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Barnacle colonization of shoes: evaluation of a novel approach to estimate the time spent in water of human remains

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Highlights

- minPMSI of a corpse may be estimated by considering the barnacle colonization of shoes
- Barnacles can colonize PLS and SS shoes within one month after placement in seawater
- Barnacle growth is affected by water temperature but not salinity
- Barnacles preference is to colonize PLS and the external parts of the shoes

Abstract

Estimating the time since death (minimum Post Mortem Interval, *minPMI*) is a necessary part of a forensic investigation. Besides considering the typical signs of death, minPMI can be estimated using the insects and other arthropods that colonize the remains (forensic entomology). In an aquatic environment, both insects and crustaceans may provide information regarding the time spent in water of the remains (minimum Floating Interval, *minFI* and minimum Post Mortem Submersion Interval, *minPMSI*), and this can also assist in determining the minPMI.

Barnacles (Crustacea: Cirripedia) are common crustaceans that colonize solid substrates in marine environments and they can be found in association with organic and inorganic remains recovered from the sea. Barnacles colonize both floating and submerged remains and their growth rate is temperature dependent. Despite their potential to be indicative of the minFI and/or minPMSI, only a few case studies have considered it for this purpose, and scant research has been conducted in this field.

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Assuming that the vast majority of the bodies found in the sea are clothed, this research is focused on the barnacle colonization of two different types of shoes placed in the sea, in order to 1) identify the colonizing species in the chosen environment; 2) identify the settlement preferences of the barnacles associated with the shoes; and 3) determine the factors affecting the growth rate of the barnacles associated with the shoes.

In April 2016 64 sport shoes (SS) and 64 patent leather shoes (PLS) were placed in the Boston Harbor (MA-USA) at 8/10 meters below sea level. Four of each shoe type were collected every two weeks for seven months. Individual barnacles from each shoe were sampled and measured to determine species and age. The overall colonization density and settlement preference was statistically analyzed.

Results show that a) *Amphibalanus improvisus* (Darwin) (Crustacea: Cirripedia: Sessilia) colonized the vast majority of shoes; b) colonization occurred in less than 30 days and continued throughout the research period; c) a significant difference in colonization densities was found between the SS and PLS, with PLS seeing higher densities; d) barnacles showed preferential colonization of specific sections on both shoe types; e) barnacle growth was found to be significantly affected by water temperature and shoe type but not by the time spent in water; f) time spent in water and shoe type had a highly significant effect on the total number of barnacles per shoe, whereas water temperature did not.

Keywords: barnacles, seawater, minPMI, minPMSI, minFI

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Introduction

Aquatic environments can be especially difficult for forensic investigations. Bodies found in or near the water can be the result of homicide, suicide, accident, or natural disaster (1-6). In instances of homicide, bodies may be left in the water after drowning or may be dumped into the water in order to cover up a crime. Mass disasters determined by natural causes, such as flooding or tsunamis, often result in large numbers of bodies becoming displaced as well as being relocated over large distances (7, 8).

To date, most research into decomposition of human bodies has been conducted in terrestrial rather than aquatic systems (9). This is possibly due to the difficulty of carrying out research projects in an environment that requires specialized personnel, expensive equipment and where remains tend to move from the initial location of the research (10). Furthermore, results of experiments may show highly variable results even in similar aquatic environments, due to the biogeography of the water body, the type of remains involved and the cause of death (3, 4, 11-14). It appears clear that due to the paucity of research available in the field, more studies from similar systems but different geographies are needed.

In general, when bodies are left in water, they go through the following stages of decomposition: 1) submerged fresh, 2) early floating, 3) floating decay, 4) bloated deterioration, 5) floating remains, and 6) sunken remains (2, 11, 15). Furthermore, the decomposition process in aquatic environments often results in the formation of adipocere, an organic substance formed by the anaerobic bacterial hydrolysis of fat tissue (16).

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Following this decomposition process, the normal texture of the body tissues will be permanently replaced by a wax-like texture (16, 17). The typical decomposition process in aquatic environments may be affected by both environmental factors and factors related to the body itself. Environmental factors include, but are not limited to, water temperature, salinity, water depth, current flow, the season of submergence, availability of the body to macro and micro scavengers, water chemistry, and aquatic microbiome (4, 11-13, 18-20). Factors related to the body remains and the decomposition process include the effects of the presence of clothing, prior trauma, causes of death and floating or heavy items connected to the body (8, 21-23). In the majority of the cases, the colder temperatures and the absence of the typical insect activity cause the decomposition to occur at a slower rate in aquatic systems than terrestrial environments (3).

Necrophagous insects (mostly Diptera: Calliphoridae, Sarcophagidae, Muscidae) are commonly used to estimate the minimum time since death (minimum Post Mortem Interval, *minPMI*) in terrestrial environments (24). In a small number of cases terrestrial insects have also been used as an indication of the minimum floating interval (*minFI*) of human remains found in inland waters (25, 26). Aquatic insects of several orders (e.g. Trichoptera, Ephemeroptera, Simuliidae, Plecoptera, Diptera: Chironomidae) use the carcass as a shelter and they are used as indicators of time spent in water (Post Mortem Submersion Interval, *minPMSI*) of the remains (2, 3, 5, 13, 24). In saltwater environments the most common arthropods of forensic importance are crustacean, such as crabs, crayfish, and barnacles (2, 10). While crabs and crayfish are motile organisms that fed on the carcass, barnacles attach permanently to substrates (sessile organisms) and they use the carcass only

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as a shelter (6, 27). Barnacles are comprised of two main orders, Sessilia (acorn barnacles) and Pedunculata (goose-neck barnacles) (28). To date, there is a paucity of case studies (1, 6, 19, 27, 29) and no research projects have estimated the time spent in water of human remains found in marine environments using the presence and the growth of barnacle species. Bytheway and Pustilnik (2013) (27) considered the glycoproteinous adhesion deposits and the size of the barnacles *Balanus improvisus* (Darwin) (Crustacea: Cirripedia: Sessilia) to estimate minPMSI of a human mandible, teeth, and knee bones examined in Texas. Based on growth rates of *B. improvisus*, the minPMSI of the colonized remains was estimated to be 375-410 days. Dennison *et al.* (2004) (29) investigated specimens of *Notobalanus decorus decorus* (Darwin) (syn. *Balanus decorus* Darwin) (Crustacea: Cirripedia: Sessilia), found on a calvaria pulled from the east coast of New Zealand. At least two years of growth was determined based on the number of rings present. Based on the barnacle growth and time needed for skeletonization, a minPMSI was determined to be 30-48 months. Magni *et al.* (2015) (6) investigated *Lepas anatifera* L. (Crustacea: Cirripedia: Peduculata) found on the clothing of a body recovered from the Tyrrhenian sea (Italy). A minFI of 65 days was calculated based on the length of the barnacles' *capitulum* (shelled body of an adult goose-neck barnacle). De Donno *et al.* (2014) (19) also studied *L. anatifera* to determine the minPMSI for an individual recovered in the Adriatic sea (Italy). The barnacle size was consistent with a minimum of 20 days of growth and this estimation was in agreement with a minPMSI estimation made using the total aquatic decomposition score. In British Columbia Sorg *et al.* (1997) (1) used the growth of *Balanus crenatus* Bruguière (Crustacea: Cirripedia: Sessilia) to estimate the minPMSI of a skull

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found at sea. The barnacle growth rates were determined to be less than a year old and this amount of time was added to the time needed for the remains to skeletonize giving an overall estimated minPMSI of approximately 20 months, part of which must have occurred in an intertidal zone.

While there is previous usage of barnacles in case studies, experimental data on barnacle colonization and growth rate in a forensic context are lacking. The present research began with the assumption that it is more common for bodies to enter the water fully clothed, including shoes, whether they enter by means of accident or homicide. Magni *et al.* (2015) (6), discussed how barnacles show a settlement preference for the surface of clothes rather than the body itself. The present research aims to identify the species, the settlement preference and the factors affecting the growth rate of barnacles colonizing two types of shoes (sport shoes VS patent leather shoes) placed in a marine environment (Boston Harbor, Boston, MA-USA). Data obtained by this research will be useful in cases in which human remains and their common garments are found in the sea, as they will provide a tool for a more accurate estimation of time spent in the water, as per minFI, minPMSI and/or minPMI.

Materials & Methods

For the purpose of this research, 64 sport shoes (SS, "Boys' Cross Trainers" by Champion®) and 64 patent leather shoes (PLS, "Boys' Grant Oxford Dress Shoe" by Smartfit®) were used. Both shoe types were all the same brand, model, colour, size 13 (US

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children size) and had laces made of cotton. While SS were made of synthetic fabric with a plastic sole, PLS were made of leather (Fig. 3).

Shoes were attached to both the inside and outside of four lobster cages (1.5x1.0x1.0 m, Sea Rose Trap Company, Glouster, MA) and each cage had 32 shoes attached using plastic zip ties (Fig. 1). Shoes were placed at a minimum of 5 cm apart from each other, in alternating order (SS-PLS-SS-PLS). In April 2016, the cages were placed in the Boston Harbor at the Massachusetts State Police Marine Unit facility, Boston, Massachusetts, located where the Charles River meets the Atlantic Ocean (Lat. 42.36700: Long. 71.06065). The cages were placed 1.5 meters from each other, at a depth of 8-10 meters below the sea level and 1.5 meters above the sea bottom. Since in this location the water is brackish and has regular average tides of 3-4 meters (data from the National Oceanic and Atmospheric Administration), the cages were attached by ropes to a floating boat pier in close proximity to the locks opening up to the Charles River. A data logger (Hobo Ware®) was attached to the outside of one lobster cage to record temperature twice a day for the duration of the research period. Salinity data were obtained from the Massachusetts Water Resources Authority Station 014.

From April 6th 2016 to May 3rd 2016 the cages and the shoes remained untouched to allow sea-life colonization to occur. Starting on May 4th 2016, 28 days from the placement of the shoes in water, a total of four of each shoe type (two from the inside of the cage and two from the outside of the cage) were randomly chosen and removed every two weeks until the end of November. This was achieved by slowly drawing the cages up to a depth of 1 meter and removing the shoes. Upon removal, shoes were individually labeled and stored

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in plastic zip bags at -20°C (Kenmore® freezer). Each shoe was photographed from above, below, and from both sides. The maximum basal diameter (mm) of both smallest and the largest barnacle on each individual shoe was measured using digital calipers (GPM®). The minimum number of barnacles adhering to each shoe was recorded. This recording was qualified as a minimum number of barnacles as newly settled barnacles could not be visually determined (<0.5mm of basal diameter). Furthermore, other barnacles were partially obscured by other adhering organisms and dense barnacle colonization, making it necessary to report the total number of barnacles per shoe as minimum number of barnacles present.

Outlines of the SS and the PLS (Fig. 2) were produced to record the species of the barnacles adhering on/in the shoes. Shoe outlines were also divided into sections to record possible settlement preferences. The sections on the SS included the insole, tongue, lacing, external part, outsole, and sole (Figure 2a), while the sections on the PLS included the insole, tongue, lacing, external part, outsole, sole, and heel (Figure 2b). Barnacles were then removed from both types of shoes using a scalpel and forceps and sent to a specialist for species identification. Barnacles were morphologically identified under a stereomicroscope (Leica® MZ8). To note, this research was specifically focused on the presence of barnacles on the shoes, however, the presence and the adhering location of other aquatic organisms in/on the shoes and the cages was recorded as well.

Statistical analyses were performed using SPSS (IBM SPSS Statistics Version 25) to establish the differences between shoe types and barnacle colonization. A univariate analysis of variance (all variances were normalized) was chosen to ascertain each effect of

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multiple factors on barnacle colonization and settlement preferences. These factors included water temperature, time spent in the water, shoe type, and salinity. This was followed by a two-tailed T-Test to discern whether there was a difference in barnacle colonization between the SS and the PLS.

Results

Barnacle and other sea-life colonization. At each collection date, shoes were selected at random from inside and outside the lobster cages. Newly settled barnacles were observed on all SS (n=4) and PLS (n=4) after 1 month following the placement of the shoes in the harbor for the entire experimental period.

All the barnacle specimens observed were morphologically identified as *Amphibalanus improvisus* (Darwin, 1854) (Crustacea: Cirripedia: Sessilia) (30-34). No other barnacle species were found adhered to any of the shoes.

Other organisms found adhered to the shoes included mussels (Mollusca: Bivalvia: Mytilidae), seaweed, fungi, bryozoans (comm. moss animals, Bryozoa), *Crepidula* sp. (comm. slipper limpets, Mollusca: Gastropoda) and ascidians (comm. sea squirts, Chordata: Ascidiacea) (Fig. 3, 4) (35). Crabs (Crustacea: Decapods) and starfish (Echinodermata: Asteroidea) were also found in close association with the shoes (Fig. 4). The breakdown of the data regarding the organisms collected at each sampling time is reported in Table 2.

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Environmental data. The average water temperature over the research period (April 2016-November 2016) was 15.0°C, with a maximum temperature of 22.0°C and a minimum temperature of 4.8°C. For the purposes of data analysis, temperature data were simplified into the average water temperature for the amount of time a single shoe was submerged in the water. The average salinity level during this time period was 29.53 practical salinity units (PSU), with a maximum of 32.31 PSU and a minimum of 15.20 PSU.

Barnacle settlement preferences. In general, the majority of barnacle colonization was found on the external part, outsole, sole, and heel (Table 1) but on one occasion barnacles were also found on the laces of one shoe belonging to each shoe type. Barnacles were found on the tongue of 11 SS and of 8 PLS. On both the SS and PLS, no barnacles were found on the insole. It was observed that in both shoe types barnacles found on the laces and tongue were generally smaller than those found on the other parts of the shoes.

Colonization and growth rate based on shoe types and on the environmental data.

The water temperature over the time the shoes were submerged was found to have a significant effect on the size of the largest barnacles ($F_{1,126} = 26.0$, $P<0.001$), but did not have a significant effect on the total number of barnacles per shoe ($F_{1,126} = 2.2$, $P>0.05$).

The time spent in the water for both shoe types had a highly significant effect on the total number of barnacles per shoe ($F_{1,126} = 9.0$, $P<0.01$), but did not have a significant effect on the largest barnacles ($F_{1,126} = 0.4$, $P>0.50$). Salinity had no effect on the size of the largest barnacle ($F_{1,126} = 1.3$, $P>0.25$).

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The type of shoe had significant effect on the size of the largest barnacle and the total number of barnacles per shoes, with both larger ($F_{1,126}$ = 4.0, P<0.05) and more ($F_{1,126}$ = 36.0, P<0.001) barnacles occurring on PLS (86 barnacles on PLS compared to 39 in SS). When water temperature is not considered, the time spent in the water had a highly significant effect on the size of the largest barnacles on both shoe types ($F_{1,126}$ = 234.5, P<0.001).

Discussion

The identification of the minPMI, minPMSI and minFI of a corpse or an item connected to a corpse may be challenging as only a limited amount of research has been conducted in the field of human decomposition and colonization in sea-water and fresh-water environments. Generally, the soft tissues of a decomposed corpse are rarely colonized due to their slimy texture or the formation of adipocere that does not facilitate the settlement of organisms (6, 16, 17). Furthermore, such tissues are often scavenged by aquatic sea-life (mostly crustaceans, fish and sharks) and sea-birds (7, 10, 36). Despite research in the area of underwater microbiomes, little is understood about how microbial communities interact with carcasses located underwater (37). However, case studies of bodies found in the marine environment reported in the literature indicate barnacles are able to colonize bones and teeth (1, 27, 29), as well as clothing and shoes associated with a decomposing body (6, 7, 19). When the environmental conditions are suitable – water temperature is between the lower and higher developmental threshold of the species and an appropriate substrate is

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available – barnacle larvae can settle on a substrate in a very short time (within a few days, depending on the water temperature) and then the growth of the juvenile and adult (based on the ecology of the species, the water temperature and the availability of nutrients) can be considered as a 'biological clock' of the minimum time spent by the remains in the water (6).

When barnacles found in association with bones are used as an indicator of minFI or minPMSI, any estimations must include both the barnacles' colonization time and barnacle growth period, as well as the skeletonization period (1, 6, 19, 27, 29). However, when barnacles colonize substrates found in conjunction with human remains, e.g. clothes or shoes, the estimation of the minFI or minPMSI eliminates the need to base estimations on the combination of barnacle life cycle and decomposition rates. The time obtained estimating the colonization rate of barnacles on a garment may therefore be a better indication of the minimum time spent in water and possibly a more accurate determination of the minPMI estimation (6).

The aim of the present research was for the first time to consider the barnacle colonization of two types of shoes placed in the sea, providing another tool for forensic investigations. Within the 164 shoes placed in the Boston Harbor, barnacles were present on all but 2 sports shoes. Furthermore, barnacles were able to colonize both SS and PLS in a very short time, as specimens were collected from shoes that spent only one month underwater. These observations clearly indicate their potential in a forensic context as an indicator of minPMSI even after a short period after death/displacement of a body wearing shoes in the marine environment.

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In this research, the only species of barnacle that was found colonizing the shoes were *A. improvisus*, commonly known as 'bay barnacles'. This species typically inhabits the Western Atlantic as well as portions of the Pacific and Australasia (30). It is most commonly found in brackish water or near the coast in water less than 10 meters deep (30). The bay barnacle commonly grows up to 10 mm in diameter and is described as having a low, cone-shape shell (38). However, several barnacles sampled in the current research had basal diameters larger than 10 mm, indicating their upper growth limit differs based on their environment. As other barnacle species, the life cycle of *A. improvisus* exhibits three main phases (35). After sexual reproduction called s*permcasting*, in which the male barnacle releases sperm into the water and the female then capture it and fertilizes her eggs, the eggs hatch into larvae (called *nauplius*) (39). The *nauplius* larvae then develop into *cyprid* larvae. During both of these swimming phases (nektonic phases), larvae swim freely seeking out potential colonization sites. These phases can last from as little as seven days up to eight months depending on species and environmental factors (1, 27). Once an acceptable site is chosen, cyprid larvae attach and metamorphose into juvenile barnacles (1, 6, 27, 40). Once attached, they remain on the chosen substrate permanently (1, 6, 27, 40, 41).

Barnacles are considered foundation species and they are typical pioneer organisms that establish colonies on substrates (42). Colonization and recruitment of barnacles are temperature dependent; colder temperatures in winter, fall, and spring lower recruitment rates (42). Continued colonization is affected by population size, not individual barnacle size. The length of colonization is dependent on the life span of the barnacle species

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because barnacles do not colonize pre-existing communities (43). Barnacles prefer to colonize dark, rough, surfaces that offer more protection and surface area (40) and their colonization is easier and faster in wave exposed areas and areas where the water column has higher concentrations of chlorophyll a (44, 45). Barnacle growth (or *barnacle age*) is dependent on the availability of food and the delivery rate of food (44-47). Higher food concentrations and increased wave action allow for higher food delivery and typically result in faster barnacle growth and reproduction (44-46). Barnacles often colonize areas in conjunction with other marine species, e.g. polychaetes, bivalves, and bryozoans (40, 41). The size and shape of acorn barnacles, in particular, can change based on the substrate to which they adhere too. Barnacles that attach to other barnacles tend to be smaller than those attached directly to substrates (47). *Amphibalanus improvisus* is known to tolerate low salinity, high levels of pollution, fresh water exposure, and strong water currents (31, 32). *Amphibalanus improvisus* is well documented to being able to colonize a variety of substratum including algae, bivalve shells and other living organisms (38, 48). This species is also documented to be a successful self-fertilizer, giving them the ability to reproduce quickly and in large quantities (48, 49). Previous research has recorded approximately 21 days between egg production and hatching at 18°C (49). Following hatching, this species goes through six nauplius stages. The time needed to complete these stages is temperature dependent, (33). Dahlstrom and collaborators reported that when kept at 26-28°C the development of *A. improvisus* from nauplius into cyprid larvae takes 6-7 days (50). Following the completion of the last stage, the nauplius metamorphoses into a cyprid larvae. The cyprid colonization is affected by light, water flow, and the type of substrate

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(51, 52). While it is believed that this species prefers to colonize dark, rough, surfaces that offer more protection and surface area (40, 53, 54), this research shows that there were higher numbers of barnacles found on the smooth PLS rather than the rough SS. Furthermore, only a small number of barnacles were found inside the shoes and on the laces. Such barnacles were found on the PLS tongues in the later sample periods, possibly due to lack of space on the more desirable regions. Being a filter feeder the internal part of the shoe is probably undesirable as there is less water flow and therefore likelihood of food being present. From an investigative perspective, with this research it is possible to state that the internal parts of shoes as well as the laces have less utility for the purpose of estimating the time spent in water of the shoes/corpse connected to it. To note, in a real case, the internal part of the shoe would be generally occupied by the corpse's foot, therefore there would be little space for the attachment of barnacles or other sea-life.

Several studies confirmed that the time of naupliar release and cyprid settlement is variable (55-58). In North Carolina, the reproductive peak of *A. improvisus* is in winter (water temperature 5.5-11°C), especially in January (water temperature 7°C) (55); in UK nauplii are released from May to late September and settlement has been recorded from May to September (56). In the Black sea nauplii start to appear when the water temperature was 10°C, reaching maximum numbers when the water temperature is 16°C (May) and disappearing when water temperature reaches 25°C (57). In Japan, instead, larvae of *A. improvisus* can be found in plankton from June, with 2-3 abundance peaks from August to October (58). Overall, in general, a water temperature within a range of 10-30°C is considered acceptable for *A. improvisus* settlement (59) and the average temperatures

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recorded in this research (average 15°C, maximum 22°C) fall within this range. This research demonstrates that the most important factor to consider when using barnacles to estimate colonization periods is the temperature of the water. As barnacle growth proceeded, and when it was compared with time, the univariate analysis revealed that temperature, not time had a more significant effect on barnacle growth. While it was apparent that barnacles grew larger the longer they were in the water, this growth is temperature dependent. If this research was carried out in waters with temperatures outside of the optimal temperature (10-30°C) range, regular growth would not be expected (59). This is very important to consider when investigating colonization intervals. These two factors need to be considered together because in case work if only one factor is considered then the resultant FI would be erroneous. However, the current literature lacks complete tables of growth for species of barnacles at different water temperatures (6). This is very much akin to insects in the terrestrial environment whereby time and temperature are integral when determining a minPMI (24).

The extent of barnacle colonization (= number of barnacles per shoes, Table 1) is, however, dependent on the amount of time a substrate spends in the water. The longer a substrate is in the water, the more chances of colonization by new waves of larvae occur. *Amphibalanus improvisus* is a highly reproductive species. The observation of small (therefore young) barnacles at each sampling time showed that multiple waves of colonization occurred throughout the research period.

Amphibalanus improvisus is known to survive in low salinity waters. Average salinity levels in the Boston Harbor are slightly lower than in other parts of the Atlantic Ocean

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(Massachusetts Water Resource Authority 2017). While this may discourage other barnacle species from colonizing in this environment, bay barnacles are very much able to thrive in Boston Harbor. However, for investigations involving more sensitive species, salinity should be considered as a factor of barnacle growth and more research should be devoted to this aspect of barnacle ecology.

Other sea life colonization included ascidians, which became most abundant in this study. Ascidians occur worldwide and are sac-like marine invertebrates and filter feeders (60). Ascidians were observed colonizing both shoe types from July and continued throughout the remainder of the study period. The heaviest colonization was recorded from August to September, for both of these shoe types and on the lobster cages (Fig. 4). A visual assessment of the colonized shoes indicated that many more ascidians colonized the SS than the PLS (Fig. 4c, 4d). It is possible that the porosity of the SS surfaces allowed for easier adherence for the ascidians. While it was originally believed that the ascidians were possibly out-competing barnacles for colonization surface, no decrease in the barnacle colonization was observed for the sample periods within the ascidian colonization time period. (Table 1). No other species, found in association with the shoe types, seemed to have any relevance on the estimation of colonization intervals or minPMSI.

Similar to any type of minPMI estimation based on insect growth, it will be necessary to collect growth data for different species in their specific living environments and in the laboratory under controlled conditions. It is well established in the field of forensic entomology that environment and temperature can have varying effects on the growth rates of species (24). This is also true for barnacles since growth is significantly affected by

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temperature (31). In order to make accurate colonization interval estimations in a forensic context based on barnacle growth, the growth of this and other common barnacle species at known temperatures must be considered.

As a final comment, not all bodies that enter the water wear shoes and in some cases shoes will be lost while the body is drifting. This is particularly true for shoes without laces. It is therefore necessary to determine barnacle colonization on other substrates such as other clothing materials. The current research occurred between spring and fall, and future research should also be conducted during the winter months as growth may subside during winter. As the data from this research indicate, water temperature is the most significant factor in affecting barnacle growth. In areas where there are large differences in marine temperature throughout the year, barnacle growth may be more variable than in areas with more consistent or uniform temperatures such as the tropics.

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Figure 1. Set up of the experimental cage prior to be placed underwater.

Figure 2. Outlines used to identify the different regions of the sport shoes (a) and patent 477 leather shoes (b).

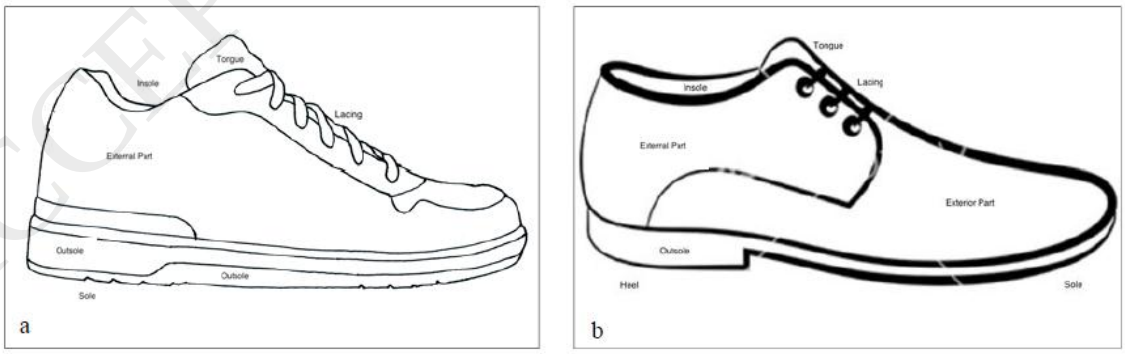

Figure 3. Fig. 3. Sea life colonization on sport shoes and patent leather shoes during the

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period of the research (May-November 2016). Sport shoes collected on a) 04.05.2016; b)
01.06.2016; c) 13/07/2016; d) 24/08/2016; e) 21/09/2016; f) 19/10/2016; g) 02/11/2016.
Patent leather shoes collected on h) 04.05.2016; i) 01.06.2016; l) 27/07/2016; m) 10/08/2016;
n) 21/09/2016; o) 19/10/2016; p) 30/11/2016.

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Figure 4. Ascidians colonization on the cage used for the research (a, b), on the sport shoes 490 (c, collected on 10/08/2016) and on the patent leather shoes (d, collected on 07/09/2016, a
491 crab is also visible inside the shoe).

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Table 1: Breakdown of data regarding the number of barnacles collected at each sampling date for the sport shoes (a) and the patent leather shoes (b). The number reported in each cell represents the total number of barnacles for the 4 shoes collected at each sampling time.

(a)

Sport Shoes *Minimum number of barnacles per shoe regions* *(data combined for the 4 shoes collected at each sampling time)*								
Sampling date	Sole	Insole	Outsole	Tongue	Lacing	External part	Total minimum number of barnacles present	total number of shoes colonized
04/05/2016	9	0	15	0	0	13	37	4
18/05/2016	48	0	45	0	0	33	126	4
01/06/2016	19	0	53	2	1	54	129	4
15/06/2016	11	0	37	0	0	13	61	4
29/06/2016	3	0	2	0	0	3	8	2
13/07/2016	11	0	46	0	0	149	206	4
27/07/2016	12	0	52	11	0	150	225	4

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10/08/2016	11	0	19	0	0	44	74	4
24/08/2016	8	0	49	0	0	74	131	4
07/09/2016	10	0	53	1	0	105	169	4
21/09/2016	32	0	76	8	0	127	243	4
05/20/2016	6	0	41	0	0	84	131	4
19/10/2016	16	0	19	7	0	91	133	4
02/11/2016	11	0	25	0	0	110	146	4
16/11/2016	13	0	32	1	0	82	128	4
30/11/2016	200	0	163	30	0	139	532	4
tot	420	0	727	60	1	1271	2479	62

(b)

Patent Leather Shoes *Minimum number of barnacles per shoe regions* *(data combined for the 4 shoes collected at each sampling time)*									
Sampling date	Sole	Insole	Outsole	Tongue	Lacing	External part	Heel	Total minimum number of	total number of shoes colonized

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								barnacles present	
04/05/2016	47	0	7	0	0	73	0	127	4
18/05/2016	30	0	2	0	0	170	0	202	4
01/06/2016	10	0	13	0	0	121	13	157	4
15/06/2016	55	0	29	0	0	82	38	204	4
29/06/2016	57	0	0	0	0	12	11	80	4
13/07/2016	22	0	12	0	0	98	13	145	4
27/07/2016	78	0	77	0	0	220	29	404	4
10/08/2016	160	0	50	0	0	176	62	448	4
24/08/2016	120	0	73	0	0	227	104	524	4
07/09/2016	129	0	30	2	1	252	40	454	4
21/09/2016	172	0	48	16	0	257	48	541	4
05/10/2016	73	0	49	12	0	210	81	425	4
19/10/2016	245	0	34	4	0	164	68	515	4
02/11/2016	126	0	30	0	0	124	34	314	4
16/11/2016	55	0	22	2	0	83	60	222	4
30/11/2016	60	0	20	0	0	650	9	739	4
total	1439	0	496	36	1	2919	610	5501	64

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Table 2: Breakdown of data collected at each sampling date including average temperature, and minimum and maximum size of barnacles present. Other organisms present per shoe type: mussels (Mollusca: Bivalvia: Mytilidae), algae, fungi, bryozoans (comm. moss animals, Bryozoa), *Crepidula* sp., (comm. slipper limpets, Mollusca: Gastropoda), ascidians (comm. sea squirts, Chordata: Ascidiacea), crabs (Crustacea: Decapods), starfish (Echinodermata: Asteroidea); n.a. = no applicable (no other organisms found).

Sampling date	**TºC**	**Sport shoes**			**Patent leather shoes**		
		Amphibalanus improvisus		Other organisms	*Amphibalanus improvisus*		Other organisms
		Total minimum number	Size (mm) min-max		Total Minimum number	Size (mm) min-max	
04/05/2016	7.5	37	0.45-2.40	n.a.	127	0.58-2.31	bryozoans
18/05/2016	8.4	126	1.35-3.50	n.a.	202	0.95-3.96	bryozoans
01/06/2016	9.4	129	0.89-4.54	mussels, algae, bryozoans	157	5.02-5.71	bryozoans
15/06/2016	10.5	61	1.64-4.94	algae, bryozoans	204	0.50-6.61	bryozoans
29/06/2016	11.5	8	1.56-4.82	*Crepidula* sp., algae, bryozoans	80	0.83-4.66	bryozoans
13/07/2016	12.2	206	1.08-8.44	crabs, *Crepidula* sp.,	145	0.68-7.21	crabs, *Crepidula* sp., algae,

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				ascidians, bryozoans			fungi, ascidians, bryozoans
27/07/2016	13.1	225	0.90-9.92	ascidians, bryozoans	404	0.94-7.23	ascidians, bryozoans
10/08/2016	13.9	74	1.52-7.95	*Crepidula* sp., algae, ascidians, bryozoans	448	0.87-8.1	slipper limpets, algae, fungi, ascidians, bryozoans
24/08/2016	14.5	131	1.44-7.45	algae, ascidians, bryozoans	524	0.95-9.05	slipper limpets, algae, ascidians, bryozoans
07/09/2016	15.0	169	1.20-8.08	algae, fungi, ascidians, bryozoans	454	0.8-7.41	crabs, slipper limpets, algae, fungi, ascidians, bryozoans
21/09/2016	15.4	243	1.97-10.68	algae, ascidians, bryozoans	541	1.06-9.85	mussels, fungi, algae, ascidians, bryozoans

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05/20/2016	15.5	131	2.46-6.79	*Crepidula* sp., algae, ascidians, bryozoans	425	0.97-11.37	algae, ascidians, bryozoans
19/10/2016	16.6	133	3.43-12.65	*Crepidula* sp., algae, ascidians, bryozoans	515	1.21-11.72	slipper limpets, ascidians, bryozoans
02/11/2016	16.4	146	0.90-8.50	*Crepidula* sp., algae, ascidians, bryozoans	314	0.50-9.63	ascidians, bryozoans
16/11/2016	16.1	128	1.6-10.3	crabs, slipper limpets, algae, ascidians, bryozoans	222	1.41-9.85	*Crepidula* sp., mussels, ascidians, bryozoans
30/11/2016	15.7	532	1.36-9.19	*Crepidula* sp., algae, ascidians, bryozoans	739	0.55-9.80	*Crepidula* sp., ascidians, bryozoans