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Discrimination of Mediterranean mussel (*Mytilus galloprovincialis*) feces in deposited materials by fecal morphology

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ABSTRACT

In the present study, we determined the common morphological characteristics of the feces of *Mytilus galloprovincialis* to develop a method for visually discriminating the feces of this mussel in deposited materials. This method can be used to assess the effect of mussel feces on benthic environments. The accuracy of visual morphology-based discrimination of mussel feces in deposited materials was confirmed by DNA analysis. Eighty-nine percent of mussel feces shared five common morphological characteristics. Of the 372 animal species investigated, only four species shared all five of these characteristics. More than 96% of the samples were visually identified as *M. galloprovincialis* feces on the basis of morphology of the particles containing the appropriate mitochondrial DNA. These results suggest that mussel feces can be discriminated with high accuracy on the basis of their morphological characteristics. Thus, our method can be used to quantitatively assess the effect of mussel feces on local benthic environments.

1. Introduction

In some marine species, many individuals gather to form large colonies. Such large colonies can sometimes have a great influence on the local marine environment and biological communities. The Mediterranean mussel *Mytilus galloprovincialis* Lmk. forms large colonies and renders habitats unsuitable for many benthic organisms by depositing large amounts of excretory substances and their mortal remains onto the sea bottom (Yamochi et al., 1995; Stenton-Dozey et al., 2001). The accumulation of substantial amounts of *M. galloprovincialis* feces and decaying dead mussels on the sea bottom results in organic enrichment (Yamochi et al., 1995; Zúñiga et al., 2014), anoxia, increase in total reducible sulfides, reduction of macrofauna biomass, and alteration of trophic groups and taxa in the sediment (Stenton-Dozey et al., 2001). This mussel is naturally distributed in the Mediterranean Sea and has been introduced into the coastal areas of South Africa, China, Hong Kong, Japan, Korea, Australia, New Zealand, Hawaii, Mexico, Russia, California, Washington, and the west coast of Canada (Carlton, 1999; Branch and Steffani, 2004). Because *M. galloprovincialis* greatly disturbs marine ecosystems and has economic effects worldwide (Branch and Steffani, 2004; Iwasaki, 2006), it is listed as one of the "100 of the World's Worst Invasive Alien Species" (Lowe et al., 2000).

Assessment of the quantity of mussels that drop from a vertical seawall is relatively easy, and such an assessment has already been performed (Miyoshi et al., 2009). The standard experimental design for in situ research to quantitatively assess the effect of mussel feces on the benthic environment is to measure the amount of sinking matter collected using a sediment trap near a hard substrate colonized by *M. galloprovincialis* (experimental group) and compare it with the amount collected in an area far from the mussel habitat (control group) (Jovanovic et al., 2009; Zúñiga et al., 2014). This sinking matter comprises a variety of materials such as feces of *M. galloprovincialis* and other animals, living plankton, dead bodies of organisms, and other organic and inorganic particles.

Hard surfaces such as port structures are inhabited not only by *M. galloprovincialis* but also by other types of animals. The mussel beds of *M. galloprovincialis* cause substantial increase in animal densities and species richness on such structures, and the community composition changes considerably (Robinson et al., 2007). Therefore, the composition and amount of matter in traps set between the experimental and control groups would differ even if the feces and mortal remains of *M. galloprovincialis* were excluded from those samples. If many feces shed by animal species other than *M. galloprovincialis* are present in the experimental group relative to the control group, then the amount of

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mussel feces will be erroneously overestimated, and thus, the effect of mussel feces on the benthic environment will also be overestimated. To avoid this problem, a method to correctly identify *M. galloprovincialis* feces in sediment is urgently required.

Although the environmental DNA (eDNA) of *M. galloprovincialis* in seawater is detectable (Nogata and Endo, 2012), eDNA analysis to compare the amounts of mussel feces between sampling points can only be used in small sites for short periods. If the sampling site is large or the sampling time differs greatly between samples, an exact comparison of the amount of mussel eDNA may be difficult because critical environmental conditions affecting the amount of eDNA, e.g., water temperature, UV-B, etc. (Strickler et al., 2015), will vary between the sampling points and sampling times. Thus, the application of the eDNA method is limited to narrow research purposes, and to conduct quantitative assessment of the effect of mussel feces on the benthic environment, another method for directly estimating the amount of mussel feces in sinking materials and measuring fecal weight is necessary. Such a method would allow the quantitative measurement of organic nitrogen in mussel feces collected in deposited materials.

M. galloprovincialis produces two types of excretory materials: feces and pseudofeces. Feces are waste matter discharged from the body through the alimentary tract. Pseudofeces are masses of particles that cannot be used as food and are ejected by the animal without passing through the alimentary tract; pseudofeces are often amorphous (Giles and Pilditch, 2004; Nagasoe et al., 2011). Because feces sink more rapidly, they tend to be deposited on the sea bottom in a smaller area than pseudofeces (Giles and Pilditch, 2004). The amount of feces produced per day and organic nitrogen content of feces is usually greater than that of pseudofeces (Arakawa, 1970; Galimany et al., 2011). Thus, the deleterious effect of feces on the local benthic environment is assumed to be more severe than that of pseudofeces. However, sinking particles also comprise feces produced by various other animal species, detritus, planktonic organisms, and organismal fragments, and these particles also accumulate at the bottom of the sea. Consequently, no method for quantitatively assessing the effect of *M. galloprovincialis* feces on the benthic environment is presently available.

Feces of aquatic animal species usually have a characteristic shape, e.g., spherical, cylindrical, elongated, or formed into strings (Wotton and Malmqvist, 2001). Classification of feces based on their morphological characteristics was proposed by Moore (1931a) and challenged by other researchers (Manning and Kumpf, 1959; Arakawa, 1970). The morphological variations in bivalve feces seem to be more closely related to feeding habits and mode of life than to systematic position (Arakawa, 1970). The shapes of feces vary depending upon the amount and kind of food, feeding conditions, and physiological conditions (Edge, 1934; Arakawa, 1970). Starved animals tend to produce thin and fragile feces that are often atypical in shape (Moore, 1931b; Arakawa, 1970).

To accurately discriminate the feces of *M. galloprovincialis* in deposited materials using a morphological approach, it is necessary to determine the species-specific morphological characteristics of this mussel's feces. Arakawa (1970) reported that the feces of *M. galloprovincialis* are a ribbon-type pellet with a groove (the cross-section of the fecal pellet is shaped like a "w"). Ovoid, ovoid to rod-like, or rod-like pellets cannot be used for specific identification because these pellet shapes are common; however, ribbon-like pellets and rods with a longitudinal groove are relatively uncommon and may allow identification of the producer (Manning and Kumpf, 1959). The shape of *M. galloprovincialis* feces might be determined by the internal structure of its alimentary canal (Arakawa, 1965). From the morphological characteristics mentioned above, it will be possible to identify the feces of *M. galloprovincialis* in deposited materials in the sea.

To demonstrate the species-specific morphology of mussel feces, it is necessary to compare the morphological characteristics of the feces of *M. galloprovincialis* with the morphological characteristics of the feces of other kinds of marine animals. Moreover, to demonstrate the advantage of using specific characteristics of mussel feces to quantitatively assess the effect of mussel feces on the benthic environment, one should be able to accurately discriminate mussel feces in deposited materials comprising feces of multiple species, detritus, etc.

In this study, we sought to clarify the common morphological characteristics of *M. galloprovincialis* feces and develop a method for discriminating mussel feces in deposited materials on the basis of these morphological characteristics. To achieve this, we first determined several common morphological characteristics of *M. galloprovincialis* feces. Next, we identified marine animal species that void feces similar to those of *M. galloprovincialis* to ascertain whether these morphological characteristics are species specific. Finally, we used mitochondrial DNA (mtDNA) analysis to determine the accuracy with which we could discriminate mussel feces in deposited materials on the basis of morphological characteristics.

2. Materials and methods

2.1. Observation of mussel fecal morphology

M. galloprovincialis specimens were collected from the ports of Kamaishi and Kagoshima (Japan) in January and February 2015 (Fig. 1). These two ports and their environments are present in distinct marine ecoregions: Kamaishi is in the "Cold Temperate Northwest Pacific," whereas Kagoshima is in the "Warm Temperate Northwest Pacific" (Spalding et al., 2007). Thus, the mussels in the two ports were considered to inhabit biogeographic provinces with distinct assemblages of natural communities and species. We searched for mussels on the side surfaces of floating piers that are continuously submerged in seawater. Mussels living under these conditions are always able to ingest food and produce feces. Consequently, they were expected to void feces within a short time after being harvested. Twenty mussels from each location were collected using our bare hands and a hand net. The collected mussels were placed in a bucket filled with natural seawater drawn from each sampling point. Twenty liters of natural seawater (salinity: 31.30 at Kamaishi and 34.14 at Kagoshima) were drawn at each sampling point. The mussels and seawater were brought to a laboratory near each sampling site.

These mussels were separately placed in 20 plastic containers, each filled with 0.5–1 L of natural seawater that was gently aerated with an air pump and air stone. The mussels began to eject feces within 5 min of being placed in the plastic containers. Three fecal pellets from the bottom of each container were transferred with a pipette to a gridded petri dish (diameter, 60 mm; grid size, 1 mm). Few pseudofeces were voided

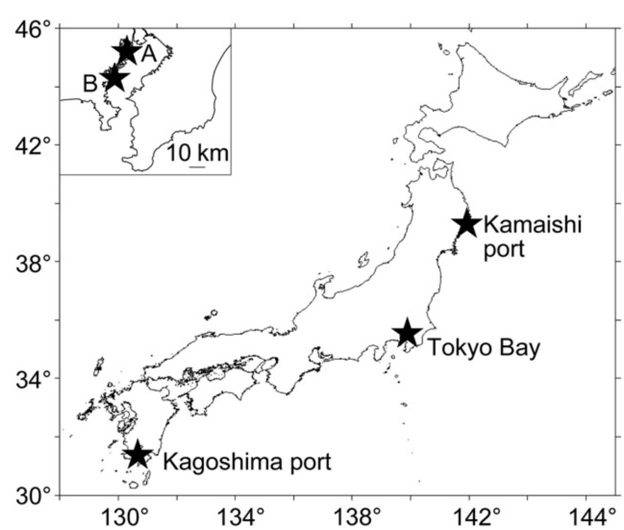

Fig. 1. Sampling sites. Magnified view of Tokyo Bay is shown at the upper left side of the panel.