009) observed that Holothurians ingest PVC fragments, nylon lines and also PVC pellets with a diameter of 4 mm. Von Moos et al. (2012) found that HDPE particles that were ingested by the blue mussel *Mytilus edulis* L. were transported to the gills and to the digestive gland after an exposure of up to 96 h. However, not only the plastic particles themselves pose a risk. They can absorb and release persistent organic pollutants such as PAHs and PCBs in the marine environment (Teuten et al. 2009; Frias et al. 2010; Rochman et al. 2013; Besseling et al. 2013; Bakir et al. 2014).

Many marine benthic organisms play an important role as a food source for human consumption. In Jakarta Bay there are many mussel cultures and the residents around Rambut Island catch fish, crabs, squids and other species. Cauwenbergh and Janssen (2014) detected microplastics in the soft tissues of *Mytilus edulis* with an amount of 0.36 ± 0.07 particles per g wet weight (ww) and in *Crassostrea gigas* 0.47 ± 0.16 particles per g ww, which are both bivalves from cultures for human consumption. If humans consume those animals, the particles may end up in the human body. Thus also impact the human utilization of marine organisms as a food source. If this happens continuously it will presumably have an impact on marine biodiversity around the island.

The physiological impact of microplastics on *H. leucospilota*

Respiration in *H. leucospilota* when tested with ANOVA and Contrast turns to the group without and with microplastics treatments were insignificant. This proved that microplastics not affected respiration rate of sea cucumbers for 60 days. Along with the increasing microplastics densities on the treatment did not showed any clear pattern illustrates the influence microplastics to respiration rate of sea cucumbers. Several possibilities may explain this result were microplastics not affected in the short time frame of their respiration system. Sea cucumbers have been previously adapted to the size of the micro-particles are sand that they
The size of the sand when compared to the size of microplastics used for sea cucumber was smaller size and the texture of microplastics itself feels smooth. Sea cucumbers are more robust than we thought to microplastics pollution threats. When compared with other species such as *Perna viridis* (green mussels) which has been investigated by Sinja (my partner GAME), showed significant results and the increasing microplastics densities effected on respiration rate of *P. viridis*. *P. viridis* is more susceptible to micro-particles when compared with *H. leucospilota*. It can be assumed filter feeder animals are more sensitive to microplastics pollution than animal deposit feeder. Animals filter feeder is an animal that catches their feed in the water column by filtering the water around the area of their life, when there is something foreign objects, shells will get a signal when they try to filter the water for plankton, there were other things that going into their respiratory tract, they can not digest it and eventually closes the airways and reduce the power level of respiring.

Generally, sea cucumbers is the one of marine invertebrates with deposit feeders in benthic environment. They does not choose their food only picking up like their desire and they will take organic matter inside the sediment and absorb nutrition for their food source. The ingestion and digestion sediment with inorganic matter will ejected by sea cucumbers if do not needed. Sea cucumber has tentacles in the anterior (oral) around their mouth, the function is for uptake food or sediment push into their mouth and ingest it. *H. leucospilota* is one of the benthic organism used the tentacles for feeding on organic matter to find in the sediment and other substrates. Sediment particles enter and through to the digestive tract then absorb organic matter such as associated bacteria, detritus where parts of the sediment indeed. Afterwards the material like anorganic matter will digested by anus from the posterior body part, then the shape form is feces pellets.

The microplastics particles grain size was smaller than the sediment. The feces production in *H. leucospilota* exposed to the microplastics showed they were ingested the microplastics proved in their feces pellets. They had possibility to take microplastics particles when uptake food for their feeding. Feces production is the one of response variables to indicate any occurences negative effect for feeding rate. During microplastics exposure for 60 days, *H. leucospilota* showed a relation between sediment without any microplastics particles and with microplastics particles. The animals exposed with microplastics indicated less produced feces following by microplastics particles density than without microplastics. The data compared at 0.3% microplastics was higher produced feces than 1% microplastics density, but if we looked at 0.03% microplastics was lower produced feces than 0.3% microplastics. Regard to the feces production could not standardized by the dry weight. The problem was directly all animals through hypoxia stress test after microplastics exposure.

There are several possible explanations relatively with not significant finding for feces production rates in all groups contaminated by microplastics. The first possibility for absence of an effect of microplastic ingestion by *H. leucospilota* can be explained by the shape of the used plastic particles and the feeding mode of the sandy organism. The alimentary tract of deposit feeders is adapted for processing a variety of indigestible particles. Microplastics particles were spherical without sharp edges and thus could probably pass...
through the digestive tract of the animals without causing any physical harm to the alimentary tract. The second possibility is may 0% group showed the highest value feces production rate. It could assume that *H. leucospilota* prefer their food with an organic material inside the sand without any pollutants.

The problem was microplastics treatment groups did not showed clear pattern following the microplastics densities. It was also happened if looked and compared at the 3% microplastics polluted with fluoranthene and 3% microplastics non polluted, there was no fluoranthene effect on *H. leucospilota*. The third possibility it might be *H. leucospilota* already suppressed under laboratory conditions during microplastics exposure for 60 days, so it would impact to their food preferences. Another possibility it may caused the method was not yet fully developed then affected the actual feces production. However, all values were in range mainly between 0.05 and 0.15 g dry weight (dw) per g body wall wet weight (ww) for all individuals in microplastics treatment groups. This suggests that the ingestion rates did not change consistently over time.

**The impact of microplastics and fluoranthene pollution in marine benthic invertebrates**

Microplastic is pollution in the form of a solid insoluble in the water and will sink and settles in the sea floor. At a certain condition where ocean has a strong tidal will bring existing microplastics which already suspended in the bottom, it will resuspend going up into the water column. This must have been the new issue of marine organisms, not only fish, but it also affects the organisms that are living in the benthic area such as benthic invertebrates. Benthic organisms were divided into two types of ways to feed, which are the filter feeders and the deposit feeders where both have their respective roles within food chains, benthic environment and an important economic value.

There are many studies already reported about the negative impact on microplastics for deposit feeders such as *Arenicola marina* L. losing their fitness and reduction in feeding activity because they ingested microplastics of polystyrene contaminated by PCBs (polychlorinated biphenyls) (Besseling et al. 2013). But unfortunately in my objective study was *H. leucospilota* did not show any clear pattern for the bad impact from microplastics used with polyvinylchloride and it has been polluted by fluoranthene. The survival during microplastics exposure for 60 days under laboratory condition showed *H. leucospilota* was 100% survived and they did not show any strange behaviour or microplastics was disrupted for their life. It could many assumptions about this, compared with *P. viridis* was more susceptible to microplastics than *H. leucospilota*. Maybe the size and the shape it might not clog or injure their digestive tract, because Holothurian already adapted by ingesting sand and other materials which is the grain size bigger than microplastics, consequently, it did not cause mortality to them and it has no negative impact on *H. leucospilota*. This is line with the findings of Haemer et al. (2014) that the marine isopod *Idotea emarginata*, there was no negative impact and no chronic ingestion of microplastics with very high concentrations of 120 particles per mg food then no distinct adverse effect on their survival.
As we heard microplastics can absorb PAHs, PCBs and many of POPs in the marine environment. Browne et al. (2013) have been investigated the toxicological effects of phenanthrene in microplastics could uptaken and transferred the additive chemicals by lugworm (*Arenicola marina*) into their guts and tissues and can cause negative effect in their ecophysiological. If compared with our results indicate that accumulation of fluoranthene only in 3 treatments group were detected fluoranthene by HPLC. The treatment groups without any microplastics, with microplastics but no fluoranthene and with both of them. Presumably, we had a problem with the HPLC process, because the result was not satisfied. It was difficult to interpret the result. If we would to say fluoranthene already contained in the body of Holothurian when we sampling them in their habitat, but not all of them detected fluoranthene in their sample tissues. When checked also only the microplastics which had incubated by fluoranthene, the result was negative.

It might several interpretations could explain the absence of detected fluoranthene in the tissue samples and different concentrations between treatment groups. It could be fluoranthene may not have been bioavailability and therefore not have been transferred into the tissues. Another possibility has occurred, it may have been detoxified subsequently metabolism (biotransformation) and excretion (elimination) without causing any bad impact to their appearances. Weston and Mayer (1998) demonstrated that the absorption efficiency of PAH solubilized by gut fluids is nearly 100%. This clearly shows that solubilization in the gut is a crucial step determining bioavailability of organic contaminants.

However, there was no clear trend with regard to a potential treatment effect from fluoranthene. Surprisingly, in our HPLC analyses we also found the chemical in untreated samples, which may be explained by a contamination of the seawater we used for the maintenance of the holothurians with fluoranthene. In contrast, fluoranthene was not detected by HPLC in microplastic we had experimentally incubated in this chemical to analyze its uptake by the PVC particles. Thus, it remains unclear to which fluoranthene concentrations the holothurians were actually exposed in my experiments, and therefore it is not really justified to draw any conclusions on the effects of fluoranthene on holothurians from my study.

It seems possible that long-term experiments might still reveal negative impacts that were not detected in the present study such as acclimation and adaptation to pollutants. In contrast, short-term experiments provide information on how a species is reacting to a manipulation at the moment. Organisms may survive or endure chronic exposure to contaminants such as microplastics and fluoranthene, the anabolic processes to maintain life, but short-term studies do not show long-term consequences for instance particular impact to their reproduction and the sustainability in the environment.

**The impact of hypoxia condition in the marine environment**

The results of the hypoxia stressed test with 0.5 mg O₂ L⁻¹ – 1 mg O₂ L⁻¹ showed significant for their survival during faced the hypoxia stressed test, but there were no differences between groups, and there was also no clear trend that
indicated microplastics densities influenced their survival during hypoxia stressed test. Organisms e.g. *H. leucospilota*, inhabiting the low tide line zone, oxygen levels under 2 mg O$_2$ L$^{-1}$, are a common stressor and the tolerance against this stressor is an important factor determining survival and mortality will start when the oxygen concentrations below 0.5 mg O$_2$ L$^{-1}$ (Diaz and Rosenberg 1995; Riedell 2012). If further environmental changes affect their performance in the natural environment, this could lead to several problems, affecting the ecosystem where *H. leucospilota* plays an important role.

During hypoxia stressed test with 0.5 mg O$_2$ L$^{-1}$ – 1 mg O$_2$ L$^{-1}$ oxygen concentration also tried looks for their physiological from behavior sides, the results showed no clear pattern for their behavior under hypoxic conditions, all of them started dying after 3 days and Almost 30 % the individulas were died on 6 days. They looked similar behavior no differences between the group without or with microplastics treatment. Holothurian such as *H. leucospilota* are considered one of the most sensitive species to low oxygen levels with severe consequences such as decreased activity and high mortality (Gray et al. 2002).

According to my measurements of the respiration rate conducted in this study, *H. leucospilota* consumes about 4 mg O$_2$ L$^{-1}$ per day. With a starting oxygen concentration of 0.5 mg O$_2$ L$^{-1}$ selected for my experiments, this already very low concentration, in fact, got further depleted by the oxygen consumption by *H. leucospilota*. This means that already after a few hours, the oxygen level got to values well below what is considered “severe hypoxia” in natural habitats (0.5 mg O$_2$ L$^{-1}$) (Diaz and Rosenberg 1995), which explains the strong reactions by *H. leucospilota*.

Hypoxia has consequences for living resources and has an impact to benthic ecosystem. Not only become a bad population to the organisms itself but has a longer impact to the whole of ecosystem in ocean life. Diaz and Rosenberg (1995) has been reported there were three kinds of condition regarding to oxygen concentration, the normoxia condition, the moderate hypoxia and the severe hypoxia. The relationship between hypoxia and holothurians behavior were strongly affected by oxygen depletion level. Astall and Jones (1991) said that holothurians reduced their metabolic system with ejected the cuvierian tubules when faced the hypoxia condition. It was relevant in (Figure 15 & 18), the lowest oxygen concentration all of holothurians ejected their cuvierian tubules during experiment.

The holothurians tried to survive during hypoxia, but unfortunately some of them died because they could not survived anymore and failed to make theirself aestivation and some of them survived until the end of experiment, its assumed that they were succed to aestivation theirself. Aestivation was the holothurians made a compensation for surviving from limit oxygen concentration and became inactive when their environment condition was not supported them (Yang et al. 2006). When they became inactive condition they were slow reaction touched, the tentacles being white some part and in long term white for overall and followed with chronic condition looked weaks and ejection of their cuvierian tubules.

It was observed the lower oxygen concentration level show higher metabolic rate, if their metabolic rate would be high they need more energy reserve to compesate it. But they saved their energy with aestivation activity and
during the dormancy process they ejected their cuvierian tubules to keep the energy. During hypoxia condition we did not fed the holothurians, they were not under starved because of holothurians ceased feeding to depress their metabolism. If they stopped to feed and they declined their metabolic system it would affect to their growth and reproduction system, because they needs nutrition for energy intake. Siikavuopio et al. (2007) has been investigated for sea urchins *Strongylocentrotus droebachiensis* that they reduced gonad growth because they unable to maintain high gonad growth and they did not intake food then had a problem for efficiency food conversion.

Therefore, Timmerman et al. (2012) said nutrient loading has a positive support to the biomass of benthic macrofaunal communities, but it also has potential to increase hypoxia which affected to benthic biomass. Hypoxia condition is a crucial event which may have a bad impact on the marine life and the diversity of marine organism. If one of the organisms in an ecosystem can not sustain their life by the breed of this could create new problems for the survival of a source of food or food web in the ecosystem. Based on researched and reviewed Long and Seitz (2008) that the bivalves *Macoma Baltica* which is a kind of food for the blue crab *Callinectes sapidus* is one of the key link of the food web. Besides that there were other factors than the loss of continuity of lower trophic levels, where the predator is getting prey as prey species are in the process of dormancy or aestivation. This is evidenced by the statement of Long et al. (2008) that the prey species will be easily found by predators because the prey has been stressed as the shells would reduced the depth to immerse theirself and dilate or expand siphon, it would easy for predators to increase the level of predation.

Holothurian not only has a commercially valuable, it also has an important role for sustain marine life. Holothurian has an ecological function in marine ecosystem, not only in the coastal ecosystem and also in the coral reef ecosystem. Schneider et al. (2011) already investigated Holothurian has a role in the coral reef ecosystem to balance CaCo\(_3\). Holothurian will process carbonate when they ingest sand and rubble through their digestive tract and dissolve CaCo\(_3\) as a part of their digestive process then digest it out and redistribute in the water column. Holothurian has a function to keep the marine ecosystem in low nutrient and becomes as a balance nutrient and also microbial in nutrient cycling. As we known that holothuria ingest sediment into their digestive tract, the unique part of that, Amaro et al. (2009) and Zhang et al. (2012) found some bacteria communities in holothurian guts, which has similar potential with the organic matter rich in sediment. It could be deduced that holothurian play an important function role in ecology of coastal ecosystem and coral reef ecosystem and it might become a marker of the existences of eutrophication within the marine environment.

**5 CONCLUSION AND FUTURE PERSPECTIVE**

**Conclusion**

The present study aimed to investigate the effects of three potential stressors on the sea cucumber *H. leucospilota*: microplastic, fluoranthene
exposure, and hypoxia. In fact, the hypoxia experiments were originally intended to analyze reduced fitness of the sea cucumbers after experimental treatment with microplastics and microplastics polluted with fluoranthene. The study revealed that short-term exposure to microplastic PVC particles does not measurably impact the analyzed physiological parameters of *H. leucospilota*, which are respiration rate, feces production and survival rate. No conclusion could be drawn on the sea cucumber’s susceptibility towards fluoranthene exposure, since the quantities of this chemical that *H. leucospilota* was actually exposed with in the course of the experiment could not be verified. A strong reaction was shown by *H. leucospilota* to hypoxia – i.e. oxygen levels that are well below what is considered “severe hypoxia” in natural habitats of the sea cucumber. Possibly because of the severity of the hypoxia conditions no effects of the prior treatments (microplastics and fluoranthene) on the fitness of *H. leucospilota* could be discerned in the course of the hypoxia experiments.

**Future Perspective**

As yet, information about microplastics and their effects on the biota in Indonesian marine habitats is very scarce. It is, therefore, important to carry out further long-term studies on possible interactions between microplastics, pollutants and sea cucumbers. Moreover, further research is needed to assess the bioavailability of organic pollutants absorbed by microplastics and their impacts on the entire ecosystem. In future experiments, the methodology for the absorption of pollutants into microplastic particles needs to be improved and less severe hypoxia conditions have to be tested to analyze the fitness of the sea cucumbers following treatment with microplastics and fluoranthene. Further studies also should not only include sediment samples but also water samples taken at the sea surface to determine the presence of this potentially harmful chemical in natural marine habitats.

**REFERENCES**


BIOGRAPHY

The author was born in Surabaya, on February 6th, 1990 as the elder daughter from Sodiqin and Rr. Soelistijaningsih. She has begun her high school, at SMU Negeri 20, in 2004, Surabaya and finished in 2007. Afterwards, she earned her Bachelor’s degree in Aquaculture from Airlangga University, Surabaya, East Java, in 2012. Her bachelor thesis versed on the potentials of leaf extract of Patikan Kebo (Euphorbia hirta) as antibacterial against Aeromonas hydrophila in vitro.

In 2013, she registered as a Master degree of Marine Science in Marine Science Program, at Bogor Agricultural University (IPB). She was selected as a member and conducted the collaboration research of Global Approach by Modular Experiment (GAME XII) project by Geomar - Germany.

Her master thesis as a part of collaborative research focused on the potential impact of microplastic particles on marine deposit-feeding invertebrates like sea cucumbers (e.g. Holothuria sp.) and filter-feeding invertebrates like mussels (e.g. Perna viridis) and she has presented about the results of the collaborative research to several Universities in Germany (Kiel Univ, Oldenburg Univ, Rostock Univ, Hamburg Univ, and Bremen Univ) in 2014. For finish her Master degree, she do a research and writing a thesis titled “The Physiological Impact of Microplastics on Holothuria leucospilota”.

She has been writing about “Diverse composition and high density of microplastics at a small island in Indonesia and their possible threat to marine biodiversity” and will submitted into Marine Pollution Bulletin, The International Journal for Marine Environmental Scientists, Engineers, Administrators, Politicians and Lawyers.