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# SOP

## Standard Operating Procedure

| Author: | Acronym: | Date last modified: |
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| **Geert Huys** | **ASIARESIST-SAMPLE** | **13-01-2003** |

Title:

# SAMPLING AND SAMPLE PROCESSING PROCEDURES FOR THE ISOLATION OF AQUACULTURE-ASSOCIATED BACTERIA

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# SOP ASIARESIST-SAMPLE

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| **Reviewed & approved by:** all ASIARESIST partners |

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# SAMPLING AND SAMPLE PROCESSING PROCEDURES FOR THE ISOLATION OF AQUACULTURE-ASSOCIATED BACTERIA

## A. PURPOSE & PRINCIPLE

The purpose of this SOP is to describe a series of procedures for collecting and processing microbial samples from aquaculture environments. Separate procedures are provided for samples of water, sediment, and cultured species. The following parameters are critical to harmonize sampling campaigns in different geographical regions:

- Accurate identification of the sampling point
- Choice of sampling equipment and storage conditions
- Sample processing protocol

## B. METHOD DESCRIPTION

### I. General considerations

- Each participating SEA lab should identify three farms representing the diversity of the economically important species cultured in the respective countries. At each farm, one pond should be identified and selected for the entire sampling campaign. At the selected pond, three easy accessible spots at some distance from the border or the edge of the culture system will be located. Preferentially, these three spots should be (i) the influent point, (ii) the middle of the pond, and (iii) the effluent point. In total, three samples will be collected per sampling occasion per sample type (water, sediment, organism). In the lab, these three samples will be pooled together to obtain one representative sample per sampling occasion per sample type.
- For each farm pond, site description data should be collected: country, water type, name of cultured species (English, local, and latin name), fishfarm site (incl. map and GPS-derived coordinates), and

farm record (including all available data on water management type, antibiotic regime, number and/or density of species cultured, etc.). *These site description data should only be submitted once to the project database.*

- For each sampling occasion, sample description data should be collected including date, depth at which water and sediment samples were taken, and physico-chemical parameters of the water (including temperature, pH, salinity, total ammonia nitrogen ($N\text{-}NH_4$) and total suspension solid (TSS)). The latter parameters are then to be averaged over the three sampling spots in a given farm pond. The sediment sample should be described according to the soil type (clay, sandy, loam, mixed). The sample description data should be submitted to the database upon each sampling occasion.
- At each site, sampling should be performed four times in a period of 8-10 months. The sampling intervals will strongly depend on the culture period of the farmed species but should in any case be at least two weeks. For shrimp, the sampling should start after one month into the culture period and subsequent sampling should be performed at three weekly intervals to guarantee that four samples are obtained over the four month culture period.
- During the first and/or the second sampling campaign, samples will also be plated on non-selective ISA medium (i.e. without CHL). The isolates that are collected in this way will serve as potential recipient strains for horizonal transfer experiments later in the project. Only for the first two sampling campaigns, ISA and ISA+CHL plates need to be prepared.

**II. Technical preparations (DAY BEFORE SAMPLING)**

- Prepare sterile glass bottles by autoclaving or make sure that pre-sterilized plastic tubes or containers are available with a sampling volume of at least 50 mL. Make sure that the sampling containers are labelled beforehand to avoid mixing of sample identity between farms, farm ponds, and sampling spots.

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- Depending on the site of isolation and the type of sediment, sediment samples will be taken using the PVC pipe system (or a Van Veen Grab). The PVC pipe can be sterilized by rinsing with a disinfectant (e.g. 5-10 ppm chlorine) or 70% alcohol after which the tube is rinsed three times with sterile water. Alternatively, the VV grab can be sterilized by soaking in 5-10 ppm chlorine. Sterilized sampling devices can be transported to the site of sampling wrapped in (sterile) aluminium foil. If appropriate, individual partners may consider sterilizing metal or glass parts of their sampling devices by autoclaving for 15 min wrapped in alumunium foil.
- Prepare the required quantity of tubes containing 4.5 or 9 mL of sterile saline solution to set up dilution series of the samples. Ideally, the amount of NaCl included in the saline solution should be adapted according to the natural salinity of the sampling site. In general, this will be in the range 0.85% (freshwater) to 3% (brackish water). *To be able to prepare the tubes beforehand, the average salinity of the site should be known prior to starting the sampling campaign.*
- Prepare the desired volume of Iso-Sensitest Agar medium (Oxoid) with or without a breakpoint concentration of 35 ppm chloramphenicol (CHL) (see §.III).
- Check the temperature of the plate incubator set at 28°C with a thermometer.

**III. Definition of chloramphenicol (CHL) breakpoint concentration**

- Prior to the actual sampling campaign, the distribution of CHL resistance frequencies needs to be determined for each sampling site in a range of increasing CHL concentrations (0-256 ppm). In this way, the selective or breakpoint concentration of CHL to be included in the isolation medium can be defined on the basis of empirical determinations. Breakpoint determinations should only be performed once for each site, and need to be carried out for both the water and the sediment phase.
- For breakpoint determinations, water and sediment samples are collected and processed essentially as described in §.IV and §.V, respectively. The major difference is that sample dilutions are inoculated in triplicate on a series of ISA plates supplemented with a two-fold increasing

concentration of CHL. In this way, heterotrophic bacterial numbers will be assessed on ISA medium containing 0, 2, 4, 8, 16, 32, 64, 128, and 256 ppm CHL. For rapid screening of sites, the concentration range can be narrowed to the 32-256 ppm range. These data should be set out in a graph (e.g. Excel format) to obtain the frequency distribution of resistant numbers along the concentration range. The modal shape of this distribution should indicate the optimum CHL breakpoint concentration, i.e. the concentration at which acquired CHL resistance can be selected for.

- Following a number of initial breakpoint determinations in SEA aquaculture environments and considering the recommended breakpoint concentrations of NCCLS, the breakpoint concentration to select for high-level CHL resistance was decided to be set at 35 ppm.
- IMPORTANT NOTE 1: it has been noticed that some sampling sites do not generate high-level CHL resistant populations in all three sample types (i.e. water, sediment, and organism samples) but only from in one or two of these sample types. In any case, the total number of colonies expected from a given sampling site (n=12, i.e. 4 isolates for three sample types) should be respected. In practice, this means that an additional four or eight colonies need to be selected from the 'positive' sample type (i.e. the sample generating high-level CHL resistant colonies on ISA+CHL plates) if the other one or two sample types remain 'negative' (i.e. no colonies on ISA+CHL plates), respectively. This may have some practical implications for the strain designations, in which colony numbers can vary from 1-12 within the same sample type of a given sample (see §.IV).
- IMPORTANT NOTE 2: In some aquaculture samples, yeasts and/or may be present in comparable numbers as the predominating bacteria. As many yeasts are intrinsically resistant to chloramphenicol, it is possible that colonies appearing on ISA+CHL medium are actually yeasts instead of bacteria. For this reason, it is advisable to incorporate 200 ppm cycloheximide to the primary isolation medium.

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**IV. Water sampling and sample processing**

- Before leaving to the site, check the necessary equipment once again: labeled sterile sampling containers, cooled storage box, plastic gloves, hand disinfectant, thermometer, salinity meter, etc.
- At the sampling site, measure and record the site description parameters (once) and sample description parameters (see §.I). At the predefined sampling spots, collect a water sample (minimum volume 50 mL) by unscrewing the sterile sampling container approximately 50 cm under the water surface. Try to collect a full bottle or tube so that a minimal volume of air is present.
- Transport the sample under refrigeration conditions and process in the lab within 3-5 hours.
- In the lab, remove the chilled ISA and ISA+CHL plates from the refrigerator and allow to adapt to ambient (bench) temperature. Open the sample containers in a sterile environment and pour out a small volume in order to obtain an air fraction in the containers facilitating homogenization of the samples. Close the sample containers again, homogenize the water samples by slowly inverting them 10 times and immediately transfer 30 mL of each individual sample (3 samples per farm) to an empty pre-sterilized bottle to obtain a pooled sample with a final volume of 90 mL. After thorough homogenization by manual inversion (10 times), transfer 1 mL of the pooled sample to a tube containing 9 mL sterile saline. Continue with this dilution to prepare a series of 10-fold dilutions up to 10E-6. Make sure that all dilutions are well homogenized. Store the dilutions @ 4°C and process within 2 hours.
- Using a micropipet, spot 100 uL of each dilution in triplicate on ISA plates and on ISA+CHL plates (thus 6 plates per sample dilution). Spread plate the suspension using a sterile glass triangle rod. Allow the plates to dry for max. 10 minutes. Longer drying times allow pre-incubation of the cells which should be avoided. Label the plates using the following designation codes:

| | |
|---|---|
| **C-S-P-p-T-D-px** | (CHL added; ISA+CHL plate) |
| or | |

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| **N-S-P-p-T-D-px** | (NO CHL added; ISA plate) |
|---|---|

with S = farm site, use two capital letters to describe the site

P = pond designation (A, B, C, etc.)

p = sampling period (1-8)

T = sample type (W for water, S for sediment, O for organism)

D = dilution (10Ex)

p = plate no (x = 1-3 for triplicate platings)

- Incubate the plates upside down @ 28°C for 24h.
- Following incubation, check both the ISA plates (code NC-) and the ISA+CHL plates (code C-) to determine the dilution that generated between 20 and 200 colonies. Plated dilutions yielding less than 20 colonies are not statistically significant, whereas counts exceeding 200 may be difficult to accurately assess. For instance, consider the following plating result of a given dilution series: 10E-1 (>1000 CFU), 10E-2 (>1000 CFU), 10E-3 (326 CFU), 10E-4 (45 CFU), 10E-5 (7 CFU), 10E-6 (0 CFU). In the above example, the 'countable' dilution would be 10E-4 (45 CFU).
- Determine total counts on the triplicate 'countable' ISA (code NC-) and ISA+CHL (code C-) plates and calculate the mean number and standard deviation. These data need to be submitted to the project database. For both ISA and ISA+CHL plates, two out of three plates are selected for colony isolation. Colonies will be picked in a semi-random way rather than basing the selection on colony morphology (which is more subjective). From the middle point of each plate, draw two perpendicular lines (one vertical and one horizontal) across the plate so that a cross-shape (+) is obtained. For selection, pick the colony that is located nearest to the four points where this cross reaches the outer edge of the plate, yielding a total of 4 colonies per plate or up to 8-12 colonies per sample (= from 2-3 plates) <u>if not all sample types generate high-level CHL resistance</u> (see §.III).

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Streak out the colonies on ISA if originating from ISA and on ISA+CHL if originating from ISA+CHL. In both cases, this step is crucial to verify whether the colony represents a single strain type. For the colonies picked from ISA+CHL medium, this step is also an additional confirmation that the selected colonies are in fact resistant to CHL and incubate at 28°C. Label each plate with the following code:

| | |
|---|---|
| **C-G-S-P-p-T-x** | (colony from ISA+CHL) |
| or | |
| **N-G-S-P-p-T-x** | (colony from ISA) |

with G = geographical origin, i.e. country (M for Malaysia, T for Thailand, V for Vietnam)

S = sampling site, use two capital letters to describe the site

P = pond designation (A, B, C, etc.)

p = sampling period (1-8)

T = sample type (W for water, S for sediment, O for organism)

x = colony number (1-12).

Number the colonies from the first plate 1-4 and from the second plate 5-8

- If colonies 1-4 (first plate) grow upon recovery on ISA and ISA+CHL medium, respectively, retain only these and do not store colonies 5-8 (second plate). However, one or more colonies numbered 5-8 can be included in case one or more of the first colonies (numbered 1-4) did not grow. In this way, a total number of four isolates per sample is guaranteed. Note: one sample type may finally yield 4 to 8-12 isolates if the other one or two sample types do not yield colonies on ISA+CHL plates.

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- Verify that the streak cultures grown on ISA or ISA+CHL are pure before storing them in cryovials. **An additional step is required for the isolates from ISA (code N-) plates**. Check growth of these NC isolates on ISA+CHL medium as described above. Only those N-isolates that do not grow on ISA+CHL medium are considered as potential recipient strains and should be stored. The other N-isolates may be discarded and will not enter the collection**. At least 10 CHL-'susceptible' (defined as: no growth on ISA+CHL) isolates per farm should be collected in this way including water, sediment, and organism isolates. Once this number is reached, there is no further need to plate samples on ISA.**
- Upon storage, submit the strain numbers to the project database and send the strains to Partner 2 (RUG) to be included in the central project collection. In all subsequent subculturing steps, it is advised to add 35 ppm to the ISA medium to avoid loss of CHL resistance determinants or reversion to the CHL susceptible phenotype.
- Using the above described procedure, each SEA lab will deliver 144 CHL resistant isolates:

**3 farms x 3 pooled samples (W, S, O) x 4 sample periods x 4 colonies/sample = 144**

In total, the three countries will deliver 432 CHL resistant isolates to the project.

- Using the above described procedure, each SEA lab will deliver 30 CHL 'susceptible' isolates (10 per farm).

**3 farms x 10 colonies from W, S, and O = 30**

In total, the three countries will deliver 90 CHL 'susceptible' isolates.

**V. Sediment sampling and sample processing**

- Before leaving for the site, check the necessary equipment once again. In addition to the standard sampling equipment described in §.IV, a pre-sterilized PVC pipe (5 cm diameter) or Van Veen Grab

is required. AAHRI will provide all participating partners with such a PVC pipe for sediment sampling where appropriate.

- At the sampling site, describe the sediment sample according to soil type (see §.I). At the predefined sampling spots, collect a sediment sample using a pre-sterilized PVC pipe or a Van Veen Grab. Transfer the upper 5-10 cm sediment (app. 100 g) to a sterile container.
- Transport the sample under refrigeration conditions and process in the lab within 3-5 hours.
- In the lab, remove the chilled ISA and ISA+CHL plates from the refrigerator and allow to adapt to ambient (bench) temperature. Open the sample containers in a sterile environment, transfer 1 g sediment sample to a tube containing 9 mL sterile saline, and homogenize the 10E-1 dilution (vortex for 1 min). After vortexing, allow the suspension to settle for 30 sec and immediately take off 3 mL of the supernatant from the middle of the tube and add to a sterile empty tube (10 mL capacity). This is performed for all three sediment samples per farm to obtain a pooled sample of 10E-1 dilutions with a final volume of 9 mL. Continue with this pooled dilution to make a series of 10-fold dilutions up to 10E-6. For this purpose, vortex the pooled 10E-1 sediment sample for 1 min, allow to settle for 30 sec, and take a 1 mL subsample of the supernatans in the middle of the watercolumn to prepare the subsequent dilution. Make sure that all dilutions are well homogenized: for dilutions higher than 10E-2, vortex for 30 sec between subsequent dilutions, allow to settle for 15 sec, and take off 1 mL from the middle of the watercolumn. Store the dilutions @ 4°C and process within 2 hours.
- Continue following the procedure described in §.IV for water sampling. Counts will be expressed as CFU/g wet weight sediment instead of dry weight sediment. Because sediment samples are pooled as dilutions, it is not possible to determine the dry weights of all individual sediment samples.

**VI. Cultured species sampling and sample processing**

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***FISH SPECIMENS***

- Before leaving for the site, check the necessary equipment once again: in addition to the standard sampling equipment described in §.IV, also include plastic bags, ice, and sterile tubes for species sampling.
- At each sampling spot, an appropriate number (depending on the relative size) of living cultured fish will be collected at the predefined sampling spots using a cast-net or hook and line. Each individual fish will be transferred to a plastic bag and covered with ice.
- Transport to the lab and process within 3-5 hours.
- In the lab, remove the chilled ISA and ISA+CHL plates from the refrigerator and allow to adapt to ambient (bench) temperature.
- Disinfect the external abdominal surface of the fish by swabbing with 70% ethanol and make an incision over the peritoneal cavity. Remove the intestine from the pyloric valve to the anus and then aseptically extrude the contents into a sterile tube. Regardless of the number of fish collected, a total weight of 0.5 g intestinal sample per sampling spot should be collected to which 4.5 mL sterile saline is added. Mix the 10E-1 dilution well (vortex for 1 min). After vortexing, allow the suspension to settle for 30 sec and immediately (avoiding the intestinal contents!) take off 3 mL of the supernatant from the middle of the tube and add to a sterile empty tube (10 mL capacity). This is performed for all three fish samples per farm to obtain a pooled sample of 10E-1 dilutions with a final volume of 9 mL. Continue with this pooled dilution to make a series of 10-fold dilutions up to 10E-6. For this purpose, vortex the pooled 10E-1 fish sample for 1 min, allow to settle for 30 sec, and take a 1 mL subsample of the supernatant in the middle of the watercolumn to prepare the subsequent dilution. Make sure that all dilutions are well homogenized: for dilutions higher than 10E-2, vortex for 30 sec between subsequent dilutions, allow to settle for 15 sec, and take off 1 mL of the supernatant from the middle of the tube. Store the dilutions @ 4°C and process within 2 hours.
- Continue following the same procedure as described in §.IV for water sampling. Counts are expressed as CFU/g wet weight intestinal sample. In this regard, it is important to determine the exact weight of the sample prior to preparing the dilution series.

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***SHRIMP SPECIMENS***

- Before leaving for the site, check the necessary equipment once again: see §.Fish Specimens sampling
- At each sampling spot, collect an appropriate number (at least three, depending on the organism's age) of living cultured adult shrimps at the predefined sampling spot using a cast-net or feed net. Each individual shrimp is transferred to a plastic bag and covered with ice. For sampling of postlarvae or juvenile shrimps, 10 organisms should be pooled and considered as one 'organism sample'.
- Transport to the lab and process within 3-5 hours.
- In the lab, remove the chilled ISA and ISA+CHL plates from the refrigerator and allow to adapt to ambient (bench) temperature.
- Disinfect the external surface of the shrimp by immersing with 70% ethanol and make an incision over the head. Remove the intestine from hepatopancreas to anus and then aseptically extrude the content into a sterile tube. Regardless of the number of shrimps collected, a total weight of 0.5 g intestinal sample per sampling spot should be collected and transferred to a tube containing 4.5 mL sterile saline. The 10E-1 dilution should be well mixed (vortex for 1 min). After vortexing, allow to settle for 30 sec and immediately (avoiding the intestinal contents!) take off 3 mL of the supernatant from the middle of the tube and add to a sterile empty tube (10 mL capacity). This is performed for all three shrimp samples per farm to obtain a pooled sample of 10E-1 dilutions with a final volume of 9 mL. Continue with this pooled dilution to make a series of 10-fold dilutions up to 10E-6. For this purpose, vortex the pooled 10E-1 shrimp sample for 1 min, allow to settle for 30 sec, and take a 1 mL subsample of the supernatant from the middle of the tube to prepare the subsequent dilution. Make sure that all dilutions are well homogenized: for dilutions higher than 10E-2, vortex for 30 sec between subsequent dilutions, allow to settle for 15 sec, and take off 1 mL supernatans from the middle of the watercolumn. Store the dilutions @ 4°C and process within 2 hours.
- Continue following the procedure described in §.IV for water sampling. Counts are expressed as CFU/g wet weight intestinal sample. In this regard, it is important to determine the exact weight of the sample prior to preparing the dilution series.

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## C. MATERIALS

- **Iso-Sensitest Agar (ISA)**
  # CM 471 (Oxoid, Basingstoke, UK)

  *ISA medium should be prepared with distilled or freshly deionised water. To avoid growth of predominant yeasts and fungi, it is advisable to add cycloheximide (a eukaryotic inhibitor, available from most popular agent manufacturers like Oxoid or Merck) in an end-concentration of 200 ppm to the medium. The cycloheximide component can be added to the medium before sterilization, BUT PLEASE BE CAREFUL IN HANDLING THIS REAGENT AS IT IS HIGHLY TOXIC TO HUMANS!!!*
  *Make sure that each bottle contains a magnetic stirrer. A known volume (approx. 500 mL) of sterilized ISA medium should be cooled down to 50-55°C. Poured plates may be stored for up to 3 days in air-tight plastic bags at 2-8°C. Immediately prior to inoculation media should be moist but free of droplets, either on the agar surface or on the petri dish lids. If necessary plates may be dried by incubation at 30-37°C or in a laminar flow cabinet for a maximum of 30 min. A representative sample of each batch of plates should be examined for sterility by incubation at 28°C for 72 h.*

- **Iso-Sensitest Agar supplemented with chloramphenicol (ISA+CHL)**
  # CM 471 (Oxoid, Basingstoke, UK)

  *ISA medium should be prepared with distilled or freshly deionised water. Make sure that each bottle contains a magnetic stirrer. A known volume (app. 500 mL) of sterilized ISA medium should be cooled down to 50-55°C. Subsequently, the chloramphenicol stock solution (100x concentrated) is added and mixed well by magnetic stirring. Try to avoid the creation of air bubbles when homogenizing the selective medium.*
  *Poured plates may be stored for up to 3 days in air-tight plastic bags at 2-8°C. Immediately prior to inoculation media should be moist but free of droplets, either on the agar surface or on the petri dish lids. If necessary plates may be dried by incubation at 30-37°C or in a laminar flow cabinet for a maximum of 30 min. A representative sample of each batch of plates should be examined for sterility by incubation at 28°C for 72 h.*

- **Chloramphenicol (CHL) stock solution**

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Preferably, all participating labs should use the same brand of chloramphenicol. In the current project, all partners have agreed to purchase CHL from Oxoid in the form of Selective Supplement vials containing 50 mg CHL each (# SR0078, Oxoid).

*The CHL stock solution needs to prepared with ethanol according to the manufacturer's instructions*
(http://www.oxoid.com/uk/index.asp?mpage=iproductdetail&pre=SR0078&l=EN&x=).
*Consequently, it is not necessary to filter-sterilize the solution as the chance that bacteria can survive in ethanol is negligible. The stock solution should be stored in the refrigerator until addition to the ISA medium.*

*For selective isolation of CHL-resistant bacteria, CHL is added to the isolation medium in an empirically defined breakpoint concentration. Prepare the CHL stock solution in a concentration that is 100 times more concentrated than the breakpoint concentration of 35 ppm to keep stock solution volumes as small as possible. In this way, a selective medium solution can be obtained by adding 5 mL 100x stock to a bottle of 500 mL ISA medium (i.e. 1/100 dilution).*

- **Saline solution**
  milli Q (deionized) water or double-distilled water containing 0.85-3% NaCl depending on the salinity of the site.