

# A New Cleaning Method for Accurate Examination of Freshwater Gastropod Shell Specimens Covered with Iron-rich Deposits

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Sodium hypochlorite has been used for cleaning specimens of freshwater and brackish water snails that are covered with deposits. Our experiments using specimens of two freshwater snail species, *Semisulcospira niponica* (Smith, 1876) and *S. reticulata* Kajiyama and Habe, 1961, showed that this traditional method could remove thin deposit layers, including algae, but was not useful for obstinate deposits. We found that a new method using ammonium thioglycolate could be applied to remove obstinate iron-rich deposits. Though ammonium thioglycolate treatment caused loss of gloss inside the aperture, this loss could be prevented by plugging a kneaded eraser into an aperture. Moreover, the new method could clean specimens with little damage of the periostracum. So as to remove deposits with the least damage to shells, 3% w/v sodium hypochlorite was useful for deposits including algae, and 20% w/v ammonium thioglycolate was suitable for cleaning specimens with iron-rich deposits. Degeneration of the microstructure of inner whorls can be avoided by plugged shell apertures with a kneaded eraser in both methods. Shell deposits that are composed of both algae and iron should be treated first with 20% w/v ammonium thioglycolate, and then with 3% w/v sodium hypochlorite to remove the deposits. Appropriate cleaning methods enable accurate examination and long-term preservation of shell specimens.

**Key Words:** deposit, Gastropoda, iron compound, kneaded eraser, periostracum, SEM observation, specimen.

## Introduction

Cleaning of specimens is one of the important procedures for examining them accurately and/or preserving them in a suitable condition over a long period. Therefore, such cleaning methods have been well developed for specimens of various metazoan groups: e.g., cleaning techniques for skeletons of mammals and birds (e.g., Dumitru et al. 2013; Larkin et al. 2015), and ultrasonic cleaning for deposits on the surface of insect exoskeletons (Hayashi 2019). Additionally, potassium hydroxide has been used for eliminating deposits on fragile specimens of arthropod families Culicidae, Isotomidae and Ephemerellidae (Schneeberg et al. 2017).

Shell cleaning is also essential for systematic studies of living and fossil molluscs, since their taxonomically important characters such as surface sculpture, as well as shell structure, color pattern and outline are usually obscured by deposits covering the shell, and thus it is often hard to observe these characters (Geiger et al. 2007; Rodrigues et al. 2012). Brushing the shell surface, ultrasonic cleaning and soaking specimens in sodium hypochlorite have been used to remove calcareous deposits on living marine shellfish (Kira 1959; Habe and Kosuge 1967; Geiger et al. 2007). These methods have also been applied for freshwater and brackish water gastropods, because sodium hypochlorite can remove algae included in deposits on their shell surfaces

(Tashiro et al. 2001; Sturm et al. 2006).

Although these traditional cleaning methods have been adopted for various gastropod species, nonetheless, the methods are known to damage or cause decomposition of gastropods' shell periostraca and microsculptures (Duncan and Ghys 2019). However, such adverse impacts on these shell features have not been verified for freshwater and brackish water species, and thus the suitability of the method has not been clarified yet. In addition to marine snail taxa such as Buccinidae and Rissoidae (Hasegawa 2017; Okutani 2017), shell surface characters of periostracum color or sculptures are critically important for systematic studies of fresh- and brackish water snails—e.g., Neritidae and Semisulcospiridae—, since these features have been treated as snails' diagnostic characters, and/or show intriguing polymorphism (Neumann 1959; Davis 1969; Pandey et al. 2019). Meanwhile, several preceding studies did not remove deposits at all, or showed incomplete removal of deposits even using sodium hypochlorite, on shells of freshwater snail type specimens that veil key taxonomic characters of these species (e.g., Kajiyama and Habe 1961; Watanabe and Nishino 1995). Therefore, there is a need to develop a method that can remove deposits with minimal damage of the periostracum and other parts of freshwater and brackish water snail specimens.

A chelating agent, ammonium thioglycolate, has been used to remove iron compounds from marble and calcare-

ous stones, and moreover, it is suggested that ammonium thioglycolate is the most efficient reagent for cleaning rust-stained marble (Leussing and Kolthoff 1953; Macchia et al. 2016). Given the similarity of the main component in marble, calcareous stones, and gastropods' shells (Wilbur and Jodrey 1952; Watabe 1974), it seems highly probable that the method would be useful for cleaning shell specimens of freshwater and brackish water snails, on which stiff deposits include iron compounds, without damaging their taxonomically important characters. In the present study, the authors aim to establish an improved shell-cleaning method by the traditional treatment using sodium hypochlorite in addition to a new treatment using ammonium thioglycolate for freshwater and brackish water gastropods.

## Materials and Methods

Two reagents, sodium hypochlorite (12% aqueous solution, Nippon Garlic Corporation, Gunma, Japan) and ammonium thioglycolate (50% aqueous solution, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), were applied to two populations of freshwater snails: *Semisulcospira niponica* (Smith, 1876) and *S. reticulata* Kajiyama and Habe, 1961. The samples of *S. niponica* were collected from pale rock bottom at water depth of 0.5 m in Lake Biwa, central Honshu, Japan, and their shell surfaces bore a thin layer of algae without iron compounds (Figs 1A, 2A). Snails of *S. reticulata* were collected from muddy bottom at the depth of 10–15 m in Lake Biwa, where iron is rich and little sunlight can reach. Therefore, stains including iron compounds were stuck to the specimens of *S. reticulata*, and these deposits contained hardly any algae (Figs 3A, 4A). In total, 60 specimens of each of the two species were examined.

We verified shells' damage from the reagents regarding three features: (1) periostracum color (hereafter PC; remained or faded), (2) periostracum layer (hereafter PL; remained on or removed from the calcium carbonate layer), and (3) gloss inside the aperture (hereafter GAP; preserved or lost). In order to examine damage to GAP caused by the reagents, initially, the specimens were divided into two groups: the aperture of the specimen was plugged with a kneaded eraser (hereafter the AP-closed group), and its aperture was not plugged with an eraser (hereafter the AP-open group) (Fig. 5). Then, five specimens each of the two species were placed individually into 100 mL of aqueous solutions with three different concentrations: sodium hypochlorite, 3%, 6% and 12% weight/volume (hereafter, w/v); ammonium thioglycolate, 5%, 10% and 20% w/v. These concentrations were chosen because 5.25% w/v sodium hypochlorite solution and 5% w/v ammonium thioglycolate aqueous solution were used for freshwater snails and marble in previous studies (Davis 1969; Macchia et al. 2016). The specimens of AP-closed group partially floated to the surface of the aqueous solution since the kneaded eraser prevented the air inside them from escaping. Accordingly, the solution surface for the group was covered with a thin plastic plate to submerge the whole specimen in the solu-

tions. The specimens in each reagent were gently agitated once per minute, and then lightly cleaned with a toothbrush when the reagent was replaced with fresh reagent once every 10 minutes. Experiments were completed when shells' deposits were fully decomposed, or when the decomposition reaction seemed to have stopped, or at 40 minutes after the specimens sank due to damage to the periostracum. Further treatment with sodium hypochlorite was conducted when algae remained after the treatment with ammonium thioglycolate. The outside of the specimen was then slightly brushed and shaken well under running tap water to rinse the reagent inside and outside the shell. The cleaned specimens were dried naturally. All experiments except for rinsing of the specimens were conducted at temperatures below 23°C in a fume hood.

After the treatments, PC and GAP of all examined specimens were assessed by visual observation. For specimens treated with the highest concentration of each reagent, the PL of the two semisulcospirids and the GAP of *S. reticulata* were also checked with a Hitachi TM1000 scanning electron microscope using a cross-section and inner surfaces of outer lips, respectively.

In addition to the two *Semisulcospira* species, specimens of other freshwater and brackish water snails—*Clithon* sp., *Heterogen japonica* (Martens, 1861), *Semisulcospira decipiens* (Westerlund, 1883), and *Stenomelania hastula* (Lea and Lea, 1851)—were cleaned by the same method using 20% w/v ammonium thioglycolate aqueous solution with plugging of apertures. After they were cleaned with the reagents, their PC and GAP were investigated by visual observation. The specimens examined in this study were deposited in the Zoological Collection of Kyoto University (KUZ) (Tables 1–3).

## Results

**Cleaning of specimens with algae-rich deposits—*S. niponica* (Table 1; Figs 1, 2, 6).** In all 12 groups, the treatments were completed within four minutes. With sodium hypochlorite, deposits were successfully removed in all six groups. PC was almost preserved by treatment with 3% solution (Figs 1B, C, 2B, C). With treatment by 6% and 12% solution, however, PC was slightly faded (Figs 1D–G, 2D–G). PL was well preserved on a layer of calcium carbonate after 12% solution treatment (Fig. 6B), as thickly as on specimens before treatments (Fig. 6A). GAP was also preserved in all six groups (Fig. 1B–G).

On the specimens treated with ammonium thioglycolate, the deposits could not be removed in all samples, and a thin layer of algae remained on the shell surface (Figs 1H–M, 2H–M). PC was almost preserved in all six groups. PL also remained after cleaning with 20% ammonium thioglycolate (Fig. 6C). GAP was almost completely preserved in all six groups. After shells were cleaned with ammonium thioglycolate, the remaining algae could be removed within 5–10 seconds by 3% sodium hypochlorite treatment with a kneaded eraser (Figs 1N–S, 2N–S).

Table 1. Results of treatment of *Semisulcospira niponica* with 3%, 6% or 12% w/v sodium hypochlorite and 5%, 10% or 20% w/v ammonium thioglycolate aqueous solution.

Reagents and concentrations (w/v)	Voucher	Group	Sample number	Number successfully cleaned	Treatment time (min)	Periostracum color	Gloss inside the aperture
hypochlorite 3%	KUZ Z3712	AP-closed	5	5	2–4	almost preserved	preserved
hypochlorite 3%	KUZ Z3713	AP-opened	5	5	2–3	almost preserved	preserved
hypochlorite 6%	KUZ Z3714	AP-closed	5	5	2–3	slightly lost	preserved
hypochlorite 6%	KUZ Z3715	AP-opened	5	5	2	slightly lost	preserved
hypochlorite 12%	KUZ Z3716	AP-closed	5	5	1–2	slightly lost	preserved
hypochlorite 12%	KUZ Z3717	AP-opened	5	5	1–2	slightly lost	preserved
ammonium thioglycolate 5%	KUZ Z3718	AP-closed	5	0	1–3	almost preserved	preserved
ammonium thioglycolate 5%	KUZ Z3719	AP-opened	5	0	1–4	almost preserved	preserved
ammonium thioglycolate 10%	KUZ Z3720	AP-closed	5	0	2–3	almost preserved	preserved
ammonium thioglycolate 10%	KUZ Z3721	AP-opened	5	0	2–3	almost preserved	preserved
ammonium thioglycolate 20%	KUZ Z3722	AP-closed	5	0	1–3	almost preserved	preserved
ammonium thioglycolate 20%	KUZ Z3723	AP-opened	5	0	1–3	almost preserved	preserved

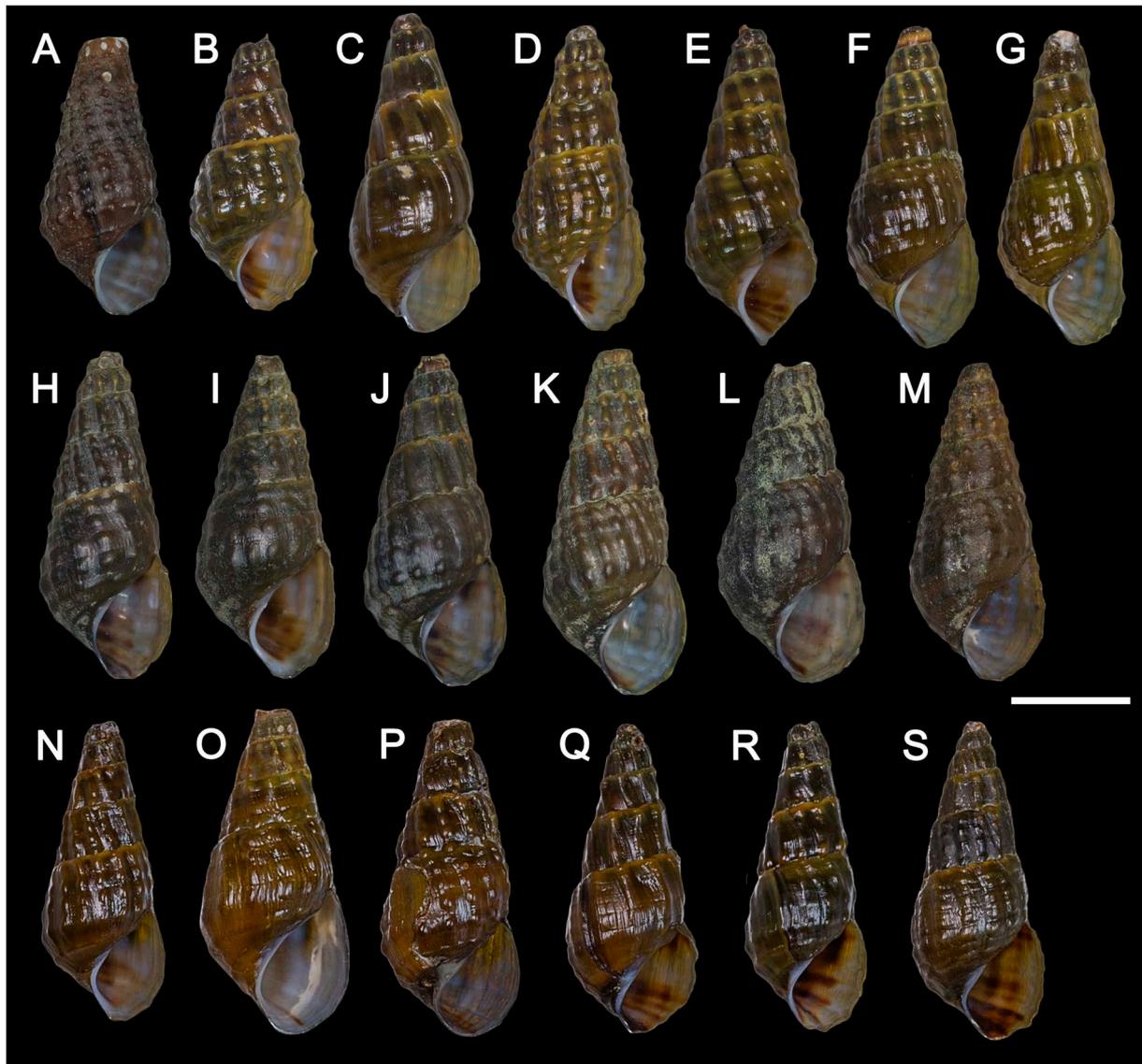


Fig. 1. Specimens of *Semisulcospira niponica* before and after treatments. A, Before treatment; B–G, After treatment with sodium hypochlorite (B, C, 3%, KUZ Z3712–Z3713; D, E, 6%, Z3714–Z3715; F, G, 12%, Z3716–Z3717; B, D, F, AP-opened; C, E, G, AP-closed); H–M, After treatment with ammonium thioglycolate; N–S, After treatment with ammonium thioglycolate followed by treatment with 3% sodium hypochlorite (H, I, N, O, 5%, KUZ Z3718–Z3719; J, K, P, Q, 10%, Z3720–Z3721; L, M, R, S, 20%, Z3722–Z3723; H, J, L, N, P, R, AP-opened; I, K, M, O, Q, S, AP-closed). Scale bar: 10 mm.

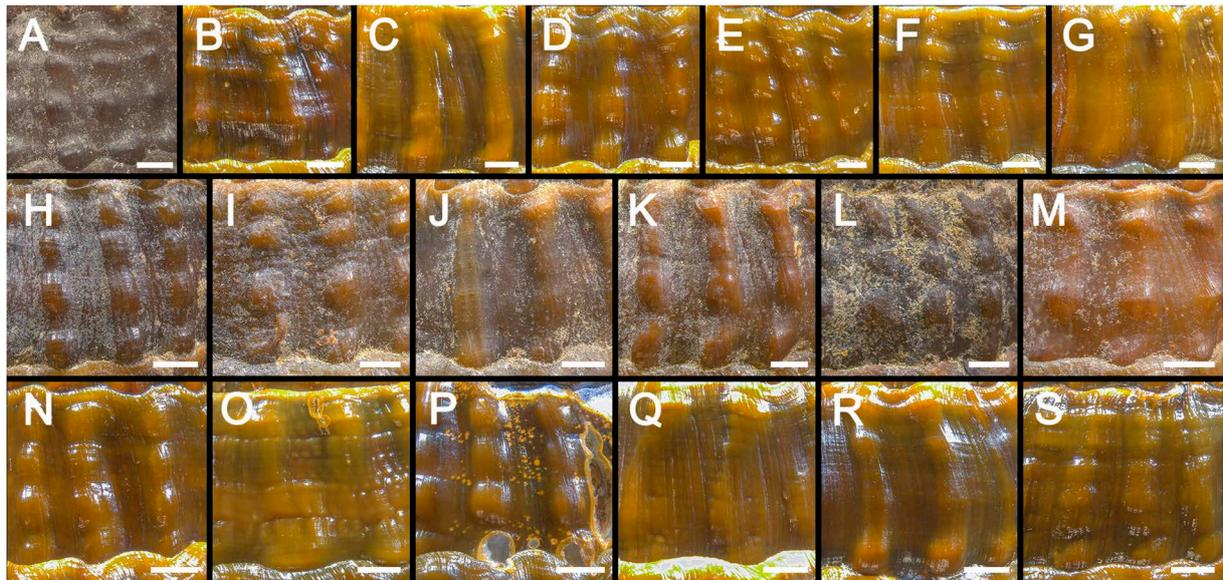


Fig. 2. Shell surfaces of penultimate whorl of *Semisulcospira niponica* before and after treatments. The specimens and their order are the same as in Fig. 1. Scale bars: 1 mm.

Table 2. Results of treatment of *Semisulcospira reticulata* with 3%, 6% or 12% w/v sodium hypochlorite and 5%, 10% or 20% w/v ammonium thioglycolate aqueous solution.

Reagents and concentrations (w/v)	Voucher	Group	Sample number	Number successfully cleaned	Treatment time (min)	Periostracum color	Gloss inside the aperture
hypochlorite 3%	KUZ Z3724	AP-closed	5	0	more than 40	considerably lost	preserved
hypochlorite 3%	KUZ Z3725	AP-opened	5	0	more than 40	considerably lost	preserved
hypochlorite 6%	KUZ Z3726	AP-closed	5	3	15–more than 40	considerably lost	preserved
hypochlorite 6%	KUZ Z3727	AP-opened	5	2	15–more than 40	considerably lost	preserved
hypochlorite 12%	KUZ Z3728	AP-closed	5	4	5–more than 40	considerably lost	preserved
hypochlorite 12%	KUZ Z3729	AP-opened	5	3	10–more than 40	considerably lost	preserved
ammonium thioglycolate 5%	KUZ Z3730	AP-closed	5	5	15–35	almost preserved	preserved
ammonium thioglycolate 5%	KUZ Z3731	AP-opened	5	5	25–40	almost preserved	lost
ammonium thioglycolate 10%	KUZ Z3732	AP-closed	5	5	20–40	almost preserved	preserved
ammonium thioglycolate 10%	KUZ Z3733	AP-opened	5	5	20–40	almost preserved	lost
ammonium thioglycolate 20%	KUZ Z3734	AP-closed	5	5	20	almost preserved	preserved
ammonium thioglycolate 20%	KUZ Z3735	AP-opened	5	5	15–20	almost preserved	lost

**Cleaning of specimens with iron-rich deposits—*S. reticulata* (Table 2; Figs 3, 4, 6).** With sodium hypochlorite, treatments took five to more than 40 minutes. Although specimens were treated for more than 40 minutes, deposits of several specimens in all six groups remained on the shell surface. PC was considerably lost in all groups (Figs 3B–G, 4B–G). PL (see Fig. 6D) was completely removed from the calcium carbonate layer after treatments with 12% solution (Fig. 6E). While GAP seemed to be preserved in all groups, microscopic holes were formed on the inner surfaces of outer lips (Fig. 6H), comparing to the specimens before treatment (Fig. 6G).

With ammonium thioglycolate, treatments were completed within 40 minutes, and took 15–40 minutes for all specimens. Deposits were completely removed in all six groups, and 20% ammonium thioglycolate removed deposits in the shortest amount of treatment time. PC was well preserved, and therefore color bands and background colors

of the periostracum were observable (Figs 3H–M, 4H–M). PL was also preserved after 20% ammonium thioglycolate treatments (Fig. 6F), and was as thick as in specimens before treatments (Fig. 6D). GAP was well preserved in the three AP-closed groups (Fig. 3I, K, M), but it was lost in the AP-opened groups (Fig. 3H, J, L). Although protrusions and cavities were formed on the inner surfaces of outer lips of the AP-opened specimens (Fig. 6J), the surface structures of AP-closed ones were similar to before treatments (Fig. 6I).

**Cleaning of other snails (Table 3; Fig. 7).** Shell surfaces of *Clithon* sp, *Heterogen japonica*, *S. decipiens* and *Stenomelania hastula* were covered with thick deposits. These deposits could be removed using 20% ammonium thioglycolate reagent. PC and GAP were well preserved in all examined specimens.

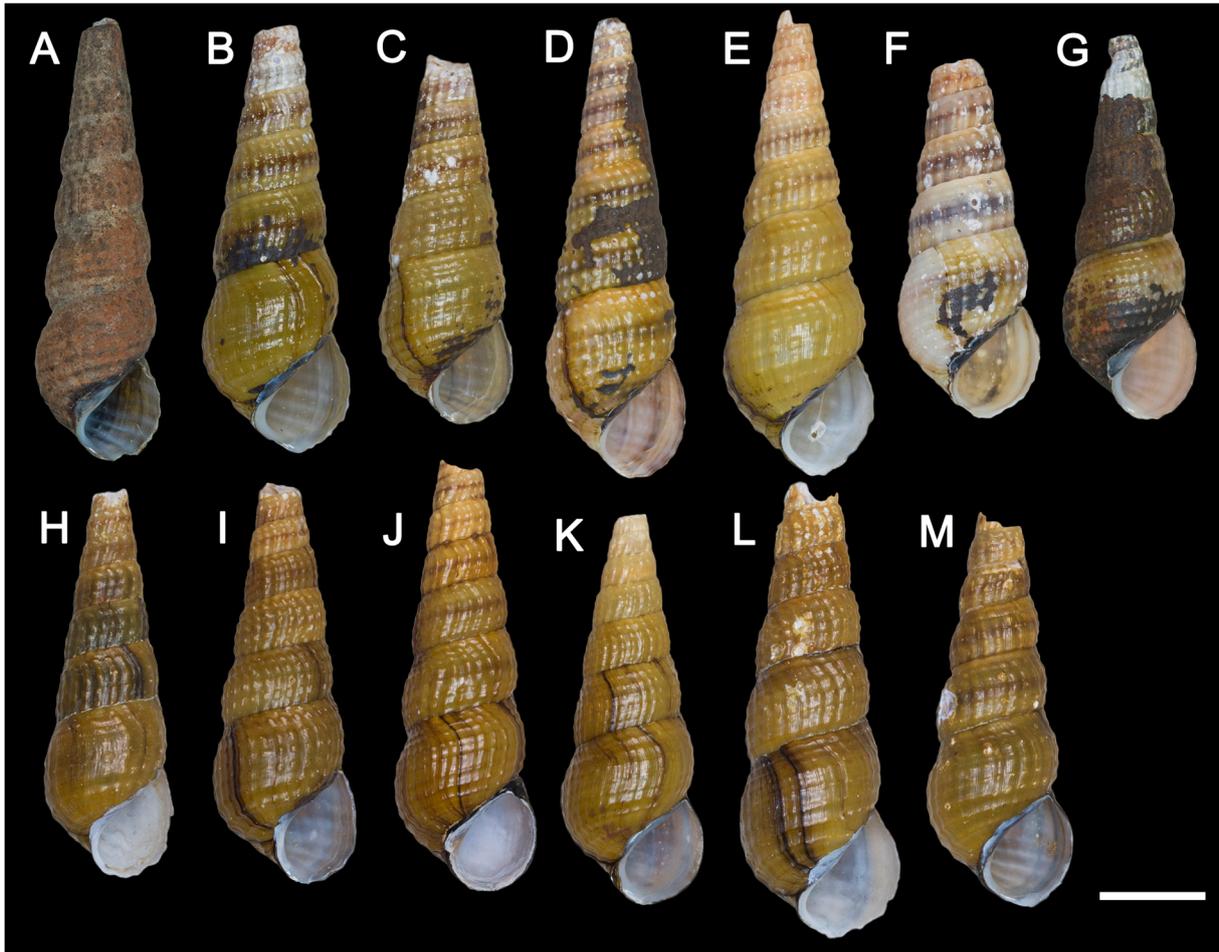


Fig. 3. Specimens of *Semisulcospira reticulata* before and after treatments. A, Before treatment; B–G, After treatment with sodium hypochlorite (B, C, 3%, KUZ Z3724–Z3725; D, E, 6%, Z3726–Z3727; F, G, 12%, Z3728–Z3729; B, D, F, AP-opened; C, E, G, AP-closed); H–M, After treatment with ammonium thioglycolate (H, I, 5%, KUZ Z3730–Z3731; J, K, 10%, Z3732–Z3733; L, M, 20%, Z3734–Z3735; H, J, L, AP-opened; I, K, M, AP-closed). Scale bar: 10 mm.

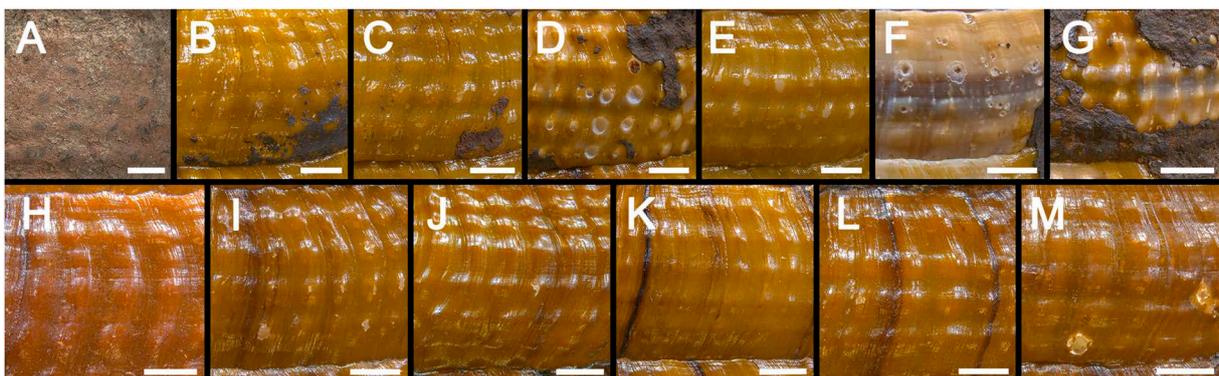


Fig. 4. Shell surfaces of penultimate whorl of *Semisulcospira reticulata* before and after treatments. The specimens and their order are the same as in Fig. 3. Scale bars: 2 mm.

### Discussion

The results obtained with *S. niponica* and *S. reticulata* showed that sodium hypochlorite seemed to be useful to clean specimens whose surface had deposits including algae,

while ammonium thioglycolate was not effective. Closing the aperture with kneaded eraser should be applied for the traditional method since microscopic holes were formed on the inner shell surfaces, despite GAP seemed to be preserved in all conditions. For shell cleaning, ca. 5% w/v sodium hypochlorite has been used in previous studies (Davis

1969; Sturm et al. 2006). However, our results indicated that highly concentrated sodium hypochlorite solution causes fading of PC, and in the worst case, completely decomposes PL of shell specimens, though it can remove deposits faster. According to our experiments, therefore, 3% w/v solution should be used so as to remove deposits with the least dam-



Fig. 5. Aperture-closing method using kneaded eraser.

age of the periostracum.

In contrast to sodium hypochlorite, ammonium thioglycolate could efficaciously decompose iron-rich deposits on the snails. The aperture of a specimen in ammonium thioglycolate should be also closed using a kneaded eraser, because GAP was removed and microstructure of inner of aperture was degenerated. In all experimental conditions—5, 10 and 20% w/v concentrations—, PC and PL were almost completely preserved, although the lowest concentration was applied for cleaning of marble in a previous study (Macchia et al. 2016). Moreover, highly concentrated ammonium thioglycolate could remove deposits faster from the shell surface. Therefore, 20% w/v ammonium thioglycolate seems to suitable for efficiently cleaning specimens with iron-rich deposits.

Our results showed that sodium hypochlorite could remove algae from deposits on the specimens that had been soaked in ammonium thioglycolate. Therefore, shell specimens with deposits composed of both algae and iron should be treated with 20% w/v ammonium thioglycolate at first, and then with 3% w/v sodium hypochlorite. We recom-

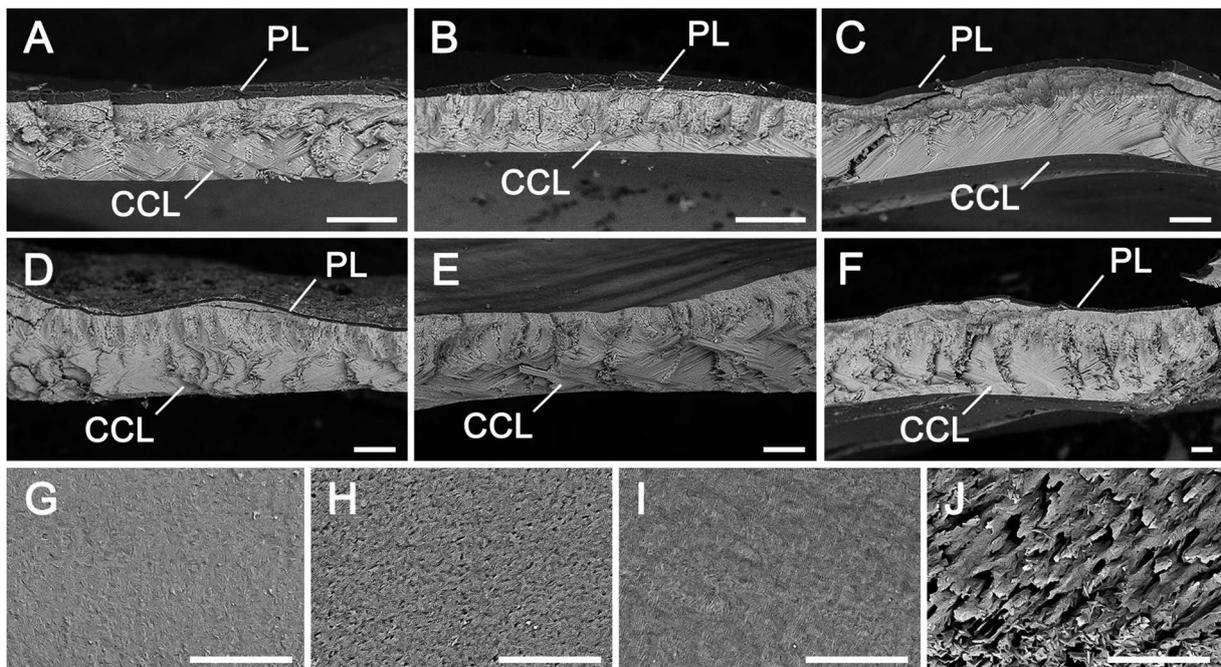


Fig. 6. SEM observations of cross sections (A–F) and inner surfaces (G–J) of outer lips. A, *Semisulcospira niponica* before treatment; B, *S. niponica* after treatment with 12% sodium hypochlorite, KUZ Z3716; C, *S. niponica* after treatment with 20% ammonium thioglycolate, KUZ Z3722; D, G, *S. reticulata* before treatment; E, H, AP-opened *S. reticulata* after treatment with 12% sodium hypochlorite, KUZ Z3728; F, I, AP-closed *S. reticulata* after treatment with 20% ammonium thioglycolate, KUZ Z3734; J, AP-opened *S. reticulata* after treatment with 20% ammonium thioglycolate, KUZ Z3735. Scale bars: 100  $\mu\text{m}$  (A–F), 10  $\mu\text{m}$  (G–J). Abbreviations: CCL, calcium carbonate layer; PL, periostracum layer.

Table 3. Results of treatment of other snails with 20% w/v ammonium thioglycolate aqueous solution.

Species	Voucher	Group	Treatment time (min)	Periostracum color	Gloss inside the aperture
<i>Semisulcospira decipiens</i>	KUZ Z3736	AP-closed	15	almost preserved	preserved
<i>Heterogen japonica</i>	KUZ Z3737	AP-closed	25	almost preserved	preserved
<i>Stenomelania hastula</i>	KUZ Z3738	AP-closed	20	almost preserved	preserved
<i>Clithon</i> sp.	KUZ Z3739	AP-closed	10	almost preserved	preserved

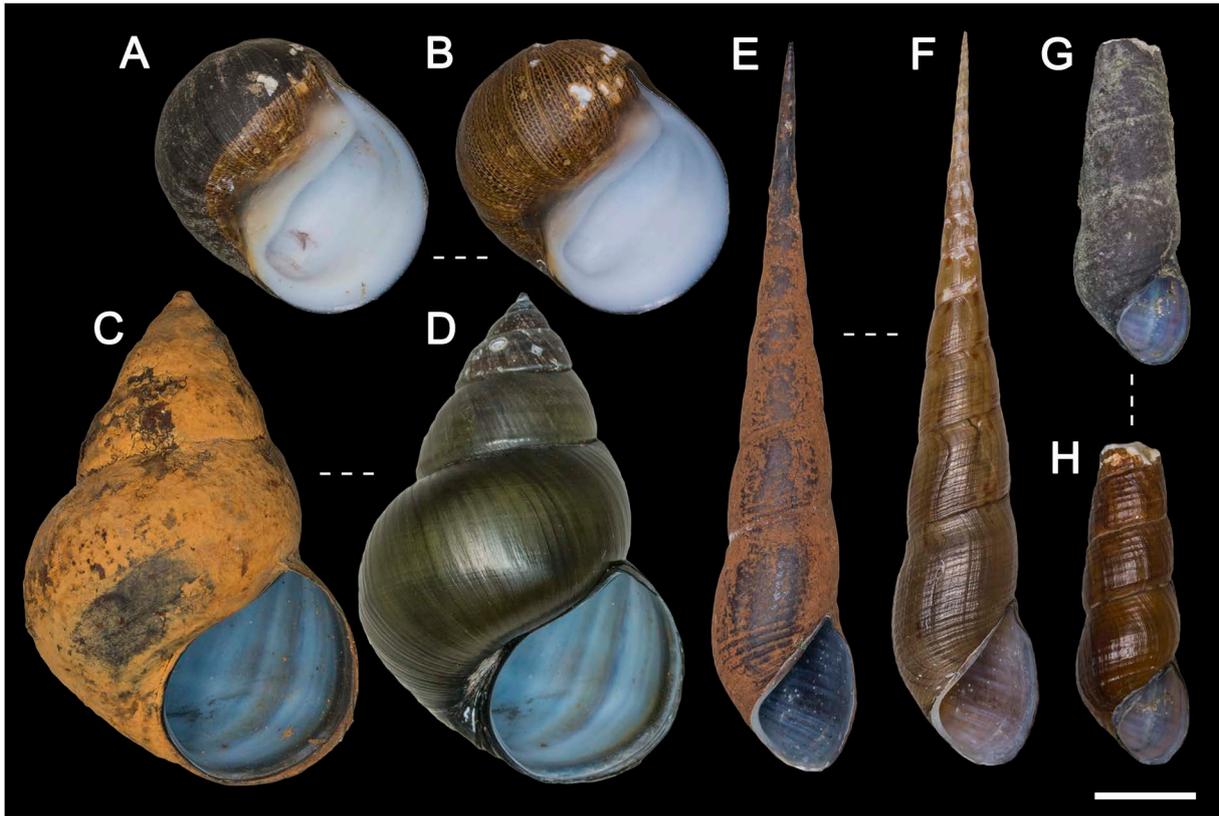


Fig. 7. Specimens of freshwater and brackish water snails after treatments with 20% ammonium thioglycolate. A, B, *Clithon* sp., KUZ Z3739; C, D, *Heterogen japonica*, KUZ Z3737; E, F, *Stenomelania hastula*, KUZ Z3738; G, H, *Semisulcospira decipiens* KUZ Z3736; A, C, E, G, Before cleaning; B, D, F, H, After cleaning. Scale bar: 10 mm.

mend our shell-cleaning method to enable accurate examination of shell specimens. However, it has been suggested that cleaning reagents that remain inside and outside of shell specimens cause long-term damage to the specimens such as discoloration and/or embrittlement of shells (Geiger et al. 2007). Accordingly, one should carefully wash shells after treatment with the reagents for taxonomic or morphological studies, so as to preserve them for a long period.

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