Procedure for Curation of Routine Microbiological Sample

Integrated Ocean Drilling Program (IODP)

Center for Deep Earth Exploration (CDEX)

Kochi Core Center (KCC)

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Following the Scientific Technology Panel (STP) recommendation and preliminary outcomes of the pilot study being conducted at the Kochi Core Center (KCC) for curation of Routine Microbiological Sample (RMS), a procedure for handling of the RMS has been drafted. This document provides an overview of the RMS curation and concisely describes various steps involved in onboard sampling, QA/QC, shipment, and onshore storage and sub-sampling of the RMS.

1. Introduction (What is RMS?)

RMS is the cored material (or portions thereof) from the subseafloor which is adequately preserved soon after recovery in frozen condition for future microbiological analyses. Figure 1 provides an overview of activities related to RMS curation. During the long history of scientific drilling, most of the cores have been stored at around 4°C in walk-in storage rooms. However, contaminating microbes from air and/or from bare hands of scientists easily grew on the cores under these storage conditions (Masui *et al.*), and thus caused serious damage to core quality. Moreover, dramatic change in ambient conditions easily kills subseafloor microbes. After cell death, fragile bio-molecules such as RNA, enzymes, sugar chains, and intact polar lipids are rapidly degraded via abiotic hydrolysis and enzymatic reactions. Thus, storage of core samples under frozen condition for preventing such degradation is of great importance to provide opportunities for future molecular analyses arising from rapid biotechnological developments (D'Hondt *et al.*).

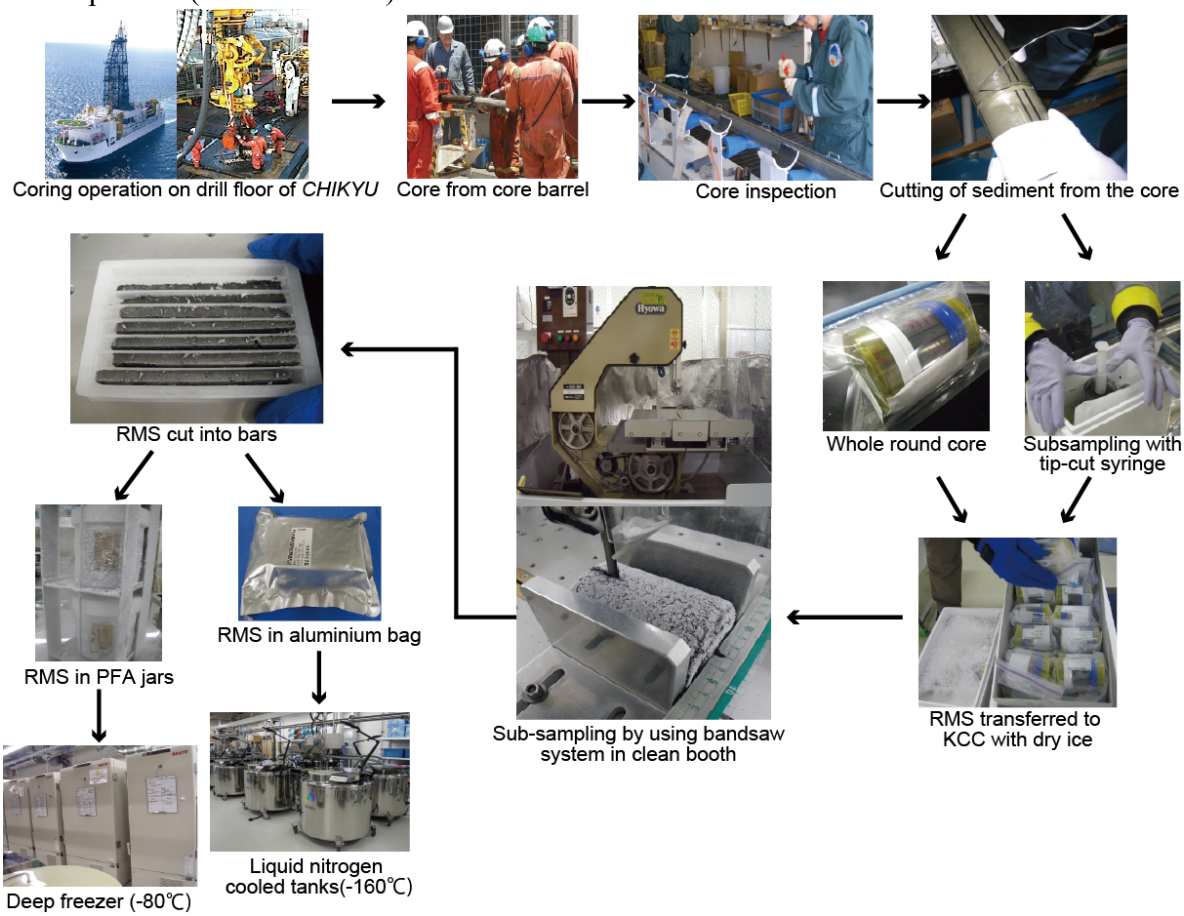

Fig. 1. Overview of RMS curation activities from onboard sampling to onshore storage

2. Onboard processing

Figure 2 shows a decision making flow chart for RMS sampling, which has been prepared in accordance with the STP recommendation 0908-09 (Appendix I). Final decision on RMS sampling and sampling method for the RMS will be made by onboard Sample Allocation Committee (SAC). Precise operational definitions of various terms relevant to handling of RMS are provided in Table T1 (Appendix II).

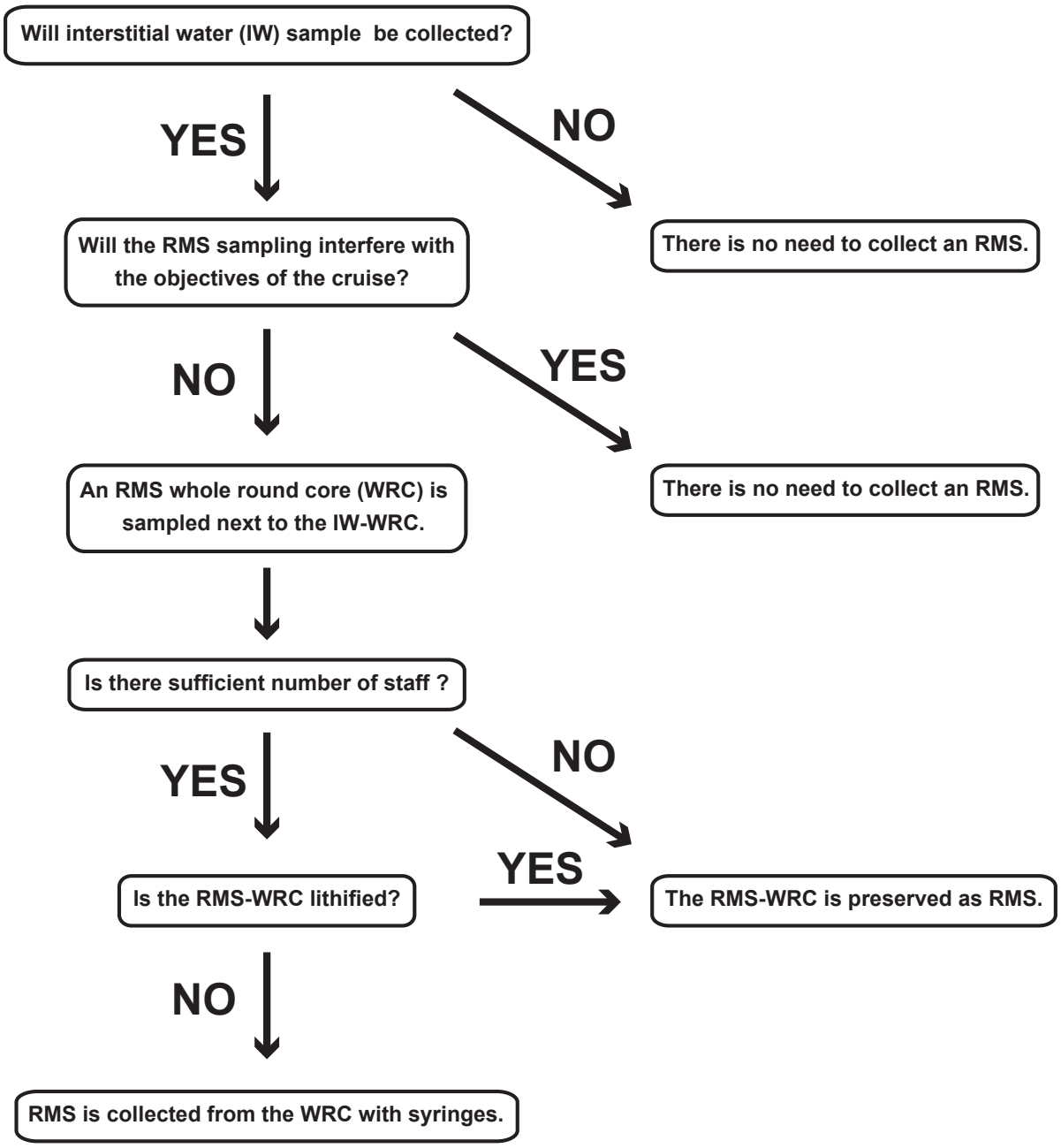

Fig. 2. Decision making flow chart for RMS sampling onboard ship

2-1. Sampling of whole round core (WRC) as RMS

2-1-1. Materials

(1) Spray bottle with 70% ethanol

(2) Clean disposable gloves

Clean disposable gloves must be used during all handling procedure (human skin is a primary contamination source).

(3) Sterilized Spatula

Rinse spatula with distilled water, wrap its head with aluminum foil, and autoclave at 121ºC for 20 min. (Fig. 3a). After autoclave, put spatula into oven (60˚C) to dry it for about 12 hours. After drying up, let spatula cool to room temperature prior to use. If autoclaving is not feasible because of high frequency of its use, spray the head of spatula with 70% ethanol, and wipe it with clean tissue paper. Perform this cleaning of spatula just before cutting the RMS-WRC.

(4) Clean end-caps

End-caps currently used cannot be sterilized by autoclaving, so these are cleaned with 70% ethanol. Rinse inside of the end-caps with distilled water to remove dust, and put on clean bench so that inside of the cap faces upward (Fig. 3b). Spray inside of the cap with 70% ethanol, and let it dry up for 1 hour by exposing to UV. After dry up, put clean caps into a clean bag, and keep it in the laboratory ready for use. Clean bag is prepared by wiping inner side of it with 70% ethanol. If above preparation is not feasible due to limited onboard staff and time, spray inside of an end-cap with 70% ethanol, and wipe it with clean tissue paper. Perform this cleaning of cap just before capping the RMS-WRC.

(5) Aluminum bag (similar to retort bag) (Fig. 3c)

Optimum bag dimensions are [210 mm(H) x 150 mm(W)].

(6) Transparent Teflon tape (e.g., NITOFLON® Adhesive Tape)

For keeping labels pasted on samples under ultra-low temperature condition

(a) 

(b) 

(c) 

Fig. 3. Tools for RMS sampling (a) Spatula sterilized with autoclave, (b) Cleaning of end-caps in clean bench, (c) Aluminum bags

2-1-2. Method

After retrieval, the core is transferred to core cutting area for cutting into sections, taking void gas and/or sample for safety monitoring, capping sections with end caps, and labeling (Fig. 4). During these processes, sections are visually inspected for their quality. If the core quality is judged to be inferior and interstitial water (IW) sample will not be collected, the RMS need not be collected. Usually, it takes approximately 15-20 min. from core retrieval ("core on deck" call) to transfer of core into core cutting area.

Fig.4. Core in core cutting area onboard D/V *Chikyu*

Sequence of steps for taking a whole round core (WRC) as RMS is shown in Fig. 5 and described as follows;

Core cutting area

(1) Select sampling point for the RMS based on discussion with onboard geochemist in order to check core quality and avoid conflict with cruise objective.
(2) Wipe the core liner around selected points (3-5 cm in width) with a tissue paper soaked with 70% ethanol.
(3) Mark the range of the RMS-IW sample with felt marker. Range for RMS is usually 5-10 cm.
(4) Cut the liner at bottom side of RMS-IW-WRC with a standard liner cutter.
(5) Cut the sediment at bottom side of the WRC with a usual spatula.
(6) Cap the bottom side of the WRC with a usual white end-cap.
(7) Cut the liner at topside of the WRC with the liner cutter.
(8) Cut the sediment at top side of the WRC with a sterilized spatula.
(9) Cap the topside of the WRC with a clean blue end-cap. Transfer the WRC to laboratory for CT scanning.

Laboratory

(10) After CT scanning, wipe the RMS-IW-WRC core liner around RMS-IW boundary (3-5 cm in width) with a tissue paper soaked with 70 % ethanol, and cut the WRC with a liner cutter and sediment in liner with a sterilized spatula.
(11) Cap the bottom side of the RMS-WRC with a clean yellow end-cap, and seal the caps and liner with vinyl tapes. Give IW-WRC to lab. Tech.
(12) Print two labels after registration of RMS-WRC in J-CORES, and paste one on end-caps and one on aluminum bag (or similar product).
(13) Cover the labels with transparent Teflon tape.
(14) Put the RMS-WRC into the aluminum bag (or similar product).
(15) Store the RMS-WRC into deep freezer (-80°C).

The RMS-WRC sampling takes approximately 30 min.

Core cutting area

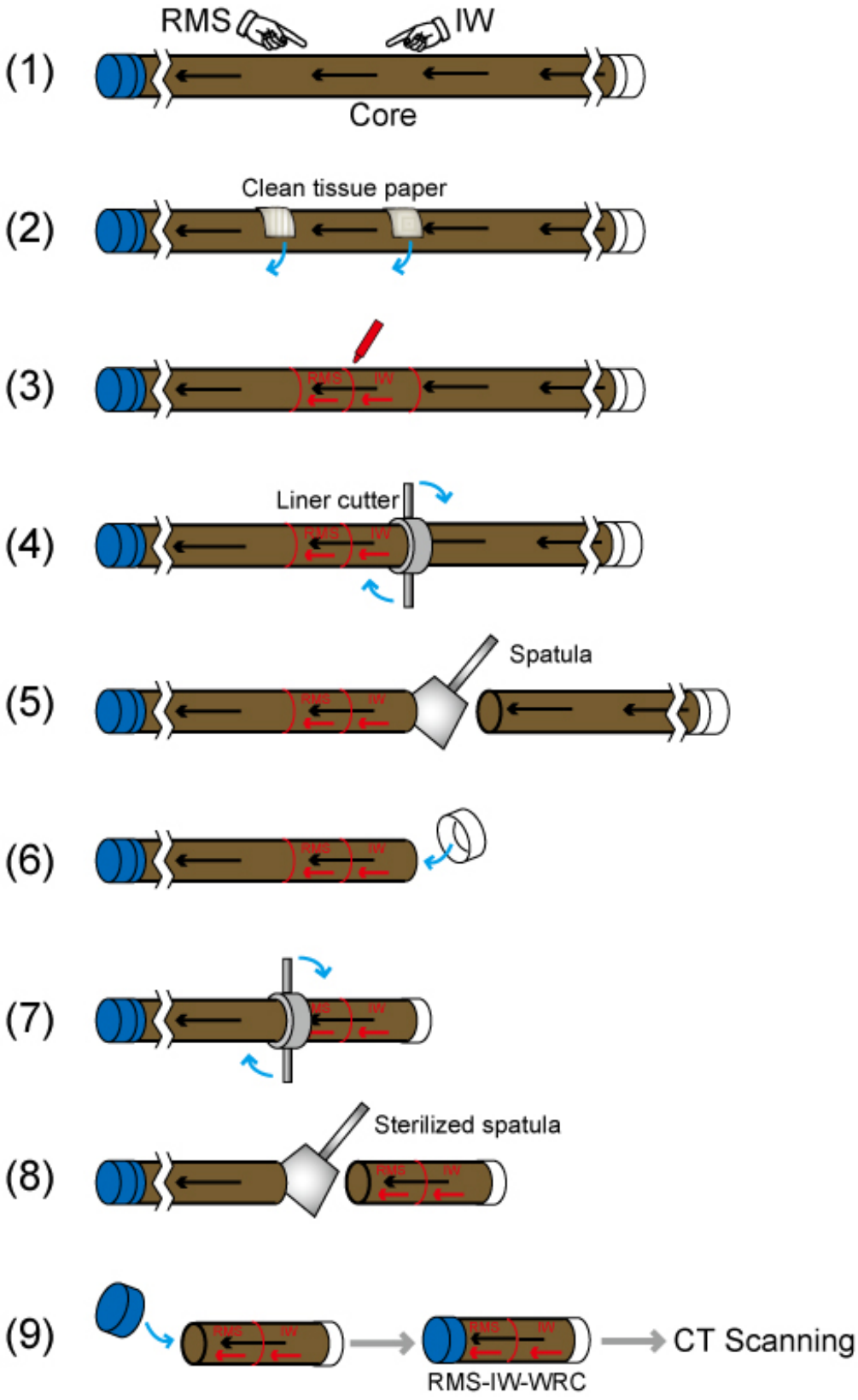

Fig. 5. Sequence of steps for taking a whole round core (WRC) as RMS at core cutting area

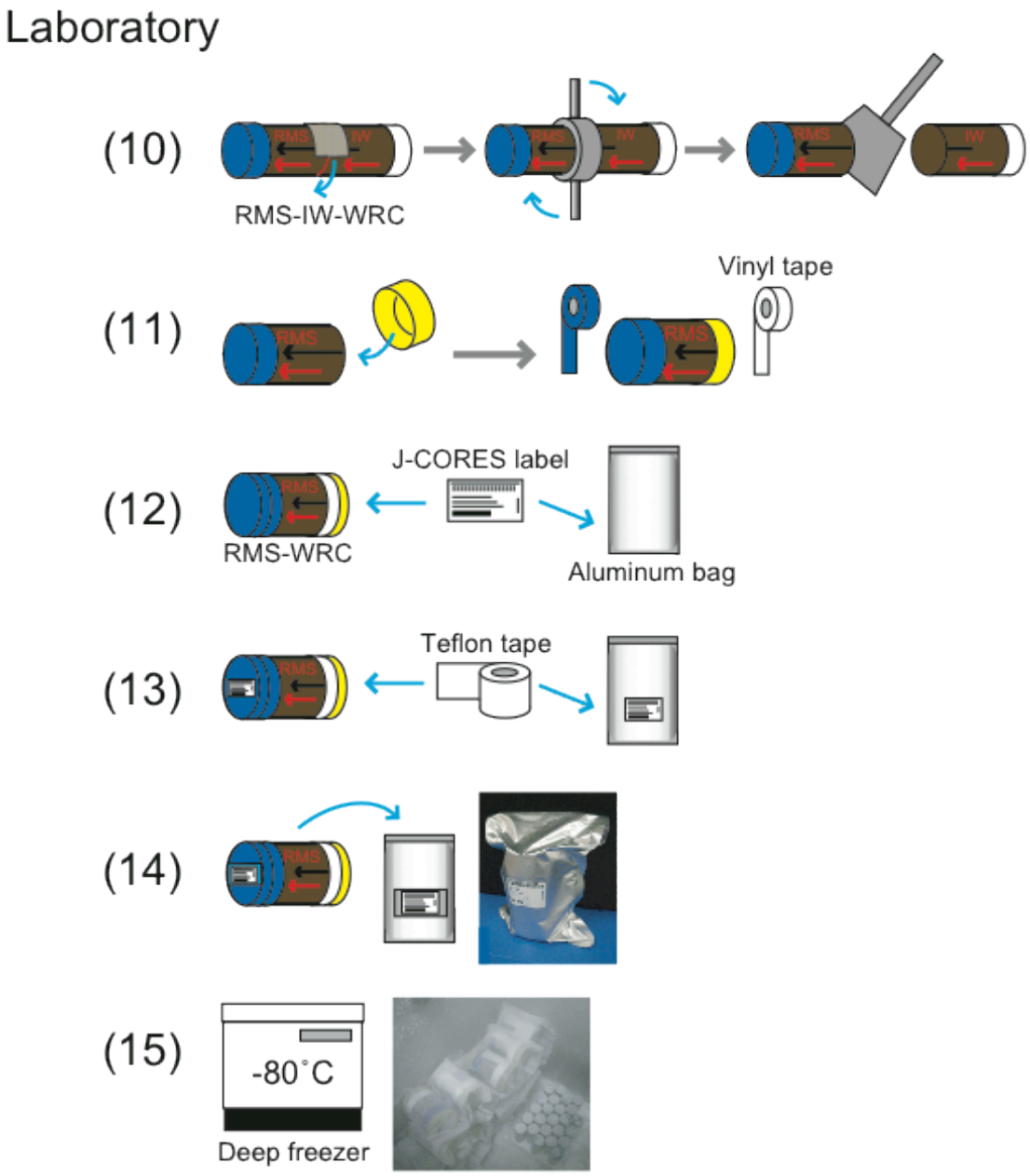

Fig. 5. (continued) Sequence of steps for taking a whole round core (WRC) as RMS at laboratory

2-2. RMS sampling from WRC by using a syringe

Core liner is not sterile and contamination of outer rim of the core cannot be avoided during drilling, therefore, it is preferable to sub-sample middle part of a WRC with a syringe with the tip removed. However, when sub-sampling is not possible (e.g., because of limited staff or indurated nature of the core), syringe sampling should be skipped.

2-2-1. Materials

(1) Tip cut-off sterilized syringes (20 and 30 cc)

Cut off the luer lok end of syringe with a heated knife. Wrap the tip cut-off syringe in aluminum foil, and autoclave at 121ºC for 20 min. After autoclaving, put syringe into oven (60˚C) to dry it up for about 12 hours. After drying up, let the syringe cool to room temperature (Fig. 6a). Store the sterilized syringes in a clean bag. If above preparation is not feasible due to limited onboard staff and time, sterilized syringes can be prepared onshore and shipped to the drilling platform.

(2) Sterile centrifuge tube (50 cc) (e.g., FALCON® tube)

For storing syringe-sampled RMS

(3) Cooling gel pack

For keeping the RMS-WRC under low temperature during sampling by tip cut-off sterilized syringe

Gel packs are to be stored at -20˚C prior to use.

(4) Styrene foam box

For keeping the RMS-WRC and cooling gel packs in place during sampling of the WRC by sterilized syringe

Inner size of box should be [250 mm (L) x 150 mm (W) x 150 mm (W)].

(5) Syringe adapter

For inserting the sterilized syringe into somewhat hard RMS-WRC (Fig. 6b)

(6) All materials mentioned in “Sampling of whole round core (WRC) as RMS”

(a)

(b)

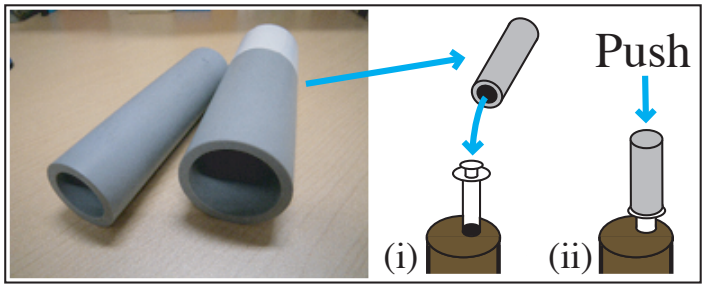

Fig. 6. Tools for RMS sampling (a) Tip cut-off sterilized syringe, (b) Syringe adapter

2-2-2. Methods

Sequence of steps for taking RMS from a WRC by using sterilized syringes is shown in Fig. 7.

Core cutting area

(1) Select sampling point for the RMS based on discussion with onboard geochemist in order to check core quality and to avoid conflict with cruise objective.
(2) Wipe the core liner around selected points (3-5 cm in width) with a tissue paper soaked with 70% ethanol.
(3) Mark the range of the RMS-IW sample with felt marker. Range for RMS is usually 5-10 cm.
(4) Cut the liner of bottom side of RMS-IW-WRC with a standard liner cutter.
(5) Cut the bottom side of RMS-IW sediment from the core with sterilized spatula.
(6) Cap the bottom side of the RMS-IW-WRC with a usual white end-cap.
(7) Cut the liner of topside of RMS-IW-WRC with the liner cutter.
(8) Cut the RMS-IW sediment from the topside core with sterilized spatula.
(9) Cap the topside of the RMS-IW-WRC with a clean blue end-cap. Transfer the WRC to laboratory for CT scanning.

Laboratory

(10) After CT scanning, cut RMS-IW-WRC with a liner cutter and sediment in liner with a sterilized spatula.
(11) Cap the bottom side of the RMS-WRC with a clean yellow end-cap, and store at 4°C.
(12) Print labels after registration of RMS-WRC in J-CORES, and paste them on blue end-cap of RMS-WRC, sterile centrifuge tubes and aluminum bag (or similar product).
(13) Cover the sample labels with transparent Teflon tape.
(14) Put the WRC into a styrene foam box with frozen cooling gel packs in clean bench. Remove the blue end-cap of the WRC.
(15) Insert three tip cut-off sterilized syringes in middle part of the WRC. Push each syringe into sediment so that it samples the entire length of the WRC (e.g., 10 cm in the case of 10 cm WRC; volume of syringe is not important.). Use syringe adapter when sediment in WRC is somewhat hard for inserting a syringe into it (see Fig. 6b). Take out the sediment-filled syringe from the WRC by gently rotating and pulling action.
(16) Transfer the sediment from the syringe into the sterile centrifuge tube, and return the syringe without piston back into the hole by gently rotating and pushing action to prevent the hole from collapsing.

(17) After taking RMS by all 3 syringes, cap the residual WRC with the blue end-cap, and seal the caps and liner with vinyl tapes.
(18) Put the centrifuge tubes with RMS and residual WRC into separate aluminum bags (or similar product), and seal them.
(19) Store the RMS and residual WRC into deep freezer (-80°C).

The syringe sampling takes 40 min to 1 hour.

Steps (1)-(10) of Fig. 5 precede this sequence.

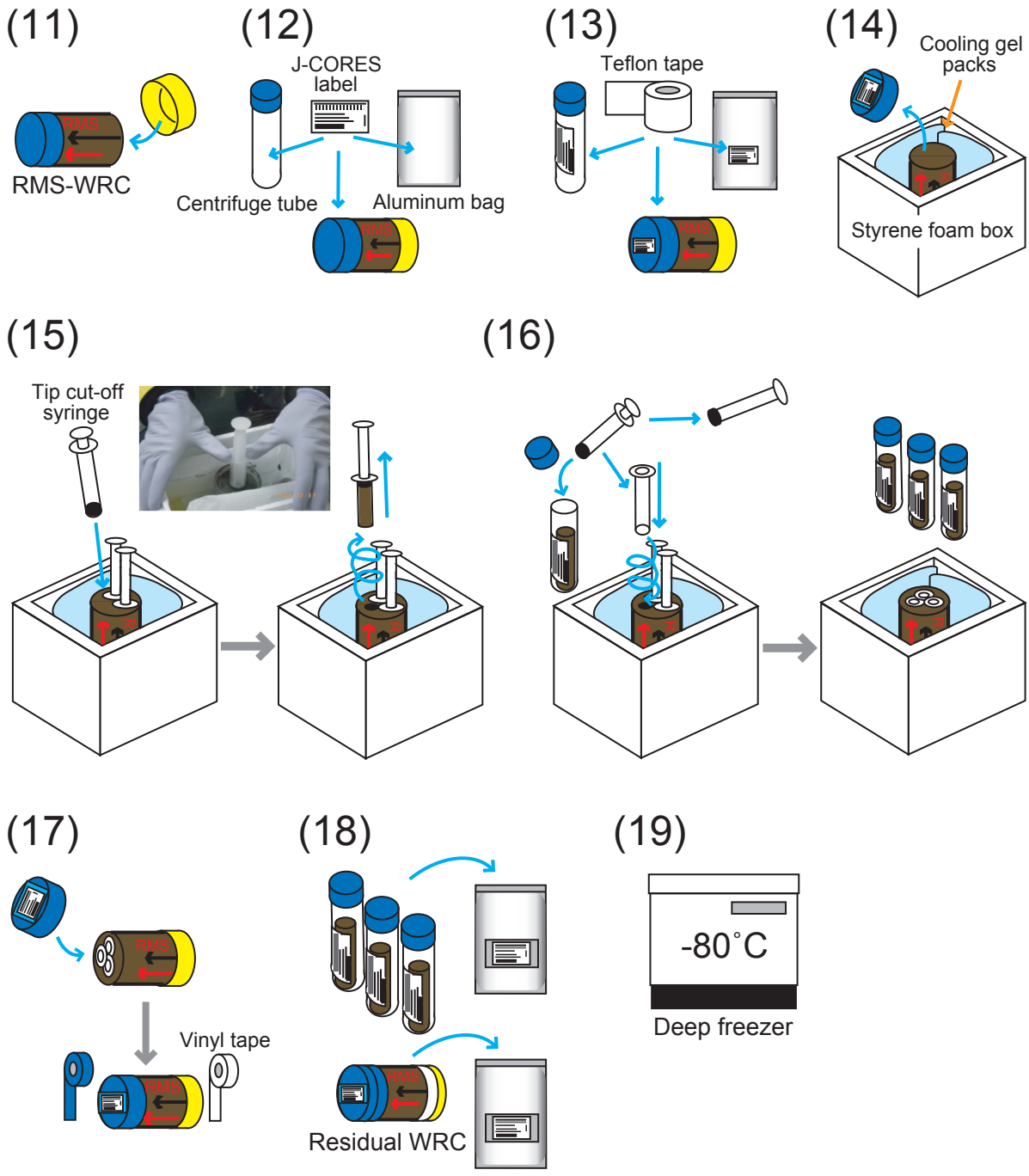

Fig. 7. Sequence of steps for taking RMS from a WRC by using sterilized syringes

Notes

- To minimize changes in quality of RMS, all handling should occur as quickly as possible.
- Safety goggles and lab coat must be put on for personal protection.
- Although steps (1) to (7) can be changed depending upon objectives of expedition, the RMS is finally sampled next to the IW-WRC.
- Depending on sequence of RMS and IW in a WRC (RMS-IW or IW-RMS), use clean end-cap of appropriate color only for the RMS-WRC (blue or yellow cap toward top and yellow or white cap toward bottom).
- Depending on shipboard freezer capacity, the frequency of RMS sampling may need to be reduced if whole-rounds or half-rounds are often collected. Final decision on sampling frequency for the RMS will be made by onboard SAC.
- In the case of larger diameter core (e.g., diameter is 10 cm), 30 cc tip cut-off sterilized syringe can be used for taking RMS from a WRC.
- In the case of sampling by syringe, if it deprives opportunities to obtain samples for onboard scientists, the residual WRC should not be frozen, and instead should be given to the curatorial staff for discrete sampling. Final decision on sampling quantity such as three 20 cc syringes or one 30 cc syringe for the RMS will be made by onboard SAC.
- Before returning the syringe without piston into the hole in a WRC, adjust the length of syringe by cutting luer lok end with a clean knife so that length of the syringe becomes nearly same as that of WRC. This will prevent sediment from collapsing inside the capped WRC. This adjustment in syringe length is not required, if WRC is 10 cm long, because syringe is originally ca. 10 cm long.

3. Shipping

RMS are shipped with dry ice in order to maintain their frozen condition. To maintain frozen condition surely, volume of dry ice should be more than twice * that of the RMS. Temperature logger may be included in each microbiological or biogeochemical shipping container to get the thermal history of the samples during shipment. Alternatively, dry ice remaining in the container at the destination can be used as an indicator of frozen condition of samples during the shipment.

* During the training cruise CK09-03, RMS-WRC were shipped with dry ice in order to maintain frozen condition (approximately -80˚C) of the samples from Shizuoka (unloading port) to KCC. Approximately 10 kg of chipped dry ice and twelve of 10 cm long RMS-WRC (approximately 5 kg) were put into a styrene foam box [dimension: 55×35×70 (cm) (Fig. 8a)] so that the WRC were surrounded by the dry ice. Next day, the samples were delivered to KCC with limited loss of dry ice (Fig. 8b).

(a)  (b) 

Fig. 8. Shipping of RMS with dry ice (a) Styrene foam boxes with RMS and dry ice, (b) Delivered RMS with limited loss of dry ice

3-1. Materials

(1) Container (Plastic cool box or styrene foam box)

The container should have ventilation so that carbon dioxide gas from the dry ice does not cause explosion of the package. Lid of the styrene foam box must be punctured (one hole of 1-2 mm diameter) to allow diffusion of the gas. Inner height of container should be more than three times that of the RMS-WRC in order to put dry ice in reasonable amount together with the RMS-WRC.

(2) Dry ice (chipped or block type)

Dry ice is lost quickly with time, so it must be made available as close to the shipping time as possible. It is preferable to use chipped dry ice for reducing the weight but increasing the volume of dry ice.

(3) Airway bill or similar document

3-2. Method

(1) Put dry ice (chipped or block type) and RMS into container so that the samples are surrounded by the dry ice (Fig. 9).

(2) Put temperature logger into the container (optional).

(3) Seal the container with the shipping tape, and paste airway bill for logistic company.

(4) Ship the container as quickly as possible.

(5) After receiving the container in repository, transfer RMS into deep freezer (-80˚C) quickly.

(6) Confirm the thermal history of container during shipment with temperature logger (optional) or by presence of remaining dry ice in the container.

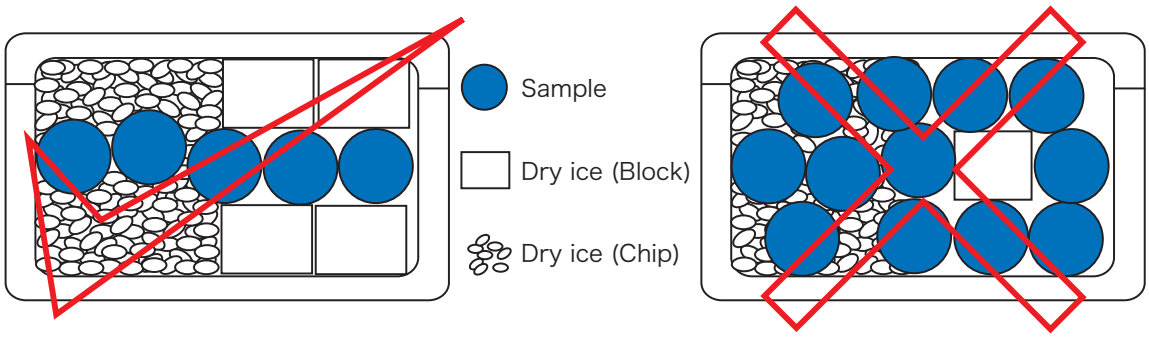

Fig. 9. Examples of good and bad packing for RMS shipping

Note

- Dry ice must be handled in a well-ventilated location because of the carbon dioxide fumes. All handling should be progressed as quickly as possible.
- Safety cold gloves must be used during handling of dry ice because it will burn human skin (cold burn).

4. Onshore curation

Storage of RMS under deep frozen condition is very important for preventing degradation of fragile bio-molecules such as DNA, RNA, enzymes, carbohydrates, and intact polar lipids caused by abiotic hydrolysis, enzymatic reaction, and possible contamination. Moreover, aseptic sub-sampling and distribution of the RMS without thawing are highly desired for quality assurance of the samples. KCC stores RMS in deep freezer (-80˚C) and/or liquid nitrogen (LN) cooled tanks (-160˚C) (Fig. 10 a, b). For sub-sampling and distribution to science community, RMS is cut with electric band saw system under HEPA-filter units (Masui *et al*.) (Fig. 10 c-f). It is possible to slice the RMS as thin as 0.5 cm slabs without thawing under clean condition, and it permits sharing the exact same horizon among multiple analyses. RMS curation data, relevant for microbiological research will be made available to the scientific community via the KCC website.

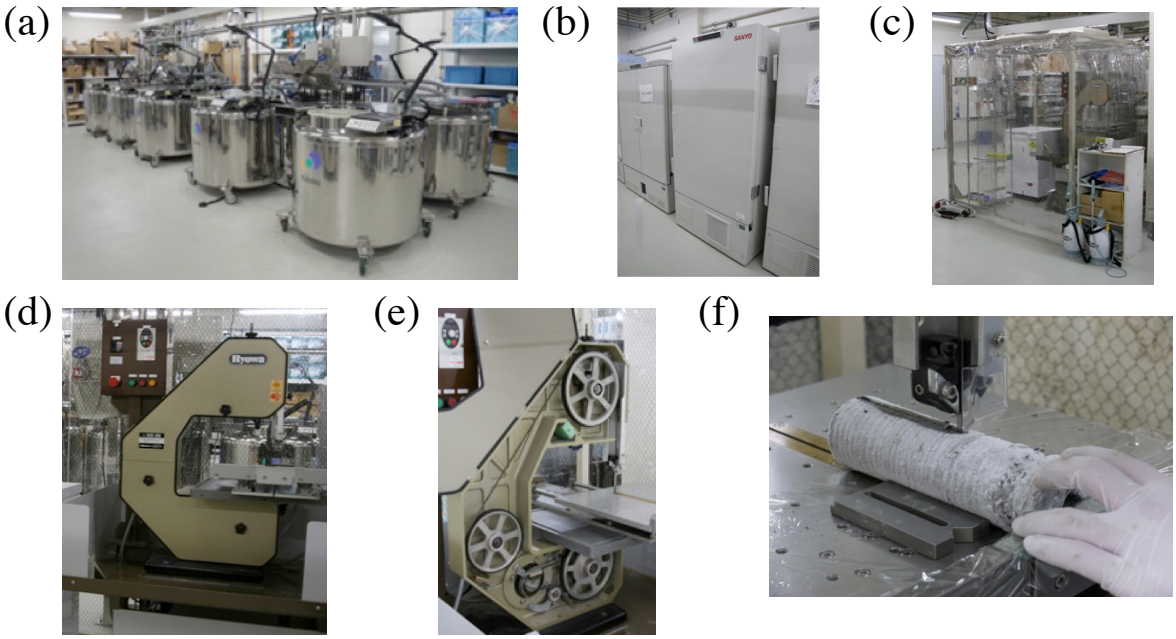

Fig. 10. Apparatuses for long-term storage and 'clean' processing of RMS in KCC (a) Liquid nitrogen cooled RMS storage tanks, (b) Deep freezers, (c) Overview of the band saw system, (d) Band saw machine in clean booth, (e) Open lid condition of the band saw machine, (f) RMS-WRC cut into half vertically

4-1. Materials

(1) Perfluoroalcoxyalkane (PFA) jar (e.g., Savillex or Sanplatec)

The PFA jar is used for storing RMS in LN cooled tank (-160˚C) (Fig. 11a). The PFA jars remain stable over a wide range of temperature from -196 to +200ºC, thus they can be autoclaved and stored in the LN cooled tanks. Roughen the outer surface of PFA jar with a sandpaper for securely pasting a sample label and preventing its detachement under ultra-low temperature condition. Autoclave the jars at 121ºC for 20 min. (the jar must not be tightly closed, otherwise it will be deformed by the vacuum effect.). After autoclaving, put the jars into oven (60˚C) for drying up over night. After drying, allow the jars to cool to room temperature.

(2) Polypropylene 8 row reservoir (e.g., Porvair sciences)

This reservoir is used for storing RMS bars in the deep freezer (-80˚C). It can be autoclaved and used for storing the bars in good order (Fig. 11b). Wrap a reservoir with aluminum foil, and autoclave it at 121ºC for 20 min. After autoclave, put the reservoir into oven (60˚C) to dry it up over night. After dry up, let the reservoir cool to room temperature.

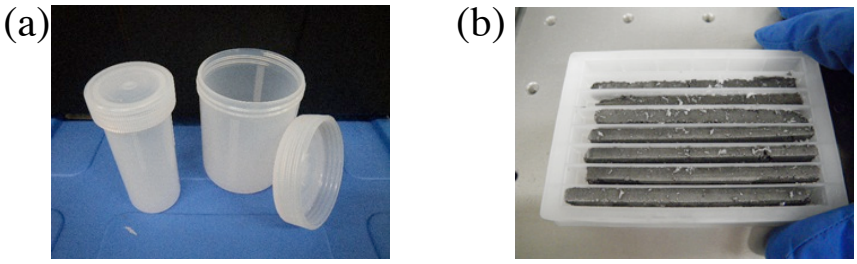

Fig. 11. Materials for storing RMS at very low temperature (a) Perfluoroalcoxyalkane (PFA) jars, (b) Polypropylene 8 row reservoir with bar shape RMS

4-2. Method

(1) Turn on two HEPA-filter units on the clean booth one hour before operating band saw system.
(2) Spray and wipe saw blade, three wheels and sample cutting stage with 70% ethanol.
(3) Fix the frozen RMS-WRC on the stage with fittings.
(4) Cut the sample into half vertically (Fig. 12a, b).
(5) Cut the halves into 10 mm thick slabs. Remove the contaminated outer edge (5 to 10 mm thick layer) from the slabs (Fig. 12c).
(6) Cut each slab into bars (5 mm x 10 mm in cross section) (Fig. 12d).
(7) Put all bars from one half into pre-labeled 8 row reservoirs in good order (Fig. 12e), and store in deep freezer (-80˚C). The bars should be put in the case in the same direction such as make the left side as top of core.
(8) Put all bars from the other half in a pre-labeled PFA jar, and store LN cooled tank (-160˚C). The bars should be put in PFA jar in the same direction such as make the top of the jar as the top of core.

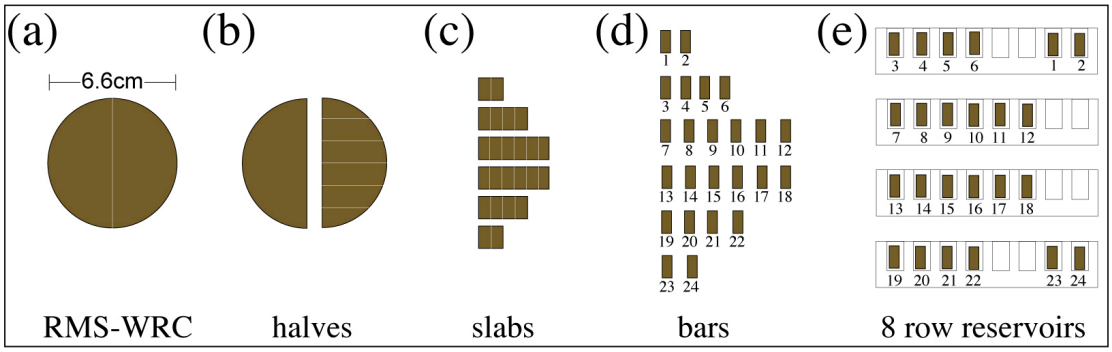

Fig.12. An example of RMS-WRC sub-sampling

(9) Clean the band saw parts, such as the saw blade, wheels and stage using a steam-heat cleaner (Fig. 13).
(10) Dry up saw's parts by wiping.
(11) Spray and wipe saw blade, three wheels and cutting stage with 70% ethanol prior to next use.
(12) Prepare curation data sheet for RMS. Format of RMS curation data sheet is shown in Table 1.

(a)  (b) 

Fig. 13. Cleaning of wheels (a) and saw blade (b) in the band saw machine

Table 1. An example of curation data sheet for RMS

Expedition	Site	Hole	Core	Type	Sec.	Interval (cm)		Volume	Depth	Contamination test		Storage temperature	Cell density	Remarks
						Top	Bottom	(cc)	(mbsf)	PFT(μg/g-sediment)	FB(Beads/g-sediment)		(total cell/g-sediment)	
322	C0011	B	9	R	2	31.0	41.0	300	414	NC	NC	-80°C	NC	WRC (already sampled by two 5 cc syringes in center)
322	C0011	B	14	R	4	36.5	46.5	300	464	NC	NC	-80°C	NC	WRC (already sampled by two 5 cc syringes in center)
3XX	C000X	A	1	H	2	30.0	40.0	350	2	NC	NC	-80°C	2.0×10^8	One 30 cc subcores, residual WRC
3XX	C000X	A	1	H	4	30.0	40.0	350	5	NC	NC	-80°C	1.8×10^8	One 30 cc subcores, residual WRC
3XX	C000X	A	2	H	3	40.0	50.0	350	10	NC	NC	-80°C	1.5×10^8	One 30 cc subcores, residual WRC
3XX	C000X	A	3	H	3	60.0	70.0	350	20	NC	NC	-80°C	8.0×10^7	One 30 cc subcores, residual WRC
3XX	C000X	A	5	H	3	85.0	95.0	350	40	NC	NC	-80°C	6.0×10^7	One 30 cc subcores, residual WRC
3XX	C000X	A	7	H	3	10.0	20.0	350	60	NC	NC	-80°C	3.0×10^7	One 30 cc subcores, residual WRC
3XX	C000X	A	9	H	3	65.0	75.0	350	80	NC	NC	-80°C	1.0×10^7	One 30 cc subcores, residual WRC
3XX	C000X	A	11	H	4	45.0	55.0	350	100	NC	NC	-80°C	9.0×10^6	One 30 cc subcores
3XX	C000X	A	18	H	4	90.0	100.0	350	200	NC	NC	-80°C	7.0×10^5	One 30 cc subcores
3XX	C000X	B	10	R	2	30.0	40.0	300	500	BD	0	-80°C/-160°C	NC	WRC (already removed scrappings from the outside)
3XX	C000X	B	20	R	2	20.0	30.0	300	600	0.05	100	-80°C/-160°C	NC	Ten 1 cc bars (After removing scrappings from the outside, WRC cut into bars)
3XX	C000X	B	30	R	1	80.0	90.0	300	700	0.08	200	-80°C/-160°C	NC	Ten 1 cc bars (After removing scrappings from the outside, WRC cut into bars)
3XX	C000X	B	40	R	2	10.0	20.0	300	800	BD	0	-80°C/-160°C	NC	WRC (already removed scrappings from the outside)
3XX	C000X	B	50	R	2	20.0	30.0	300	900	BD	0	-80°C/-160°C	NC	WRC (already removed scrappings from the outside)
3XX	C000X	B	60	R	1	120.0	125.0	150	1000	0.06	100	-80°C/-160°C	NC	Ten 1 cc bars (After removing scrappings from the outside, WRC cut into bars)

Note: PFT = perfluorocarbon tracer. FB = fluorescent beads. NC = not conducted. BD = below detection.
Cells in red frame show the RMS curation data relevant for microbiological analyses.

Notes

- In advance, J-CORES labels must be pasted on PFA jars and 8 row reservoir. The labels must be covered with transparent Teflon tape.
- Clean disposable gloves must be used during all handling procedure.
- Safety goggles and earplug must be put on for personal protection during cutting and cleaning operation.
- Cutting of RMS should be progressed as quickly as possible in order to avoid thawing.
- The WRC-half and slabs are stored temporarily in -50˚C freezer in the clean booth to avoid unintentional thawing. PFA jars and 8 row reservoirs should be pre-cooled in this freezer so that no unintentional warming of the bars takes place.

• In the case of syringe RMS, cut it into halves, and then cut each half into nearly equal volume bars. Store half of the bars in deep freezer (-80˚C) and the remaining bars in LN cooled tanks (-160˚C).

Optional: Chemical fixation procedure

Cell counts on fixed samples is an IODP standard measurement for microbiological QA/QC of samples. Therefore, it is preferable to collect a sub-sample from RMS-WRC for chemical fixation. Final decision on sampling for chemical fixation will be made by Implementing Organization (e.g., CDEX).

Materials

(1) Phosphate Buffer Saline (PBS buffer)(pH 7.2) (e.g., Ambion or Invitrogen)
Dilute commercial 10 x PBS pH 7.2 with distilled water, and autoclave or filter to sterilize.

(2) 4% performaldehyde solution (50 ml)
Preparation
(i) Heat to 60˚C 30 ml of distilled water in Duran glass bottle.
(ii) Add 2 g of paraformaldehyde with a stirring bar to the water.
(iii) Transfer the solution to fume hood, maintain at 60˚C on a hot plate and keep stirring (Be careful not to overheat the solution!).
(iv) Add 1.5 µl of 10 N NaOH to the solution.
(v) Add 15 ml of distilled water to the solution.
(vi) Remove from hot plate and add 5 ml of 10 x PBS (Confirm the pH with pH test paper).
(vii) Filter the mixture with a 0.2 µm filter.
(viii) Cool the filtrate to room temperature or to 4˚C.

(3) Tip cut-off of sterilized syringe (1 cc) (Fig.14)
Cut off the luer lok end of syringes with a heated knife. Wrap the tip cut-off syringe with aluminum foil, and autoclave at 121˚C for 20 min. After autoclave, put syringe into oven (60˚C) to dry it up for about 12 hours. After drying, let syringe cool to room temperature prior to use. This preparation can be done onshore, too.

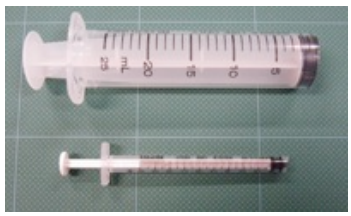

Fig. 14. One cc tip cut-off syringe (below) and 20 cc syringe (above)

(4) 13 cc round-bottom centrifuge tube with screw cap (e.g., Sarstedt)

(5) 100 % ethanol

(6) Centrifuge machine (Rotor for 15 cc tube, 3000 g)

(7) Micro pipet and sterilized tip (5 cc)

For adding reagents such as PBS, 4% performaldehyde solution and 100 % ethanol into centrifuge tube

Method

(1) Print a label after registration of RMS-WRC in J-CORES, and paste it on a 13 cc round-bottom centrifuge tube with screw cap.
(2) Cover the label with transparent Teflon tape.
(3) Add 4.5 ml of Phosphate Buffer Saline (PBS buffer) to the tube.
(4) Sub-sample sediment from middle part of RMS-WRC with a 1 cc tip cut-off sterilized syringe so that it samples entire length of the WRC.
(5) Add 1 cc of sample to the buffer tube. Mix vigorously.
(6) Add 4.5 ml of 4% performaldehyde solution in draft chamber. Mix immediately.
(7) Incubate at 4 ˚C for 12 hours.
(8) Centrifuge the tubes at 3,000 g for 10 minutes at 4°C.
(9) Store supernatant in waste container and add 9 ml of PBS buffer to the tube in draft chamber. Mix vigorously.
(10) Repeat steps (8) and (9) twice to remove performaldehyde.
(11) Centrifuge tubes at 3,000 g for 10 minutes at 4°C.
(12) Remove supernatant by decantation and add 4.5 ml of PBS buffer to the tube. Mix vigorously.
(13) Adjust to 10 ml with 100 % ethanol. Mix vigorously.
(14) Store these buffered samples at –20°C.

Time requirement: Steps (1) to (6) 20 min., steps (8) to (14) 40 min.

The buffered frozen samples can be brought to an onshore laboratory for further processing for cell counting by following the procedure described by Morono *et al*.

Notes

- As performaldehyde may cause sensitization of skin and eye, and may be harmful if inhaled and ingested, clean disposable gloves, safety goggle and mask are to be used during all handling procedure.
- As performaldehyde solution does not last long, prepare it at least once per week.
- Liquid wastes containing performaldehyde are separately stored in a Polypropylene bottle. They should not be flushed into chemical drain.

References

D'Hondt, S., Inagaki, F., Ferdelman, T., Jørgensen, B. B., Kato, K., Kemp, P., Sobecky, P., Sogin, M., and Takai, K. (2007) Exploring subseafloor life with the Integrated Ocean Drilling Program. *Sci*. *Drilling*, 5, 58-69.

Masui, N., Morono, Y., and Inagaki, F. (2009) Bio-archive core storage and subsampling procedure for subseafloor molecular biological research. *Sci*. *Drilling*, 8, 35-37.

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Appendix I

STP *Recommendation* 0908-09: STP Recommendations for Routine Microbiological Sampling on IODP Expeditions. The STP recommends the following approach to assist routine microbiological sampling on IODP expeditions (including those for which microbiology is the primary scientific objective) so that samples are adequately and consistently preserved for future microbiological analysis. The panel further recommends that a microbiologist sail as part of the science party with each expedition in order to oversee the proper sampling, preservation, and integration of these materials into specific expedition objectives.

To assist the IOs in implementing the collection of routine microbiological samples (RMS) on IODP expeditions, the STP recommends the following:

- **Recommendation 1**. On IODP expeditions, a minimum of one RMS should be collected per IW sample acquired from each site that is sampled[1]*. The RMS should be collected in close proximity (within a few centimeters) to the IW sample. For reference, see STP Consensus Statement 0807-12. The RMS sampling interval should be included in the scientific prospectus for the expedition. Consideration of the scientific objectives of each cruise is encouraged in order to determine how microbiological analysis of RMS can help to accomplish IODP objectives.

The candidate sampling procedure, based upon the Subseafloor Life Task Force (SLTF) recommendation (STP consensus statement 0807-12), is as follows:

1) Bulk sediment should be collected in triplicate (e.g., with 30 cc sterile cut-off syringes; thereafter frozen) and also scrapings from the outside of the core to be placed into a centrifuge tube (for use as an indigenous tracer) where possible, or
2) When #1 is not possible (e.g., because of limited staff or indurated nature of the core) then successive whole- or half-round cores should be preserved as deep frozen samples. Depending on shipboard freezer capacity, the frequency of RMS sampling may need to be reduced if whole-rounds or half-rounds are often collected.
3) If the core quality is judged to be inferior and an IW sample will not be collected, or if the RMS sample will interfere with the objectives of the cruise (e.g., if the sample would be taken near a critical interval), then there is no need to collect an RMS.

For preservation of RMS, the contents of the sterile syringes should be extruded into separate centrifuge tubes followed by storage at -80 C. The whole- or half-rounds should be packaged in alumibags (or similar product) and frozen at -80 C. Freezing of the RMS should occur as soon as possible after subcoring or collection (D'Hondt et al. 2007).

- **Recommendation 2.** An on-board microbiologist or a technician trained in aseptic sampling techniques should implement these recommendations and the RMS sampling itself. The technician will be responsible for maintaining the equipment required for routine sampling, maintaining a clean microbiology working space, and for maintaining an adequate inventory of needed sampling supplies on board.
- **Recommendation 3**. Regular (e.g., annual) review of the methods adopted for RMS should be performed by the SLTF. As part of the review, if new methods come to light in relation to the collection and preservation of samples, these should be considered for adoption.
- **Recommendation 4.** Questions related to implementation of any of these recommendations should be directed to the SLTF for guidance.
- **Recommendation 5.** Clarification of the final archival disposition of the RMS and the shipping procedure at the conclusion of the expedition needs to be addressed by the IODP Curators.

[1] * Based on three sites each from ODP Leg 202 & IODP Expedition 307, tying this RMS scheme to the collection of IW samples will entail taking ~20 to 40 samples (range = 19-59, mean = 35) for each category at the average site focused on paleoceanography or sedimentary processes. This will entail removal of sample plugs from the center of, or whole rounds totaling, ~3.5 m of core at each site. Assuming three full-length holes per site (range = 3-5 for 202 and 307) and very conservative 70% coring recovery, sediment will be removed from 3.5 of 504 m of core (~0.7%) per site. Much of the sediment in these 3.5 m will remain after sampling. (These residual sediment samples may not be of optimal use.) A 24 ft^3 -80C freezer will hold ~430 packages of triplicate 30cc samples. This is almost four times the capacity required for IODP Leg 307 and about 1.33 times the capacity required for ODP Leg 202.

Appendix II

Table T1. Definitions of terms used in microbiology sampling.

Term	Definition
Aseptic	A clinical definition referring to freedom from pathogens. Clinical conditions are not applicable to ODP operations.
Sterile	The most stringent category used during Leg 201. Sterile indicates the absence of contaminating prokaryotic cells that are capable of metabolism and growth and their nucleic acids.
Clean	Defined as giving careful consideration and effort to avoid cross-contamination. May involve ethanol washing, flaming instruments, etc., and may approach sterile conditions.
Anaerobic	A property of organisms indicating the ability to live without oxygen. Also a property of a method ("anaerobic cultivation") or of laboratory cultivation equipment ("anaerobic chambers").
Anoxic	Property of an oxygen-free environment such as used in a glove bag or N_2-flushed sampling bucket.

Shipboard Scientific Party
Chapter 5, explanatory notes, P 81.

Proceedings of the Ocean Drilling Program, Initial Reports Volume 201. Published in 2003.

Appendix III

Coring contamination tests by using fluorescent microspheres during CK09-03 Expedition 904

Kochi JAMSTEC　Noriaki Masui

Onboard processing

Contamination tests were conducted for two coring systems (HPCS and EPCS) by attaching plastic bags containing 50 ml of fluorescent microspheres suspension (approximately 1×10^{10} microspheres/mL) inside a core catcher (Fig. 1). Fluoresbrite 0.5 μm microspheres (Polysciences Inc.) were used as particulate tracer of contamination.

In the HPCS coring, the test was done for Core C9010D-3H. Ruptured empty plastic bag was found in the core liner of Core 3H (Fig. 2). In the EPCS coring, the experiment was done for Core C9010D-13X. Ruptured empty bag was found at Female Quick Release at the top of the lower inner barrel assembly. Ruptured bags prove that microspheres were successfully released in core barrel during coring activities. Samples for contamination tests were taken as 10 cm whole round core (WRC) per section from the 2 above mentioned cores. Each WRC was sealed in a Ziploc bag and stored in -20□C freezer.

Fig.1. Plastic bags containing suspension of fluorescent microspheres, attached in core catcher

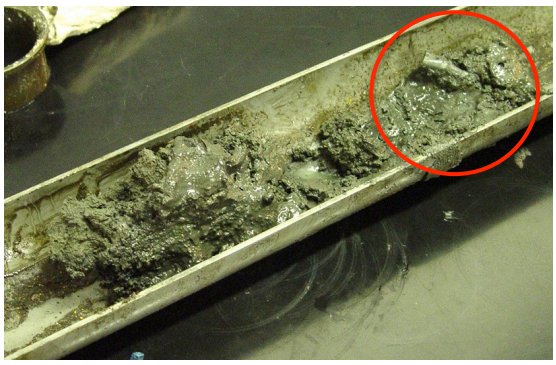

Fig.2. Ruptured plastic bag (in red circle) in a recovered core

Sample collection and counting procedure

Sample collection procedure is shown in Fig. 3. A round slice sample (10 mm thick) was sub-sampled from WRC by using a band-saw system (Fig. 4). Three portions of sliced sample (center, intermediate and outer edge) were sampled by using tip-cut syringe. Each sediment sample was then mixed with an equal volume of saturated sodium chloride solution. The solution was centrifuged (5 min, 2800 x g), and the supernatant was filtered onto black polycarbonate filters (0.2-μm pore size). Fluorescent microspheres on the filter were counted under ultraviolet (UV) light, and data were reported as numbers of microspheres per gram of sediment.

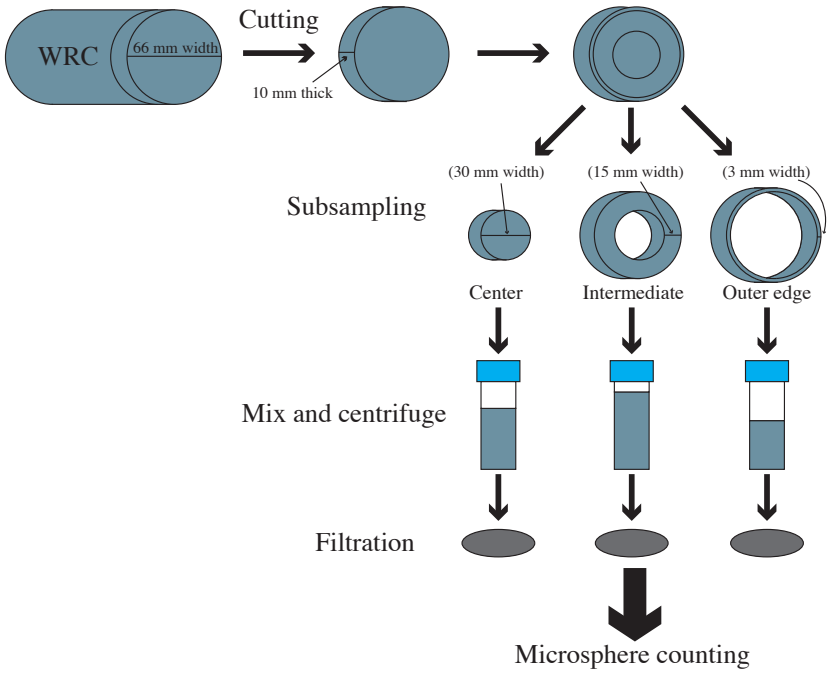

Fig. 3. Procedure of sample collection and counting

Fig. 4. Cutting core sample by using a band-saw system

Fig. 5. WRC C9010D-3H-4 covered with sandy particles

Contamination assessment for samples obtained by HPCS and EPCS coring system

Results of contamination assessment are shown in Table 1. Microspheres were detected from the outer edge of all samples. In contrast, no microsphere was detected from center and intermediate zone of samples except for WRC C9010D-3H-4, which was mainly composed of silt and covered with sandy particles (Fig. 5). Most of the WRC, in which the microspheres from center and intermediate zones were not detected, were composed of mainly clay or silt. Therefore, microspheres detected in center and intermediate zones of the C9010D-3H-4 seem to have diffused in from the sandy core surface. When band-saw system is used to subsample cored material, contamination is likely to spread among subsamples. However, in the case of WRC subsampled by using the band-saw, microsphere was not detected from center and intermediate zones. This suggests that band-saw system will not cause cross-contamination during subsampling of a frozen sample. Sample processing by band-saw system in the Kochi Core Center shows minimum risk of cross-contamination (Fig. 4).

There is no influence of difference of coring system (HPCS and EPCS) on contamination of core sample, especially in the intermediate and central zones. Number of microspheres from outer edge of C9010D-13X-1 and 13X-2 (8.5×10^2 and 9.0×10^2 (microspheres/g)) are lower than that of WRC C9010D-3H ($2.5\sim7.5\times10^3$ (microspheres/g)). The lower number of microspheres in outer edge of C9010D-13X can be attributed to washing and scraping of the core during drilling and recovery. Application of chemical tracer such as perfluorocarbon may demonstrate a different level of contamination of samples in contrast to that observed here by using microspheres because chemical tracers are much smaller in size, and therefore are easy to diffuse into sediment, than the microspheres.

Table 1. Fluoresent microspheres in C9010D cores

	Number of microspheres (microspheres/g)		
Core section	Outer edge	Intermediate	Center
HPCS			
C9010D-3H-1	3.5×10^3	ND	ND
C9010D-3H-2	2.5×10^3	ND	ND
C9010D-3H-4*	7.5×10^3	1.0×10^2	1.0×10^2
C9010D-3H-8	4.5×10^3	ND	ND
EPCS			
C9010D-13X-1	8.5×10^2	ND	ND
C9010D-13X-2	9.0×10^2	ND	ND

ND = None Detected

Delivery of microspheres was confirmed by detecting microspheres in scrapings and water samples from inner wall of core liner.

*Surface of WRC C9010D-3H-4 was covered with sandy particles.