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10	Abstract (100 words)	MoES has been implementing a national programme on Coastal Ocean Monitoring and Prediction System (COMAPS) since 1990. The collection of reliable data requires time tested sampling and analytical protocols, the expert committee constituted a group of participating institutes to prepare a draft on sampling and analytical protocols for adoption by the participating institutes. The content of this manuscript will be useful in the monitoring and assessment of health of coastal waters of India.
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- **Project proposal** = **PP**
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सत्यमेव जयते

भारत सरकार
Government of India
पृथ्वी विज्ञान मंत्रालय
Ministry of Earth Sciences

एकीकृत तटीय एवं समुद्री क्षेत्र प्रबंधन (इकमाम) परियोजना निदेशालय
Integrated Coastal and Marine Area Management (ICMAM) Project Directorate
(पृथ्वी विज्ञान मंत्रालय की अनुसंधान तथा विकास ईकाई एवं सम्बद्ध कार्यालय)
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PREFACE

Coastal waters are highly productive and support rich fishery resources. However, pollution through both domestic and industrial wastes, either as point or non-point sources, affects the coastal waters. The problem is further aggravated due to lack of proper sewer systems and discharge of untreated/semi-treated sewage and effluents from many coastal towns/cities into coastal environment. Monitoring the health of coastal seas is highly essential to assess the status of environmental quality, to detect radical changes and to alert government and public institutions, of their implications. In view of this, Ministry of Earth Sciences (MoES) formerly Department of Ocean Development (DOD) has been implementing a national programme on Coastal Ocean Monitoring and Prediction System (COMAPS) along Indian coastal areas since 1990. The programme has been implemented with the participation of national R&D laboratories, institutes and universities.

On 22 September 2010, an Expert Committee meeting was held at Ministry of Earth Sciences, New Delhi, under the Chairmanship of Dr. S.Krishnaswami, INSA Senior Scientist, Physical Research Laboratory, Ahmedabad. Dr. Shailesh Nayak, Secretary, MoES also participated in the meeting. The Committee discussed on the Draft Report on the Assessment of Assessment of coastal waters of India prepared by ICMAM Project Directorate, based on long term data collected under COMAPS programme. Secretary mentioned about core parameters to be measured and sampling and analytical protocols. Recognizing that the collection of reliable data requires time tested sampling and analytical protocols, the committee constituted a group of Participating Institutes already engaged in COMAPS programme to put together detailed sampling and analytical protocols for various parameters. The group was requested to prepare a draft on sampling and analytical protocols for adoption by the participating institutes. I thank the group members and their team and the expert for the advice, in bringing out this manuscript. I am sure that the contents of this manuscript will be useful in the monitoring and assessment of health of coastal waters of India.


(B. R. Subramanian)

15 November 2012

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**COASTAL WATER QUALITY MEASUREMENTS PROTOCOL
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COASTAL WATER QUALITY MEASUREMENTS FOR COMAPS PROGRAMME
MINISTRY OF EARTH SCIENCES (MOES),
GOVERNMENT OF INDIA

S. No.	CONTENTS	Page No.
1.	Preface	1
2.	Parameters to be analysed	2
	2.1 Water Quality	2
	2.2 Sediment	2
	2.3 Dissolved alkali and alkaline metals	2
	2.4 Biological parameters	2
3.	Basic concepts in sampling and analysis	3
	3.1 Shipboard sampling	3
	3.2 Sampling and sub sampling	4
	3.3 Filtration	5
	3.4 Collection of sediment samples - (Grabs and Corer)	5
	3.5 Storage and Preservation of samples	6
4.	Quality Evaluation, Standardization and Intercalibration	7
	4.1 Standardization	7
5.	Analysis of Physico-chemical parameters	8
	5.1 Temperature	8
6.	pH (Hydrogen ion concentration)	9
7.	Alkalinity	9
8.	Chlorinity / Salinity	12
9.	Dissolved Oxygen (DO)	14
10.	Biochemical Oxygen Demand (BOD)	16
11.	Determination of Nutrients in seawater	18
	11.1 Origin of Phosphorus in seawater	18
	11.2 Inorganic Phosphate (PO ₄ -P)	18
	11.3 Origin of various forms of nitrogen in sea water	21
	11.3.1 Determination of Ammonia – Nitrogen	22
	11.4 Determination of Nitrate and Nitrite	26
	11.5 Nitrite – Nitrogen (NO ₂ -N)	28
	11.6 Nitrate – Nitrogen (NO ₃ -N)	30
	11.7 Determination of Total Nitrogen (TN) and Total Phosphorus (TP)	34
	11.8 Genesis of silicate in sea water	39
	11.8.1. Determination of Reactive Silicate	39
12.	Determination of Particulate Organic Carbon (POC) and Particulate Nitrogen (PN)	42

S. No.	CONTENTS		Page No.
13.	Determination of Dissolved metals and ions in Seawater		45
14.	Determination of Petroleum Hydrocarbons in Seawater		45
15.	Determination of Total Organic Carbon		47
16.	Determination of Heavy metals in Sediment and Biological Samples		47
17.	Biological Parameters		49
	17.1	Determination of Chlorophyll	49
		17.1.1 Spectrophotometric Method	49
		17.1.2 Outline of the Method	49
		17.1.3 Apparatus and Equipment Required	49
		17.1.4 Sampling Procedure and Storage	49
		17.1.5 Procedure	50
		17.1.6 Calculation and Expression of Results	50
		17.1.7 References	52
	17.2	Determination of Phaeophytin Pigments	53
		17.2.1 Spectrophotometric Method	53
		17.2.2 Outline of the Method	53
		17.2.3 Apparatus and Equipment Required	53
		17.2.4 Sampling Procedure and Storage	53
		17.2.5 Procedure	54
		17.2.6 Calculation and Expression of Results	55
		17.2.7 References	56
	17.3	Determination of Chlorophyll and Phaeophytin Pigments	56
		17.3.1 Fluorometric Determination	56
		17.3.2 Principle of Analysis	56
		17.3.3 Apparatus	56
		17.3.4 Sample Collection and Storage	57
		17.3.5 Procedure	57
		17.3.6 Standardization	57
		17.3.7 Calculation and Expression of Results	58
		17.3.8 References	59
18.	Marine Phytoplankton sampling Methods and Analysis		59
	Collection, Preservation, Identification and Enumeration		59
	18.1	Introduction	59
	18.2	Sample Collection	60
		18.2.1 Qualitative and Quantitative Sampling	60
		18.2.2 Surface Sampling	60
		18.2.3 Sub-surface and Deep Water Sampling	60

S. No.	CONTENTS		PagNo.
	18.2.4	Collection using Nets	60
	18.2.5	Standard Plankton Net	61
	18.2.6	Calculation of Volume of Water Filtered	62
	18.2.7	Concentration of Sample	62
18.3	Sample Preservation		62
	18.3.1	Preservation with Lugol's Iodine Solution	63
	18.3.2	Preservation with Formaldehyde	63
18.4	Storing of Phytoplankton Sample		64
18.5	Labelling of Sample		64
18.6	Sample Processing		64
	18.6.1	Sedimentation	64
	18.6.2	Preparation of Chamber	64
	18.6.3	Filling up of Utermöhl's Chambers	65
18.7	Counting		65
	18.7.1	Sedgewick-Rafter Counting Slide	65
18.8	Sample Identification		66
	18.8.1	Microscopy	66
18.9	References		67
18.10	Suggested Readings		68
19.	Marine Zooplankton Sampling Methods and Analysis		69
	19.1	Introduction	69
	19.2	Methods of Collection	70
	19.2.1	Sampling by Bottles	70
	19.2.2	Sampling by Pumps	71
	19.2.3	Sampling by Nets	71
	19.2.4	Various Types of Plankton Sampler	73
	19.2.5	Flow meter Calculation	75
	19.3	Method of Zooplankton Preservation	76
	19.4	Method of Zooplankton Analysis	77
	19.4.1	Measurement of Biomass	77
	19.4.2	Enumeration of Zooplankton Population	78
	19.4.2.1	Sub-sample for Zooplankton Analysis	79
	19.4.2.2	Enumeration of Zooplankton Under Microscope	80
	19.4.2.3	Expression of Zooplankton Population Result	81
	19.5	References	82
20.	Marine Benthos Sampling Methods and Analysis		83
	20.1	Introduction	83

S. No.	CONTENTS		Page No.
	20.2	Sampling Methods	84
	20.2.1	Macrobenthos - Sub-tidal Sampling	84
	20.2.2	Macrobenthos - Inter-tidal Sampling	86
	20.3	Laboratory Analysis	94
	20.4	Meiobenthos	95
	20.5	Biomass	95
	20.6	Calculation	95
		20.6.1. Macrobenthos	95
		20.6.2. Meiobenthos	96
	20.7	References	97
21.	Monitoring of Microbiological Parameters in Marine Environments (Water And Sediments)		99
	21.1	Introduction	99
	21.2	Enumeration of Marine Bacteria	101
	21.3	Total Viable Counts (TVC) (retrievable count (RC)/ colony forming unit (CFU)	102
	21.3.1	Plate method (Culture method)	102
	21.3.2	Membrane filter method	106
	21.4	Enumeration of Health Indicator Bacteria	106
	21.5	Isolation and Purification of Bacterial Strains	107
	21.6	Preparation of Culture for Sending	109
	21.7	Colour Chart for Identification of Hicrome Media Recommended	109
	21.8	References	109

1. PREFACE

The coastal water quality measurements, erstwhile in operation under the name COMAPS for the last two decades have been reviewed annually and the data generated by different participating institutions are deposited with INCOIS of MoES. The data generated are made available through web site of the Ministry for Public dissemination as well as through user agencies such as Pollution Control Boards, Fisheries, Environment, etc. The recent review on the assessment of the assessment by the committee recommended the following areas for routine sampling. As per the recommendations a sampling protocol has been prepared for implementation from January 2011 onwards.

S. No.	Monitoring Institute	Monitoring coast	Locations
1.	Regional Centre, National Inst of Oceanography (NIO) Mumbai	Gujarat	Vadinar, Veraval, Hazira including Tapi river mouth
		Maharashtra	Mumbai (Thane creek), Worli outfall, Malvan
2.	National Institute of Oceanography, Goa	Goa	Sea off Mandovi including Mandovi river mouth
3.	Centre for Earth Science Studies, Trivandrum	Karnataka	Mangalore
		Kerala	Kochi including mouth of Kochi backwaters
		Lakshadweep	Kavaratti
4	Centre for Advanced Studies in Marine Biology, Parangipettai	Tamilnadu	Ennore coast including mouth of Ennore creek, Tuticorin and Puducherry
5.	Regional Centre, NIO, Visakhapatnam	Andhra Pradesh	Kakinada , Visakhapatnam
6.	Institute for Minerals and Materials Technology, Bhubaneswar	Orissa	Paradip
7	Dept. of Marine Sciences, University of Kolkata	West Bengal	Sandheads, Hooghly mouth
8.	Andaman & Nicobar Centre for Ocean Science and Technology, NIOT, Port Blair	Andaman	Port Blair

Sampling Locations and Periodicity: The samples to be collected at the mouths of estuaries/creeks/lagoons, shore (0.5 km), nearshore (2 km) and offshore (5 km), both during low and high tides, for water analysis. Collection of surface waters from all the three zones (shore, nearshore and offshore) and bottom waters from near and offshore transects is mandatory. Sampling should be carried out for 48 hrs (3 hourly) at creek/river/backwater mouths and during low and high tides at 2 km on either side of the shore station (along the shore). The 48hrs sampling is mandatory and both surface and bottom samples need to be analysed. The samples should be collected for 4 seasons which include three dry seasons and one wet season with an interval of 3 months for each sampling. It is essential that sample should be collected separately for low and high tides. Seasonal sampling should indicate month, date and tidal condition and need to be maintained for comparison of data during past year.

2. PARAMETERS TO BE ANALYSED

The recommended core parameters are:

2.1. Water Quality: Atmospheric and water Temperature, Dissolved oxygen, salinity, pH, (upto 4 decimals mandatory), alkalinity, Total dissolved nutrients (Nitrogen and Phosphorus), ammonia, nitrite, nitrate, total nitrogen, inorganic phosphate, total phosphorus, silicate and Petroleum Hydrocarbons.

2.2. Sediment: Total Organic carbon, texture, iron, manganese, zinc, aluminium, copper, lead, cadmium, chromium, nickel and mercury. One bivalve for estimation of trace metals (aluminium, copper, lead, cadmium, zinc, chromium, nickel and mercury).

2.3. Dissolved alkali and alkaline metals: Sodium, potassium, calcium, magnesium, chloride, sulphate, iron, manganese, aluminum, copper, lead, cadmium, zinc, chromium and nickel.

It is recommended that all institutions follow the same sampling and analytical protocols and make use of modern techniques for analysis. To minimize errors arising from sample handling and analysis preference should be for automated methods over manual approaches. Towards this Auto Analysers, CTD with necessary sensors to be provided to above institutions as required.

2.4. Biological Parameters: Total viable counts, *E.coli*, *Streptococcus faecalis* (in water and sediment), Chlorophyll *a,b,c*, Phaeophytin, phytoplankton species (numbers and diversity), Zooplankton species (numbers, biomass and diversity) and macro and meiofauna

species in beach and sea sediments (macrofauna species: numbers, biomass, diversity; meiofauna species: numbers, diversity).

3. BASIC CONCEPTS IN SAMPLING AND ANALYSIS

The committee recommends that at a few select locations a few additional properties of water be monitored.

	Monitoring Institute	Monitoring coast	Locations
1.	NIO, Goa	Goa	ΣCO_2 , POC, copper, cadmium, lead and mercury at all transects off Mandovi
2.	Regional Centre, NIO, Visakhapatnam	Andhra Pradesh	ΣCO_2 and POC at all transects off Vizag
3	Dept. of Marine Sciences, University of Kolkata	West Bengal	ΣCO_2 and POC at all transects off Sandheads
4.	ICMAM Project Directorate, Chennai	Tamilnadu	Copper, cadmium, lead and mercury at all transects off Ennore
5	Centre for Advanced Study in Marine Biology, Parangipettai	At all COMAPS locations specified, with samples obtained from institutions	Dissolved and sediment Polyaromatic Hydrocarbons

In order to accomplish the above task, a working manual has been prepared for circulation among the participating institutions. This manual covers different aspects such as Sampling and preprocessing both for water quality and biological parameters. Detailed methods of analysis of all the parameters in terms of chemical and biological are provided.

3.1. Shipboard sampling

For accurate measurements of the *in situ* properties and composition of seawater proper sampling is of utmost importance. It is essential to ensure that the sampling is contamination free and all the samples are appropriately sub-sampled and preserved to avoid/minimize changes in the water composition during storage. The program involves measurements of hydrographic and chemical properties of sea water in coastal and near coastal waters, therefore much of the sampling will rely on boats and the measurements in

shore based laboratories. Adequate samples have to be collected for duplicate and repeat analysis.

3.2. Sampling and sub sampling

Prior to sampling, the sampler and sampling bottles should be acid washed with 1N HCl in the laboratory. Sample bottles should be rinsed 2 times with the environmental sample and then water sample should be collected. The desired samples should be collected from the place away from where the sampler and sample bottles were washed. Care should also be taken to avoid the sewage flush out from the boats/ships at the time of sampling. For plankton sampling, the nets should be thoroughly washed and air dried to avoid clogging of meshes in net and flow meter should be calibrated at regular intervals.

Water samples are to be collected using Teflon coated Niskin samplers (avoid any form of metal contact with samples). It should be noted that these samplers have to be used even for surface water samples, not a bucket as often done by many researchers. Once the sample is collected with a help of a 5litre Niskin sampler, all water samples must be collected from the knob of the Niskin sampler using silicon rubber tubes. The prioritized individual subsampling steps should be followed as given below:

(1) for dissolved gases, alkalinity and pH, (2) for nutrients and physical parameters, (3) trace metals, (4) for biological Chlorophylls and (5) for bacteria.

Collection of samples for measurement of DO, other gases like CO₂, pH and alkalinity must avoid atmospheric contamination during sampling. For dissolved oxygen, the samples need to be fixed by employing Winkler's reagent on board vessel itself. Temperature and pH are to be measured immediately after collection.

Nutrient measurements should be done as soon as possible, within a few hours of collection. Till such time, samples should be stored in an ice box for transportation. If environmental water is turbid, then filtration is required before analysis.

Trace metal samples should be collected in acid-washed and precleaned high density polyethylene (HDPE) bottles. Separate good quality glass bottles should be used for mercury sample. Disposable, clean gloves need to be used while sampling and handling samples for trace metals and mercury analysis. All samples should be kept in a cool condition away from light to avoid evaporation. All samples (trace metals & Hg) bottles should be stored in metal free plastic bags till analysis, so as to avoid contamination.

Immediate filtration is required for Chlorophylls. Sampling for bacteria should be carried out soon after Chlorophyll sampling. It is suggested to filter a minimum of 1Litre for Chlorophyll.

Sediment samples should be stored in metal free plastic bags for trace metals and in aluminium foils for organic constituents. These samples are also need to be stored in ice boxes for transportation and put to dry at low temperatures (about 60 degrees C) in petridishes.

Surface and bottom water samples are to be collected separately for the near shore and offshore. The processing protocol described above should be meticulously followed for individual samples.

3.3. Filtration

The question, which has been often posed, is about filtration of samples to remove flora, fauna and other suspended material. For coastal monitoring, filtration is necessary. Filtration is a serious source of contamination. Adequate rinsing of the filter and the Buchner flask should be done as given below:

Type of sample	Acid wash	Filter paper
Nitrogen compounds sample	1N HCl	0.45 μ M; dia: 47mm washed using MilliQ water
Dissolved metals sample	1N HNO ₃	0.45 μ M dia: 47mm washed using MilliQ water
Biological sample	Not required	Combusted at 300 °C for 2 hrs GF/F filter paper

The filtration can be done only for analyzing the dissolved nutrients and not for total nitrogen and phosphorous. Sufficient care should be taken during filtration for collection of dissolved alkali and alkaline metal samples. No filtration is required for sensitive parameters like DO, pH, alkalinity and dissolved gases. A check should also be made to see whether the filter used contribute nutrients. Where facilities are available, centrifugation should be preferred over filtration.

3.4. Collection of Sediment Samples (Grabs and Corer)

Van Veen grab with a sampling area of 0.1 m² can be employed as a standard sediment sampler, since it is (i) an efficient sampler for the range of soft sediments

encountered in the near shore area, (ii) reliable and simple to operate and (iii) widely applied, which allows data comparison with other marine areas. Van Veen grab should be made of stainless steel or Teflon coated so as enable for sediment collection for metal analysis without contamination. Grab should be equipped with hinged inspection ports. The biting depth of grabs can vary with sediment conditions. Weights coated with Teflon can be added to adjust according to the sediment conditions.

3.5. Storage and Preservation of Samples

It should be understood that the concentration of nutrients is bound to change with time, due to the biological activity of the microorganisms present in the seawater. Therefore, they should be analysed immediately. Where immediate analysis is not possible, methods recommended include freezing and poisoning with sulphuric acid, chloroform or mercuric chloride. A quick note for Sample collection and Preservation is given below.

Sl. No	Samples types	Actions	Preservation method
1	Nutrients (Nitrate, Nitrite, Phosphate, Silicate)	Filter immediately with 0.45 μ M filter paper, if water is more turbid.	Don't add mercuric chloride. Keep the sample in chill condition (i.e refrigerator or ice) till the samples arrive up to laboratory.
2	TP and TN	No filtration required. Collect water with cleaned plastic bottles (250 ml capacity)	Keep the sample in chill condition (i.e refrigerator or ice) till the samples arrive up to laboratory.
3	DO	Collect the bubble free water and fix with winkler A & B	Not required
4	pH and alkalinity	Collection the water with sufficient avoiding the atmospheric contaminants and measured immediately. <ul style="list-style-type: none"> • pH can be measured by pH electrode with pH meter standardised and calibrated with 4.00, 7.00 and 10.00 prior to the sample measurement. • Measure the alkalinity 	Not required
5	Sediment	Collect the samples with stainless or Teflon coated grab and collect the sediment with a scoop (not in hand) and kept in metal free plastic bag with proper labelling.	Keep the sample in chill condition (i.e refrigerator or ice) till the samples arrive up to laboratory.
6	Dissolved metals and ions	More chances for contaminations. Collect the water with sufficient avoiding the atmospheric as well as metal	Keep the sample in chill condition (i.e refrigerator or ice) till the samples arrive up

		contaminants and filter immediately and kept in a HDPE bottles.	to laboratory.
7	Chlorophylls	On board, collect immediately and filter with GF/F paper. Otherwise, add 1 ml saturated MgCl ₂ per litre of sample and keep sample in chill condition.	The GF/F paper should be preserved with liquid nitrogen till transported to laboratory.

4. QUALITY EVALUATION, STANDARDIZATION AND INTERCALIBRATION

All analysts have to participate in national and international intercalibrations programs. Data quality to be tested by carrying out analysis of reference standards, simultaneously with the samples. Reference standards should be procured from NRC, Canada. NRC-Canada: Seawater: MOOS-1 (*Seawater Certified Reference Materials for Nutrients*), Trace metals: NASS-5 (*Seawater Reference Materials for Trace Metals*).

4.1. Standardization

It is better to realize that, in the case of nutrients, the calibration slope may change depending on salinity. This is especially true in determining ammonia. Therefore, the standardization should be undertaken in nutrients matrix, similar to the samples analyzed.

This matrix (synthetic sea water) can be prepared by ‘spiking’ low nutrients seawater (LNSW) with standard solutions of respective nutrient salts. The spiking should be done, just before standardization, to prevent any reaction with microorganisms that may still be present in the seawater. LNSW can be conveniently collected after the main plankton bloom, when all the nutrients have been used up. This seawater in a polyethylene carboy is kept in sunlight for a week, to deplete any nutrients that may still be present. After about four weeks, the supernatant can be siphoned of and stored in polyethylene containers.

There are various sources of contamination especially while analyzing for nutrients, which are normally present at trace levels. Distilled/demineralised water should be checked for the nutrients. The atmosphere of a laboratory is a highly potential source of contamination especially of ammonia. Similar is the contribution by the chemist who performs the analysis, without proper awareness.

The chemicals used for the determination of nutrients should be of the highest purity. Consider, for example, the bias from the use of impure reagents on the determination of phosphates. Suppose the contribution from the reagents is equivalent of 1

$\mu\text{g P/ml}$ *ie.* $33 \mu\text{mol/l}$ of phosphate. If one analyses a seawater sample containing $1 \mu\text{mol/l}$ of phosphate, the value obtained would be reported as $34 \mu\text{mol/l}$ which is seriously in error. Even if one corrects for the blank, the value obtained would not be reliable.

The standardization should preferably be done at concentrations, near to that of the samples, to be analyzed. Also it is a good practice to take three aliquots of the same sample and assess the precision or repeatability of the measurement. The international standards are available for nutrients in seawater to check the accuracy of analytical data, to get the true value.

One good practice can be noting down the make/grade/impurities of the chemical used or paste a copy of the label on the bottle and stick it in the laboratory work book. This helps in sorting out any complication/confusion that may arise later while checking /using the data.

5. ANALYSIS OF PHYSICO-CHEMICAL PARAMETERS

All the samples have to be analysed in duplicate positively.

5.1. TEMPERATURE

Aim: To determine temperature of seawater.

Outline of the method

A calibrated thermometer is allowed to stand in seawater sample and the reading is recorded.

Equipments

Certified thermometer: $0\text{-}50^{\circ}\text{C}$ with 0.1° accuracy

Thermometer: $0\text{-}50^{\circ}\text{C}$ having mercury thread, calibrated with the certified thermometer.

Procedure

Dip the thermometer in the seawater sub-sample drawn for temperature measurements. This sub-sample to be drawn immediately after retrieving the samples onboard. Record the temperature after 2 min.

Results

6. pH (HYDROGEN ION CONCENTRATION)

Aim: To determine pH of seawater.

Outline of the method

The electrodes of a pH meter duly calibrated at desired pH range is introduced in seawater sample and pH is recorded.

Equipment

Portable pH meter/pH meter with glass calomel electrode: Accuracy 0.1.

Standards

Buffer solution of pH 7.0 and 9.4/10.0

Procedure

1. Calibration: Rinse the electrode with deionised water (DW) and calibrate at pH 7.0 and 9.4/10.0.
2. Sample analysis: Introduce the electrode in seawater sample after rinsing with DW.
3. Allow the reading to stabilize for 1min. Read the pH of seawater. Rinse electrode with DW after each use.

Note: In case of slow response of pH meter, wash the electrode with HCl followed by DW till Re calibrate with pH buffer, rinse and use for measuring seawater pH.

Results

7. TOTAL ALKALINITY

The natural water alkalinity is due to three constituents OH, CO₃ and HCO₃. The total content of substances in water that cause an increased concentration of hydroxide ions either upon dissociation or as a result of hydrolysis is called alkalinity of water. Total alkalinity of water is determined by the amount of acid H₂SO₄ neutralised together with a phenolphthalein and methyl orange, which gives a pink colour in the presence of OH and normal carbonate. Mixed indicator consisting of methyl orange and bromocresol green in which the colour change is from blue green to orange, when HCO₃ is titrated with acid. Phenolphthalein gives a pink colour only with OH and CO₃. End point will be from pink to colorless, at a pH of 8.3. The mixed indicator indicates the HCO₃ titration at a pH of 4.3. In

general CO_3 and HCO_3 may exist together so also are OH and CO_3 . But OH and HCO_3 cannot co-exist. Therefore, if there is any phenolphthalein alkalinity, it may be due to OH or CO_3 or both. Similarly if there is methyl orange alkalinity it may be due to HCO_3 or CO_3 and HCO_3 . Total alkalinity of water is not only due to the presence of bicarbonate, carbonate, hydroxide ions, but also to other ions that react with acid, including humates. The presence of salts of some weak organic acids (humates) also leads alkalinity to natural waters.

Reagents

1. 0.02N H_2SO_4 : Dilute 6 ml of con. H_2SO_4 (35N) with distilled water under cooling and make up to one liter with more water in a volumetric flask. This stock gives 0.2N H_2SO_4 . Dilute 100ml of this 0.2N H_2SO_4 with 1000ml DW to get a 0.02N H_2SO_4 in a volumetric flask. Standardise this acid against a standard solution of sodium carbonate.
2. Sodium carbonate solution (0.02N): Dissolve 0.108 g of sodium carbonate Na_2CO_3 in distilled water and make up to 100ml.

Find out the exact normality of H_2SO_4 (N) from the relation.

$$N_1 V_1 = N_2 V_2$$

N_1 is the normality of Sodium carbonate solution (0.02N)

V_1 is the Volume of Sodium carbonate solution (0.02N) = 10ml

V_2 = Volume of H_2SO_4 used in Standardization

$$\text{Normality of } \text{H}_2\text{SO}_4 \text{ } N_2 = \frac{\text{Normality of } \text{Na}_2\text{CO}_3 * 10}{\text{Volume of } \text{H}_2\text{SO}_4 \text{ used in Standardization}}$$

3. Phenolphthalein indicator: Dissolve 0.25g of Phenolphthalein powder in 50ml of 50% alcohol.
4. Methyl orange indicator-bromocresol green: Dissolve 0.01g methyl orange and 0.05 g of bromocresol green in 100ml of water. Colour change at end point (pH4.3) is more sharper than that shown by methyl orange alone.

Procedure

100ml of filtered sample is taken in a conical flask and add few drops of alcoholic phenolphthalein indicator. If a pink colour develops, titrate the solution with 0.02N H₂SO₄ until it becomes colorless. This is the phenolphthalein alkalinity (PA). Note the titre value. If the sample was colorless after the addition of phenolphthalein indicator, added few drops of aqueous methyl orange indicator and titrate against the acid till an end point (blue green to orange). This is the total alkalinity (TA). The nature of alkalinity was then predicated from the titration as follows:-

1. If the titration to the phenolphthalein end point was zero, alkalinity was regarded as due to bicarbonate alone.
2. When there was no further titration to the methyl orange end point after the phenolphthalein end point, the alkalinity was only due to hydroxides.
3. When the phenolphthalein end point titration was half the total titration, only carbonate alkalinity was expected to be present.
4. When the phenolphthalein end point titration was greater than half the total titration, the alkalinity is due to both carbonate and hydroxides.
5. When the phenolphthalein end point was less than half the total titration, the alkalinity was due to carbonate and bicarbonates.

Calculation of alkalinity

Volume of water sample taken = 100ml

Volume of 0.02N H₂SO₄ used for phenolphthalein end point = Xml

Volume of 0.02N H₂SO₄ used for methyl orange end point = Yml

Volume of 0.02N H₂SO₄ required for neutralize the carbonate alone = 2Xml

Volume of 0.02N H₂SO₄ required for neutralize the bicarbonate alone = (Y – 2X)

Normality of H₂SO₄ (0.02N)

$$\text{CO}_3^{2-} \text{ (mg L}^{-1}\text{)} = \frac{(2X) * \text{Normality of H}_2\text{SO}_4 * 30}{100} \times 1000$$

$$\text{HCO}_3^- \text{ (mg L}^{-1}\text{)} = \frac{(Y - 2X) * \text{Normality of H}_2\text{SO}_4 * 61}{100} \times 1000$$

8. CHLORINITY/SALINITY

Aim: To determine chlorinity and salinity of seawater by Mohr-Knudson argentometric titration method.

Definitions

1. Chlorinity: Chlorinity is defined as the mass in grams of pure silver necessary to precipitate the halogens in 0.3285255 kg of seawater (All weights are vacuum weights).
2. Salinity: Salinity is defined as the weight in grams (in vacuo) of solids that can be obtained from 1 kg of seawater (also measured in vacuo), when all of the carbonate has been converted to oxide, the bromine and iodine replaced by chloride, all organic matter oxidized, and the residue dried at 480°C to constant weight.

Outline of the method

Standard solution of silver nitrate is used to precipitate halide ions in seawater using potassium chromate as an indicator, to form silver halides. When a slight excess of silver ions are present, red silver chromate is formed.

Reagents

1. Standard solution of seawater (SSW): Use known chlorinity (19.375×10^{-3})/ salinity (34.99 ppt) or as quoted for seawater (SSW) supplied by the institute of oceanographic science in Wormley, Godalming, Surrey, (U.K.) in sealed glass ampules for standardizing silver nitrate solution.
2. Silver nitrate solution: Dissolve 25 g silver nitrate (AR) in 1000 ml DW. Store in an amber glass bottle.
3. Potassium chromate solution: Dissolve 8 g potassium chromate (AR) in 100 ml DW. Store in a stoppered glass bottle.

Apparatus

1. Burette: 25 ml, accuracy 0.1 ml
2. Bulb pipette: 5 ml, Accuracy 0.1 ml
3. Conical flask: 50 ml
4. Magnetic stirrer
5. Magnetic needle

Procedure

1. Standardisation of silver nitrate solution

Pipette out 5.0 ml SSW into a clean conical flask, add 6 drops of potassium chromate indicator and titrate with silver nitrate solution from the burette while stirring vigorously on a magnetic stirrer. Clean the inner wall of the flask with a jet of distilled water frequently and continue the titration. When colour change is observed, slow down the addition of titrant to drop by drop till colour change is observed from yellow to dirty orange. Repeat the standardization at least thrice and find out the mean of burette readings [BR (SSW)]. Find out the standardization factor F as follows:

$$F = \frac{\text{Chlorinity of SSW}}{\text{Mean BR (SSW) (ml)}}$$

2. Sample Analysis

Pipette out 5.0 ml sea water sample into a clean conical flask. Add distilled water (25 ml along the wall of the flask). Add 6 drops of indicator. Titrate against silver nitrate in the same manner as described in above step (1). Obtain the reading (ml) [BR(s)].

Calculations

Calculate the “normalised volume” (V) from the Equation

$$V = \text{BR(s)} \times F$$

Obtain the correction factor (k) corresponding to V from table 4.1 and then calculate the chlorinity (Cl) and salinity (S) by using the relations

$$\text{Cl} = V + k$$

$$S = 1.80655 \times \text{Cl}$$

If salinity of SSW is given, calculate salinity of samples as follows:

$$F = \text{salinity of SSW}$$

$$\text{Mean BR (SSW) (ml)}$$

$$\text{Salinity (ppt)} = F \times \text{BR(s) (ml)}$$

Results

Note: The salinity can be measured *insitu* by CTD probes/ dedicated salinometer / hand held refractometer etc. However, the argentometric, titrimetric procedures can be employed for finding out the instrumental error if any. The titrimetric procedure can be improved by using autotitrators.

9. DISSOLVED OXYGEN (DO)

Aim: To determine Dissolved Oxygen (DO) in seawater by Winkler method.

Outline of the method

DO in water reacts with manganous (II) hydroxide in strongly alkaline medium forming manganese (III) hydroxide (cloudy precipitate). When acidified to pH less than 2.5, the manganese (III) hydroxide is dissolved to liberate manganese which is a strong oxidizing agent in acidic media. It reacts with iodide ions (previously added), liberating equivalent amount of free iodine, which is titrated against standard thiosulphate solution using starch as an indicator.

Reagents

1. Winkler –A (WA): Dissolve 400g manganous chloride in 1000ml DW and store in a polyethylene bottle.
2. Winkler –B (WB): Dissolve separately and mix together, 360 g potassium iodide and 100g sodium hydroxide in 1000ml DW. Store in polyethylene bottle.
3. HCl (50%): Carefully add 50ml conc. HCl to 50ml DW. Store in a ground stoppered glass bottle.
4. Sodium thiosulphate solution (0.02N): Dissolve 5g sodium thiosulphate in DW and make up to 1000ml in a volumetric flask. Store in a ground stoppered glass bottle.
5. Starch solution (indicator): Disperse 1g starch in 100ml hot DW, quickly heat the suspension to boiling (complete dissolution of starch) and cool. Store in a ground stoppered glass bottle. This solution should not be kept for more than a week.

6. Standard iodate solution (0.02N): Dissolve 0.3567g potassium iodate (AR) in 500ml DW in a volumetric flask.

Apparatus

1. Burette: 25ml, accuracy of 0.05ml.
2. Conical flask: 100ml.
3. Magnetic stirrer.
4. Graduated pipettes: 1 and 5ml and bulb pipettes (10 and 50ml).

Procedure

1. Reagent blank: Pipette out 50ml DW in 100ml conical flask. Add 3ml of 50% hydrochloric acid and mix well. Add 1ml of WB and mix. Add 1ml of WA and again mix well. Add 1ml starch indicator to get blue colour. Titrate against thiosulphate from the burette. Note down the burette reading when blue colour disappears. Repeat the experiment three times and find out the mean burette reading [BR (b)].
2. Standardization of thiosulphate solution: Follow the steps in the same order as given under step (1). Add 10ml of 0.02N potassium iodate solution. Mix well and keep in dark for 5min to allow iodine to liberate. Titrate liberated iodine with sodium thiosulphate solution from the burette till the solution turns pale yellow. Add 1ml of starch indicator and continue titration drop by drop till the colour changes from blue to colourless and remains colourless atleast for 30s. Repeat the experiment three times and find out the mean burette reading [BR (st)].

Find out the exact normality of sodium thiosulphate (N) from the relation

$$N = \frac{10 \times 0.02}{BR(st) - BR(b)}$$

3. Sample Analysis: Add 3ml of 50% hydrochloric acid, by inserting the pipette tip close to the settled precipitate in DO bottle. Stopper the bottle immediately and shake vigorously till all precipitate dissolve. Pipette out 50ml of the clear solution in the conical flask and titrate against thiosulphate solution from the burette using starch as given in step (2). Note down the burette reading [BR (s)].

4. Calculation

The amount of DO in one liter of sample is given by

$$\text{DO (ml/l)} = 5.6 \times N \times [\text{BR(s)} - \text{BR (b)}] \times \frac{V}{V-1} \times \frac{1000}{a}$$

$$\text{DO (mg/l)} = \text{DO (ml/l)} / 0.7$$

Where N = normality of thiosulphate

BR(s) = titre value of the sample (mean)

BR (b) = titre value of the blank (mean)

V = volume of the sample bottle (125ml)

a = volume of the sample titrate (50ml)

10. BIOCHEMICAL OXYGEN DEMAND (BOD)

The biochemical oxygen demand (BOD) is an empirical test to assess water quality in terms of “Organic Matter” as an approximate index. This test measures the oxygen utilized for the biochemical degradation of organic materials (carbonaceous demand) and to oxidize inorganic materials such as sulfides and ferrous ion, over a specified time. This test also measures the oxygen used to oxidize the reduced forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor. 5 day BOD is in routine practice and the duration should be modified to shorter or longer period as to the condition of sample water. The BOD incubation has to be carried out at a specific temperature of 20°C.

In practice, this method involves diluting certain portions of waste water samples with oxygen saturated water and seeded with a micro-organism culture, if necessary. The dissolved oxygen is analyzed both at the start and after a specific time of incubation, usually five days, at 20°C in a BOD incubator. The dissolved oxygen in the bottle can be measured either by Winkler’s titration method or by an oxygen analyser.

Necessary precautions should be followed while diluting the samples as it may lead to more dilution of pollutants, which may affect the growth of micro-organisms. Nutrients such as nitrogen and phosphorus are basic and essential and the normal required ratio is B.O.D.: N:P is 100:5:1 and this could be achieved by adding buffer. The trace elements are also required for microbial growth.

Apparatus

1. BOD incubation bottles (250 to 300 ml capacity)
2. Incubator: Thermostatically controlled at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ without any light source.

Reagent

1. Manganous sulphate $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ --- 480 g/l
(or)
Manganous chloride $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ --- 600 g/l
2. Alkaline Iodide NaOH --- 500 g/l
NaI --- 150 g/l
(or)
KOH --- 700 g/l
KI --- 150 g/l
3. Sulphuric acid H_2SO_4 Conc.
4. Sodium thiosulphate (0.1 N) --- 24.82 g/l
5. Potassium dichromate (0.01 N) --- 0.49 g/l
6. Starch indicator --- 1 g in 100 ml

Procedure

Transfer the known quantity of sample into the BOD bottle (250 ml or 300 ml capacity). Prior to transfer, provide sufficient oxygen by aerating the samples for 5 or 10 minutes. Dilution and seeding of the sample can be made, if necessary, by using buffers and microbial culture. Check the initial oxygen level and incubate the sample for five days at a constant temperature of $20 \pm 1^{\circ}\text{C}$ after five days, check the oxygen level. Subtract the final oxygen value from the initial, which is equal to the amount of oxygen consumed for biological oxidation purposes.

Calculation

Calculate the BOD in the sample in mg/l as follows

$$\text{BOD mg/l} = \frac{[A-B]N \times EX \ 1000}{V_s}$$

Where:

A = Milliliters of $\text{Na}_2\text{S}_2\text{O}_3$ solution required for titration of the initial level
(Before incubation).

B = Milliliters of $\text{Na}_2\text{S}_2\text{O}_3$ solution required for titration of the final level
(After incubation)

N = Normality of $\text{Na}_2\text{S}_2\text{O}_3$ used (0.1).

E = Equivalent weight of oxygen (8).

VS = Volume of the sample (750 ml).

11. DETERMINATION OF NUTRIENTS IN THE SEA WATER

The biological productivity of seawater depends on the available nutrients and organic matter. A measurement of water quality parameter essentially involves the analysis of nutrients such as nitrogen, phosphorus and silicon. All these nutrients are predominantly utilized by the primary producers as inorganic forms. Thus the measurements need to be carried out both inorganic and organic form of nutrients. A brief outline is given below about the importance of the nutrients and basic principles involved in analyzing these parameters. However, a detailed step wise methodology is given below.

11.1. Origin of phosphorus in seawater

Weathering of rocks liberates phosphorus as soluble alkali phosphates and colloidal calcium phosphates are carried by many sources which reaches the sea. In addition to this, anthropogenic inputs of fertilizers (superphosphate), and detergents (alkyl phosphates) which increase amount of phosphorus in sea water.

In general, two forms of phosphorus exist in sea water such as - inorganic and organic. Inorganic phosphate exists as PO_4^{3-} ions and HPO_4^{2-} ions. The condensed phosphate as P_2O_7 ions exists in estuarine and coastal waters and plays an important role in energy transformation processes in the biological systems and the organic phosphates exists as phospholipids, phosphonucleotides originated from decomposition and excretion of organisms. Therefore, determination of Phosphates includes two phases such as determination of inorganic phosphate-P and total phosphorus that includes all forms of Phosphorus.

11.2. Inorganic Phosphate ($\text{PO}_4\text{-P}$)

The inorganic phosphate ions in seawater react with acidified molybdate reagent to yield phosphomolybdate complex, which is reduced to molybdenum blue. The colour is measured spectrophotometrically at 880 nm.

The mixed reagent is of ammonium molybdate in sulphuric acid, containing antimony in the bivalent state. The presence of antimony ions leads to a rapid reaction resulting in the formation of phosphor antimony molybdate complex (P: Sb as1:1), which yields a heteropoly blue complex on reduction. The reducing agent favored is ascorbic acid. The method is essentially based on the Murphy and Riley procedure. It has been demonstrated that acid/molybdenum ratio is crucial, in determining the form of the reduced complex and in controlling the kinetics. To obtain a rapid color development and to suppress the interference of silicate, the final pH should be less than 1 and that the ratio of sulphuric acid to molybdate should be between 2 and 3, when the concentrations are given in normality and percentage respectively.

Sensitivity

The major absorptivity is around 23,000 at 880 nm. A sample of seawater having a phosphate concentration of 1.0 $\mu\text{mol/l}$ gives an absorbance of around 0.1, using a 50 mm path length. Therefore, the use of smaller path lengths, for measurements of phosphate, when the normal concentrations are 1.0 $\mu\text{mol/l}$ should be completely discouraged.

Aim

To determine the amount of dissolved inorganic reactive phosphate –phosphorus in seawater.

Principle

Phosphate in seawater is allowed to react with acid Ammonium molybdate, forming a phosphomolybdate complex, which is reduced by ascorbic acid, in presence of antimonyl ions (to accelerate to reaction) to a blue coloured complex containing 1: 1 atomic ratio of phosphate and antimonyl ions. The blue colour forming through the reaction is measured at 880nm using 1cm cell (cuvette). To avoid interference by silicate the pH is kept below 1.

Reagents preparation

- 1. 9 N Sulphuric acid:** Add carefully 250ml conc. H_2SO_4 into a 1 liter volumetric flask containing 750ml Milli-Q water.
- 2. Ammonium molybdate solution:** Dissolve 12.5 g of ammonium molybdate tetrahydrate in 125ml MQ water and store it in a clean plastic or glass bottle.
- 3. Potassium antimonyl tartrate solution:** Dissolve 0.5g potassium antimonyl tartrate in 20ml MQ water and store it in a glass bottle.

4. **Mixed reagent:** Add slowly while stirring 125ml molybdate solution to 350ml 9N sulphuric acid. Then add 20ml of tartrate solution, mix well by proper shaking and store in an amber glass bottle. It is stable for more than a month.
5. **Ascorbic acid solution:** Dissolve 10g of ascorbic acid in 50ml MQ and add 50ml of 9N H₂SO₄. Store the reagent in an amber coloured bottle, in a refrigerator.
6. **Phosphate standard stock solution:** Weigh accurately 0.1361g of potassium dihydrogen phosphate (KH₂PO₄), add 1 ml of 9N H₂SO₄ and make up the volume up to 100ml with MQ water in a standard flask. Mix thoroughly by proper shaking. (prior to weighing, dry the potassium dihydrogen phosphate in oven at 110°C and cool by placing in a dessicator). This stock solution contains 10 mmol/L PO₄³⁻-P (or) 10,000 μmol/L PO₄³⁻-P.

Apparatus required

1. Spectrophotometer: With 1 cm path length cell.
2. Stopper glass tubes: With marking at 25 ml volume.
3. Standard flasks: 100ml and 25ml
4. Standard pipettes: 1ml, 2 ml, 5ml and 10ml.

Procedure

a) Preparation of working standard solution:

- Transfer 1ml of phosphate stock solution into a 100ml volumetric flask and dilute to the mark with MQ water. This solution contains 100 μmol PO₄³⁻-P/L.
- Again transfer 1ml of the above 100 μmol solution into another 100ml volumetric flask and dilute to the mark with MQ water. This solution contains 1 μmol PO₄³⁻-P/L. Prepare similarly for following standards as 2, 3, 4 and 5 μmol PO₄³⁻-P/L concentrations from above said stock solution.

$$0.1361\text{g in }100\text{ml}=10,000\ \mu\text{mol PO}_4^{3-}\text{-P/L}$$

$$1\ \text{ml from }10,000\ \mu\text{mol solution in }100\text{ml}=100\ \mu\text{mol PO}_4^{3-}\text{-P/L}$$

$$1\ \text{ml from }100\ \mu\text{mol solution in }100\text{ml}=1\ \mu\text{mol PO}_4^{3-}\text{-P/L}$$

$$2\ \text{ml from }100\ \mu\text{mol solution in }100\text{ml}=2\ \mu\text{mol PO}_4^{3-}\text{-P/L}$$

$$3\ \text{ml from }100\ \mu\text{mol solution in }100\text{ml}=3\ \mu\text{mol PO}_4^{3-}\text{-P/L}$$

- b) Calibration of standards and blank:** Measure out 25 ml of MQ water for blank in triplicate. Similarly measure out 25 ml of working standard solutions in clean stopper glass tubes in triplicate. Add 0.5 ml of ascorbic acid solution to each tube and mix well. Then add 0.5ml of mixed reagent, mix well and wait for 10 min for the development of blue complex. Measure the absorbance of blank (b) and standard solutions (st) in a spectrophotometer using 1 cm cell at 880 nm using MQ water as reference.
- c) Sample analysis:** Measure out 25ml of the sample in a clean stopper glass tube and add 0.5 ml of ascorbic acid solution to each tube and mix well. Then add 0.5ml of mixed reagent, mix well and wait for 10 min for the development of blue complex. Measure the absorbance of the sample in 1 cm cell at 880 nm (As).

Calculation

Calculation for Factor value (F):

$$F = \frac{\text{Conc. of standard solution}}{A(\text{st}) - A(\text{b})}$$

Where A (st) = Mean absorbance of standards.

A (b) = Mean absorbance of blanks.

Calculate the concentration of phosphate –phosphorus present in the sample

$$\text{PO}_4^{3-} - \text{P } \mu\text{mol/L} = F \times A(\text{s}) - A(\text{b})$$

Where A(s) = Mean absorbance of samples.

A(b) = Mean absorbance of blanks.

Results

11.3. Origin of various forms of Nitrogen in seawater

Nitrogen in the atmosphere converted to nitrate by lightning and it is carried to the sea by rain and river run off. In addition to this, anthropogenic inputs of nitrate as fertilizer which makes various forms of nitrogen in seawater. Nitrate is reduced to nitrite, and ammonia under anoxic condition. It is the amino acid compounds, which are the synthetic blocks of proteins and cells of the biota.

Nitrate is considered a micronutrient, controlling primary production in the euphotic surface layers. If there is sufficient light penetration in the water, the uptake by the primary producers is much faster than processes transporting nitrate into surface layers. Therefore, nitrate concentrations can be as high as $50\mu\text{mol/l}$ in the Indian Ocean.

Nitrite is an intermediate compound in the microbial reduction of nitrate or oxidation of ammonia. The natural level of nitrite in seawater is very low ($0.1\mu\text{mol}$ per litre). Upwelling leads to higher values of nitrite ($1-2\mu\text{mol/l}$ per litre). Pollution leads to very high values.

The concentration of ammonium nitrogen varies considerably. Nitrate is reduced to ammonia before transformation to amino acids. Ammonia is also excreted directly by animals. In oxygenated unpolluted waters, NH_3 and NH_4 together rarely exceed $5\mu\text{mol/l}$ per litre. But in anoxic waters, the amount of ammonia can be as high as $100\mu\text{mol/l}$.

11.3.1. DETERMINATION OF AMMONIA - NITROGEN

Ammonia reacts with hypochlorite to form monochloramine, in moderately alkaline medium. In presence of phenol and nitroprusside, indophenols blue is formed. The absorbance is measured at 630 nm. Nitroprusside ions catalyse the reaction. The reaction mechanism is complicated and has not been fully explained.

The formation of monochloramine requires a pH between 8 and 11.5. To prevent the precipitation of hydroxides of calcium and magnesium at the higher pH, citrate is generally added to complex calcium and magnesium.

Particular care is to be taken to keep the ratio of phenol to active chlorine constant and close to 25 w/w. Otherwise there is variation in absorbance.

Care should also be taken to keep a set of glassware exclusively for the determination of ammonia. There should be a well ventilated laboratory, where no ammoniacal solutions are stored.

The distilled water needed for preparation of reagents and for dilutions, should obviously be free of ammonia. This is carried out by a second distillation, in presence of alkaline $\text{K}_2\text{S}_2\text{O}_8$. Once the distillate is collected it should be stored in tightly closed polyethylene bottles.

Instead of thermostating at $37 \pm 10^\circ\text{C}$, the solutions of the sample, after mixing with citrate buffer, phenol and hypochlorite, can be kept in the dark for at least 3h. The measurement for absorbance can then be carried out at 630 nm.

Sensitivity

The molar absorptivity is around 10,000 at 630 nm, much lower than that of the method for nitrite / nitrate. For eg. a sea water with an ammonical nitrogen of $1.0 \mu\text{mol}$ at N/l would give an absorbance of 0.05, even with a 50 mm path length cell.

Aim

To determine the amount of Ammonia – Nitrogen in seawater.

Principle

The ammonia in the seawater reacts with sodium hypochlorite and phenol in alkaline condition to produce indophenols blue. Sodium nitroprusside is used as a catalyst and colour intensifier.

Reagents preparation

1. Phenol alcohol:

Dissolve 10 g of phenol in a mixture (100 ml) consisting of ethyl alcohol (95 ml) and n- propanol (5 ml).

2. Sodium nitroprusside:

Dissolve 1 g of sodium nitroprusside in 200 ml of MQ water and store it in an amber glass bottle.

3. Sodium citrate:

Dissolve 20 g of sodium citrate and 1 g of sodium hydroxide in 100 ml of MQ water.

4. Sodium hypochlorite:

Commercially available Sodium hypochlorite (“Chlorox”) of 1.5 N.

5. Oxidizing reagent

Mix 100 ml of sodium citrate solution and 25 ml of sodium hypochlorite solution just before use.

Apparatus required

Spectrophotometer: With 1 cm path length cell.

Stopper glass tubes: With marking at 25 ml volume.

Standard flasks: 25ml

Standard pipettes: 1ml, 2 ml and 5ml.

Procedure

Preparation of standard solution:

- Dissolve 0.5349 g of Ammonium chloride (AR) in 100 ml of MQ water. This solution contains 10,000 $\mu\text{mol/L}$. (Stock solution).
- Transfer 1ml of the above 10,000 μmol solution into another 100 ml volumetric flask and dilute to the mark with ammonia free MQ water. This solution contains 100 $\mu\text{mol/L}$.
- Transfer 1ml of the above 100 μmol solution into another 100 ml volumetric flask and dilute to the mark with ammonia free MQ water. This solution contains 1 $\mu\text{mol/L}$.

$$0.5349 \text{ g in } 100\text{ml} = 10,000 \mu\text{mol/L}$$

$$1 \text{ ml from } 10,000 \mu\text{mol solution in } 100 \text{ ml} = 100 \mu\text{mol NH}_4 \text{-N /L.}$$

$$1 \text{ ml from } 100 \mu\text{mol solution in } 100 \text{ ml} = 1 \mu\text{mol/L.}$$

$$2 \text{ ml from } 100 \mu\text{mol solution in } 100\text{ml} = 2 \mu\text{mol/L.}$$

$$3 \text{ ml from } 100 \mu\text{mol solution in } 100\text{ml} = 3 \mu\text{mol/L.}$$

Calibration of standard and blanks

Measure out 50 ml of working standard solutions in clean stopper glass tubes in triplicate and 50 ml of MQ water in clean stopper glass tubes as blank. Add 2 ml of phenol alcohol solution and mix well. Then add 2 ml of sodium nitroprusside solution followed by 5 ml of oxidizing solution and mix well once again.

Cover the flasks with polythene sheet and wait for 1 hour and measure the absorbance of standard solutions $A(\text{st})$ and blanks $A(\text{b})$ in a

spectrophotometer using 1 cm cell at 640 nm against ammonia free MQ water as reference.

Sample analysis

Measure out 50 ml of the sample (triplicate) in a clean stopper glass tube and add 2 ml of phenol alcohol solution and mix well. Then add 2 ml of sodium nitroprusside solution followed by 5 ml of oxidizing solution and mix well.

Cover the flasks with polythene sheet and wait for 1hour and measure the absorbance of sample in a spectrophotometer using 1 cm cell at 640 nm against ammonia free MQ water as reference.

Calculation:

Calculation for Factor value (F):

$$F = \frac{\text{Conc. of standard solution}}{A(\text{st}) - A(\text{b})}$$

Where A (st) = Mean absorbance of standards.

A (b) = Mean absorbance of blanks.

Calculate the concentration of Ammonia - Nitrogen present in the sample

$$\text{Ammonia } \mu\text{mol/L} = F \times A(\text{s}) - A(\text{b})$$

Where A(s) = Mean absorbance of samples.

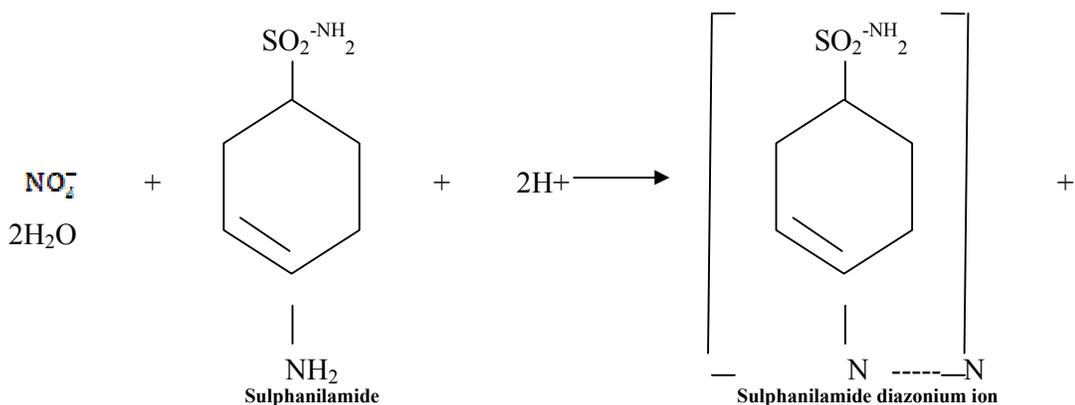
A(b) = Mean absorbance of blanks.

Results

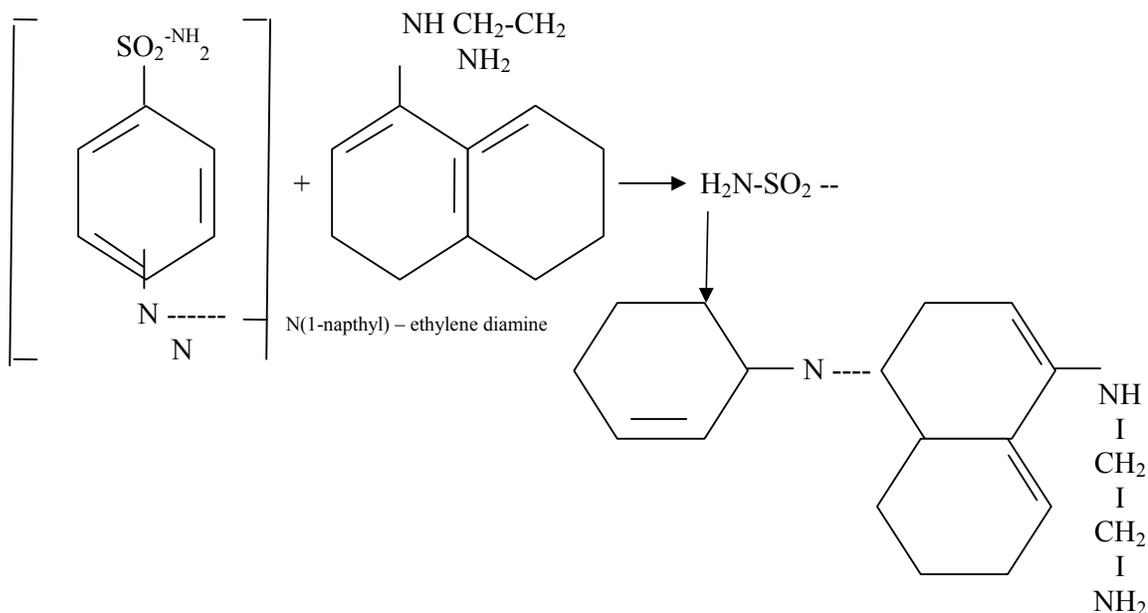
11.4. DETERMINATION OF NITRATE AND NITRITE

The method of nitrite determination depends on reaction with an aromatic amine, sulphanilamide, which is then coupled with N(1-Naphthyl) – ethylene diamine dihydrochloride, to form an azo dye. The absorbance of the dye is measured spectrophotometrically at 543 nm. Basically, this method is same as the one suggested by Bentschneider and Robinson.

Formation of Diazonium ion



a. Formation of azo dye



The nitrate is reduced to nitrite before determination. The reduction is carried out in a reductor of Cd granules. The Cd is either coated with Hg or Cu. The conditions are so adjusted that the nitrate is quantitatively reduced to nitrite and not further to NO. The standard potentials are very close in both reactions.



However, in a neutral or alkaline solution, the standard potential is



There is a possibility that cadmium ions formed during reduction is precipitated as the hydroxide and thus reducing the efficiency of the reductor column. Therefore, the solution should be buffered to prevent this efficiency loss. Seawater, of course, has a limited buffering capacity, but this is not sufficient. Ammonium chloride is added as a buffer and as a complexing agent.



Thus the two H⁺ formed neutralize the two OH⁻ ions formed, as in equation above. The Cd⁺⁺ combines with ammonia to form the complex.

Still it is necessary to control the rate of passage of seawater through the reductor, to prevent any further reduction of nitrite, already present. The efficiency of the reduction of nitrate should be maintained close to 100% by frequent standardization.

Taking through the procedure at least three solutions of different concentrations, bracketing the concentration of the sample solution should carry out the standardization. As mentioned under phosphate, this would give information on the repeatability of measurements.

Sensitivity

The molar absorptivity of the azo dye formed from nitrite is 50,000. Therefore, a sample of sea water having a nitrate + nitrite concentration of 1.0 μmol at (NO₃ – NO₂) N per litre should have at absorbance of 0.250, using a 50 mm path length cell. For nitrite, which is normally present at sub microgram atom per litre, the use of a 50 mm path length is a must.

11.5. NITRITE – NITROGEN (NO₂-N)

Aim

To determine the amount of Nitrite – Nitrogen in seawater.

Principle

The Nitrite in seawater is diazotized with Sulphanilamide at pH 1.5 to 2.0 and then resulting diazo compound is coupled with N – (1- naphthyl) – Ethylene diamine to form a highly coloured azodye with absorption maxima at 540nm.

Reagents preparation

(1) Sulphanilamide:

Dissolve 1 g of Sulphanilamide in 10 ml conc. HCl and make up to 100ml with MQ water and store in an amber glass bottle.

(2) N – (1- naphthyl) – Ethylene diamine dihydrochloride (NEDA) :

Dissolve 0.1 g of NEDA in 100ml of MQ water and store it in an amber glass bottle.

(3) Nitrite stock solution:

Anhydrous Sodium nitrite (NaNO₂) is dried at 110°C for some hours. Weigh accurately 0.069g of NaNO₂ and dissolve in 100 ml of MQ water up to the mark in 100 ml standard flask. This stock solution contains 10 mmol/L NO₂ - N (or) 10,000µmol/L NO₂ -N.

Apparatus required

Spectrophotometer: With 1 cm path length cell.

Stopper glass tubes: With marking at 25 ml volume.

Standard flasks: 100ml and 25ml

Standard pipettes: 1ml, 2 ml, 5ml and 10ml.

Procedure

Preparation of working standard solution:

- Transfer 1ml of Nitrite stock solution into a 100ml volumetric flask and dilute to the mark with MQ water. This solution contains $100\mu\text{mol NO}_2\text{-N/L}$.
- Again transfer 1ml of the above $100\mu\text{mol}$ solution into another 100ml volumetric flask and dilute to the mark with MQ water. This solution contains $1\mu\text{mol NO}_2\text{-N/L}$. Prepare similarly for following standards as 2, 3, 4 and 5 $\mu\text{mol NO}_2\text{-N/L}$ concentrations from above said stock solution.

$$0.069\text{g in } 100\text{ml} = 10,000 \mu\text{mol NO}_2\text{-N /L}$$

$$1 \text{ ml from } 10,000 \mu\text{mol solution in } 100\text{ml} = 100 \mu\text{mol NO}_2\text{-N /L.}$$

$$1 \text{ ml from } 100 \mu\text{mol solution in } 100\text{ml} = 1\mu\text{mol NO}_2\text{-N /L.}$$

$$2 \text{ ml from } 100 \mu\text{mol solution in } 100\text{ml} = 2\mu\text{mol NO}_2\text{-N /L.}$$

$$3 \text{ ml from } 100 \mu\text{mol solution in } 100\text{ml} = 3\mu\text{mol NO}_2\text{-N /L.}$$

- Calibration of standards and blank:** Measure out 25 ml of MQ water for blank in triplicate. Similarly measure out 25 ml of working standard solutions in clean stopper glass tubes in triplicate. Add 0.5 ml of sulphanilamide to each tube and mix well. After 1 min reaction time, add 0.5ml of NEDA solution and mix well once again and allow to proceed for 15 min. Measure the absorbance of blank A(b) and standard solutions A (st) in a spectrophotometer using 1 cm cell at 540 nm against MQ water as reference.
- Sample analysis:** Measure out 25ml of the sample (triplicate) in a clean stopper glass tube and add 0.5 ml of sulphanilamide to each tube and mix well. Then add 0.5ml of NEDA, mix well and wait for 15 min. Measure the absorbance A (s) of the sample in 1 cm cell at 540 nm.

Calculation

Calculation for Factor value (F):

$$F = \frac{\text{Conc. of standard solution}}{A(\text{st}) - A(\text{b})}$$

Where A (st) = Mean absorbance of standards.

A (b) = Mean absorbance of blanks.

Calculate the concentrations of Nitrite-Nitrogen present in the sample

$$\text{NO}_2\text{-N } \mu\text{mol/L} = F \times A(\text{s}) - A(\text{b})$$

Where A(s) = Mean absorbance of samples.

A(b) = Mean absorbance of blanks.

Results

11.6. NITRATE – NITROGEN (NO₃-N)

Aim

To determine the amount of Nitrate – Nitrogen in seawater.

Principle

Nitrate in seawater is quantitatively reduced to nitrite by heterogeneous reduction involving copper – cadmium granules. Nitrite thus produced is determined by diazotising with Sulphanilamide and coupling with N – (1- naphthyl) – Ethylene diamine through the column without change. Hence correction should be made for nitrite present in the sample.

Reagents preparation

(1) Ammonium chloride buffer:

Dissolve 10 g of ammonium chloride in 1000 ml of MQ water. Store in a polythene bottle and the pH adjusted to 8.5 with liquid ammonia.

(2) Sulphanilamide solution (1%):

Dissolve 1 g of Sulphanilamide in 10 ml conc. HCl and make up to 100ml with MQ water. Store in an amber coloured glass bottle.

(3) N – (1- naphthyl) – Ethylene diamine dihydrochloride (NEDA) :

Dissolve 0.1 g of NEDA in 100ml of MQ water and store it in an amber glass bottle.

(4) Cadmium metal granules :

0.3 – 1.6 mm (Merck, Germany, product No – 1.02001.0250)

(5) Copper sulphate solution (CuSO₄·5H₂O):

Dissolve 1 g of CuSO₄·5H₂O in 100 ml of MQ water.

(6) Nitrate stock solution:

Dissolve 0.1011g dry potassium nitrate (NaNO₃) (priorly dried at 110°C for some hours) in 100 ml of MQ water up to the mark in 100 ml standard flask.

This stock solution contains 10 mmol/L NO₃ -N (or) 10,000µmol/L NO₃ -N.

Preparation of working standard solution

- Transfer 1ml of nitrate stock solution in to a 100ml volumetric flask and dilute to the mark with MQ water. This solution contains 100µmol NO₃ -N/L.
- Again transfer 5 ml of the above 100µmol solution in to another 100ml volumetric flask and dilute to the mark with MQ water. This solution contains 5µmol NO₃ -N/L. Prepare similarly for following standards as 10, 15, 20 and 25 µmol NO₃ -N/L concentrations from above said stock solution.

0.1011g in 100ml=10,000 µmol NO₃ -N /L

1 ml from 10,000 µmol solution in 100ml=100 µmol NO₃ -N /L.

5 ml from 100 µmol solution in 100ml=5µmol NO₃ -N /L.

10 ml from 100 µmol solution in 100ml=10µmol NO₃ -N /L.

15ml from 100 µmol solution in 100ml=15µmol NO₃ -N /L.

Nitrate activator solution

Transfer 20 ml of 100µmol/L to a 100ml of volumetric flasks and make up to the mark. This solution contains 20 µmol NO₃ -N /L. It is used as a nitrate activator solution.

Preparation of reduction column

E Merck grade **Cd** granules can be used for packing the column. Remove any iron particles from the filings with the help of magnet. Wash the granules with acetone to remove oil and grease. Next wash with 2N HCl and then with ambient amount of MQ water.

Place cadmium granules (70-80 g) in a stopper glass bottle (125ml) and fill the bottle with 1% copper sulphate solution. Stopper the bottle taking care to see that no air bubbles are trapped in the bottle. Shake the bottle for 10 min and keep for 90 min. Open the bottle and drain out the copper sulphate solution taking care that cadmium metal does not come in contact with the air. Fill MQ water up to the rim of the bottle.

Packing the reduction column

- Place a plug of cotton (or) glass wool at the bottom of the column and the reservoir filled with MQ water.
- Transfer the copperised cadmium into the column reservoir and allow the granules to fall freely into the column taking care that no air cavities are formed.
- After transferring the filings in the column, place another plug of cotton (or) glass wool on the top of the filings.
- The cadmium filings should be under water to avoid getting dried.
- Adjust the flow rate to 6 to 7ml per minute and pass the buffer solution (pH-8.5) through the column.

Activation of column

- Pass 50 ml of activator solution + 50ml of buffer through the column at a flow rate of 6 – 7 ml per minute and discard the elute.
- Pass 50 ml of MQ water + 50ml of buffer through the column. Stop the elution, leaving the buffer water just above the filings in the column.

Apparatus required

Spectrophotometer: With 1 cm path length cell.

Stopper glass tubes: With marking at 25 ml volume.

Standard flasks: 100ml and 25ml

Standard pipettes: 1ml, 5ml, 10ml, 25ml and 50ml.

Measuring cylinders: 25 ml, 50 ml and 100 ml.

Procedure

(1) Calibration of standards and blank:

Prepare the following standards from the stock solution as described earlier *i.e.*, 5, 10, 15, 20 and 25 $\mu\text{mol NO}_3^-/\text{L}$. Take 50 ml of this diluted nitrate standard solution + 50 ml of buffer in a 100 ml standard flask and pass through the column. Discard the first 50 ml and collect next portion of 25 ml elute in the stopper glass tubes for further analysis. Add to each tube 1ml of sulphanilamide and mix well. After 1 min reaction time, add 1ml of NEDA solution and mix well once again and allow for 15 min reaction time. Measure the absorbance A(st) in a spectrophotometer using 1 cm cell at 540 nm against MQ water as reference.

Blank

Take 50 ml of MQ water + 50 ml of buffer in a 100 ml standard flask and pass through the cadmium column. Discard the first 25 ml and collect next two portions of 25 ml in the stopper glass tubes for determining the absorbance of blank A (b).

(2) **Sample analysis:** Measure out 50 ml of the sample (triplicate) + 50 ml of buffer in 100 ml standard flask and pass through the column, discard the first 50 ml and the next two portions be preserved. Pass the samples through the column and proceed as before for the standards. Measure the absorbance A (NO_2+NO_3) in 1 cm cell at 540 nm against MQ water as reference. Analyze the same sample for nitrite and determine the concentration of nitrite in $\mu\text{mol/L}$.

Calculation

Calculation for Factor value (F):

$$F = \frac{\text{Conc. of standard solution}}{A(\text{st}) - A(\text{b})}$$

Where A (st) = Mean absorbance of standards.

A (b) = Mean absorbance of blanks.

Calculate the concentrations of Nitrate + Nitrite

$$C(\text{NO}_2 + \text{NO}_3) = F \times [A(\text{NO}_2 + \text{NO}_3) - A(b)]$$

Correct the values for nitrate by using this formula

$$C(\text{NO}_3) \mu\text{mol/L} = C(\text{NO}_2 + \text{NO}_3) - C(\text{NO}_2)$$

The (NO_2) concentration of nitrite was the in $\mu\text{mol/L}$ determined in the same sample earlier.

Results

11.7 TOTAL PHOSPHORUS AND NITROGEN (TP, TN)

Aim: To determine total dissolved phosphorus and nitrogen in seawater.

Outline of the Method

Total dissolved parameters (TP or TN) include all forms of dissolved inorganic and organic species. Filtered water sample is oxidized with the help of strong oxidizing agent (alkaline persulphate) by autoclaving in closed conditions. The organic forms of Phosphate and Nitrate and also their inorganic forms in lower oxidation states are finally oxidized to inorganic Phosphate and Nitrate respectively.

Reagents

1. Sulphuric acid (9.0 N): Add carefully 250 ml concentrated sulphuric acid to a 1 litre volumetric flask containing 750 ml MQ water.
2. Ammonium molybdate solution: Dissolve 12.5 g ammonium molybdate tetra hydrate (AR) in 125 ml MQ water. Store in a plastic or glass bottle.
3. Potassium antimonyl tartrate solution: Dissolve 0.5 g potassium antimonyl tartrate (AR) in 20 ml MQ water. Store in glass bottle.
4. Mixed reagent: Add slowly while stirring 125 ml molybdate solution to 350 ml 9.0 N H_2SO_4 . Then add 20 ml tartrate solution. Mix by shaking and store in a glass bottle.
5. Ascorbic acid solution: Dissolve 10 g ascorbic acid in 50 ml distilled water and add 50 ml 9.0N H_2SO_4 . Reagent should be stored in an amber coloured bottle, in refrigeration, and could be used for a week.

6. Ammonium chloride buffer: Dissolve 10.0 g of ammonium chloride (AR) in 1000 ml MQ water. Store in a polyethylene bottle and adjust the pH to 8.5 with ammonia.
7. Sulfanilamide solution (1%): Dissolve 1 g sulfanilamide in 10 ml conc. hydrochloric acid and make up to 100 ml with MQ water. Store in an amber coloured glass bottle.
8. N-(1-naphthyl) – ethylene diamine dihydrochloride (1%): Dissolve 0.1 g in 100 ml MQ water and store in an amber coloured glass bottle.
9. Cadmium metal filings: 0.3 to 1.6mm (E Merck-Product No. 1.02001.0250)
10. Copper sulfate solution $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1%): Dissolve 2.5g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 250ml of MQ water.
11. Nitrate Activator Solution: Transfer 20ml of 100 $\mu\text{mol/L}$ to a 100ml of volumetric flask and make up to the mark. This solution contains 20 $\mu\text{mol/L}$.

Preparation of Reduction column:

The size of the Cd granules should be between 0.5 to 1.0 mm. E Merck grade Cd granules need to be used for packing the column. Remove any iron particles from the filings with the help of magnet. Wash the filings with acetone to remove oil and grease. Next wash them with HCl (2 N) and then with copious amounts of MQ water. Place Cadmium filings (70 – 80 g) in a stoppered glass bottle (125 ml) and fill the bottle with 2% Copper sulphate. Stopper the bottle taking care to see that no air bubbles are trapped in the bottle. Shake the bottle for 10 min and keep for 90 min. Open the bottle and drain out the copper sulphate solution taking care that cadmium metal does not come in contact with the air. Fill MQ water up to the rim of the bottle.

Packing the Reduction column:

- Place a plug of glass wool at the bottom end of the column and the column and the reservoir filled with MQ water.
- Transfer the copperised cadmium into the column reservoir. Allow the filings to fall freely into the column taking care that no air cavities are formed.
- After transferring the filings in the column, place another piece of glass wool on the top of the filings.
- The cadmium filings should be kept under water always to avoid getting dried.

- Adjust flow rate to 8 ml for 1 min. Pass the buffer solution (pH 8.5) through the column.

Activation of column

- Pass 50 ml of activator solution plus 50 ml of buffer through the column at a rate of 6-8 ml in 1 minute and discard the elute.
- Pass 50 ml MQ water plus 50 ml buffer through the column. Stop the elution, leaving the buffer water just above the filings in the column.

Standard stock organic nitrogen solution:

Dissolve EDTA disodium salt (0.1862 g) in ammonia free distilled water (100 ml) and store in a glass bottle in refrigerator. The standard contains 10 mmol /l organic nitrogen/l or 1000 μ mol. From 10,000 μ mol, 1ml in 100ml gives 100 μ mol /L. From 100 μ mol 5ml in 100ml gives 5 μ mol /L. From 100 μ mol 10ml in 100ml gives 10 μ mol /L. From 100 μ mol 15ml in 100ml gives 15 μ mol /L. From this standard concentrations of (5, 10, 15 μ mol /L), 50ml is transferred into a oxidation flask.

Phosphate standard solution:

Weigh 0.1361g of potassium dihydrogen phosphate (AR) already dried at 110 °C in an oven and cooled in a dessicator and dissolve in 100 ml DW containing 1 ml of 9.0 N H₂SO₄. This solution contains 10,000 μ mol PO₄⁻³/L. From 10,000 μ mol, 1ml in 100ml gives 100 μ mol PO₄⁻³/L. From 100 μ mol, 1ml in 100ml gives 1 μ mol PO₄⁻³/L. From 100 μ mol, 2ml in 100ml gives 2 μ mol PO₄⁻³/L. From 100 μ mol, 5ml in 100ml gives 5 μ mol /L. From this standard concentrations of (1, 2, 5 μ mol /L), 50ml is transferred into a oxidation flask.

Apparatus

1. Autoclave/Pressure cooker
2. Oxidizing flasks

Procedure

Oxidation:

Measure out Ammonia free distilled water (50 ml) in clean oxidation flask. Mark the bottle as Blank. Similarly measure out (50ml) organic nitrogen standard solution i.e., 5, 10, 15 μ mol /L and Phosphate standard solution i.e., 1, 2, 5 μ mol /L in duplicate in another two

oxidation flasks. Mark the bottles separately. Take sample also in the same manner and mark as W1, W2. To each of the bottles, add 5 ml oxidizing agent (Total 55ml). Wrap the bottle neck with aluminum foil and autoclave for 30 min. Take out the flasks from autoclave, swirl to dissolve any precipitate and open the flasks to release any over pressure. Allow the flasks to cool.

Calibration and determination of blank for TN analysis:

Take stock solution i.e., 5, 10, 15 μmol TN concentration. Take 55 ml of oxidised diluted nitrate standard solution plus 50 ml buffer in a 100 ml standard flask and pass through the column. Discard the first 50 ml and collect next portion of 25 ml eluate in the stoppered glass tubes for subsequent analysis. Add to each tube, 1 ml of Sulphanilamide and mix well. After 1 min reaction time, 1 ml of NEDA solution is added, mix well and allow a reaction time of 15 min. Measure the absorbance A (st) in 1 cm cuvette at 540 nm against MQ water as reference within 1h.

Blank:

Take 55 ml of oxidized Blank water plus 50 ml buffer in a 100 ml standard flask and pass through the column. Discard the first 25 ml and collect next two portions of 25 ml in the stoppered glass tubes for determining the absorbance of blank A (b).

Sample analysis:

Take 5 ml of oxidized sample make up to 50ml plus 50 ml buffer in 100ml standard flask and pass through the column, reject the first 50 ml and the next one portion is preserved. Pass the sample through the column and proceed as before for the standards. Measure the absorbance A (TN) in a 1 cm cuvette at 540 nm against MQ water as reference. Analyze the same sample for Nitrite and determine the concentration of nitrite in $\mu\text{mol/l}$.

Calibration and determination of blank for TP analysis:

Take stock solution i.e., 1, 2, 5 μmol TP concentration. Take 25 ml of oxidized diluted phosphate standard solution in the stoppered glass tubes for subsequent analysis. Add 0.5 ml of Ascorbic acid reagent to each tube and mix well. Add 0.5 ml of mixed reagent, mix and wait for 10 min to allow for the development of blue complex. Measure the absorbance of blank and standards in the Spectrophotometer using 5 or 1 cm cell at 880nm with D.W as reference.

Sample:

Measure out 25 ml of oxidized portion of sample in glass tube and add reagents in the same order describe above. Measure the absorbance of sample in the Spectrophotometer using 5 or 1 cm cell at 880nm with D.W as reference.

Calculations

Calculate the calibration factor for total Phosphorus by the relation

$$F (TP) = \frac{\text{Conc. of standard solution}}{A (st) - A (b)}$$

Where A (st) = Mean absorbance of standards

A (b) = Mean absorbance of blanks

Calculate the concentrations of total Phosphorus from the relation

$$\text{Total P } \mu\text{mol/l} = F (TP) \times [A (s) - A (b)]$$

Where A (s) = Mean absorbance of sample

A (b) = Mean absorbance of blank

Calculate the factor F for total Nitrogen from the relation

$$F (TN) = \frac{\text{Conc. of standard solution}}{A (st) - A (b)}$$

Where A (st) = Mean absorbance of standards

A (b) = Mean absorbance of blanks

Calculate the concentrations of total Nitrogen from the relation

$$\text{Total N } \mu\text{mol/l} = F (TN) \times [A (s) - A (b)] \times 10$$

Where A (s) = Mean absorbance of sample

A (b) = Mean absorbance of blank

Results

Report the results up to two places of decimal.

11.8. GENESIS OF SILICATE IN SEAWATER

The element silicon is the most abundant element in the universe. During weathering of rocks, silicate is brought into solution and thus is present in sea water. The average concentration is around 1 mg/l, much below its saturation value of 50 mg/l. In addition silica is also present in particulate, in varying quantities.

Many believe that the distribution of silica in sea water is controlled by processes involving organisms. Thus, hydrated silica is a major constituent of diatoms, which form a large proportion of phytoplankton. When the organisms die, silicon is liberated. The element passes through these cycles many times, in one season. It is estimated that approximately 120 million tonnes of silicon is removed from sea per annum by the growth and sedimentation of phytoplankton.

11.8.1. DETERMINATION OF REACTIVE SILICATE

The determination is based on the formation of a yellow silicomolybdic acid when an acidified solution of the sea water is treated with molybdate. This complex exists in two isomeric forms, depending on pH, which differ in their hydration. The α isomer is formed at pH 3.5 – 4.5 and is very stable, once formed. On the other hand, the β form is rapidly formed in the pH range 0.8 – 2.5, but it is much less stable. However, the latter has a higher molar absorptive.

Since both the isomeric forms have only low intensity absorbance, several methods have been developed to reduce the complexes to intensely coloured blue complexes. For the purpose, several organic and inorganic reducing agents have been used. In the present method, the use of oxalic acid has been recommended.

Sensitivity

The molar absorptive is around 19,000 in sea water, lower than that in distilled water of 22,000.

Aim

To determine the reactive silicate concentration in seawater.

Principle

The seawater sample is allowed to react with molybdate under conditions which result in the formation of silicomolybdate, phosphomolybdate and arsenomolybdate

complexes. A reducing solution, containing metol and oxalic acid, is then added which reduces the silicomolybdate complex to give a blue reduction compound and simultaneously decomposes the phosphomolybdate or arsenomolybdate eliminating the phosphate and arsenate interference.

Reagents preparation

1. Molybdate reagent:

Dissolve 4 g of ammonium paramolybdate ($\text{NH}_4\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$) in about 300 ml of MQ water and add 12.0 ml of conc. HCl, mix well and make the volume to 500 ml with MQ water. Store it in a polythene bottle.

2. Metol – Sulphate solution:

Dissolve 1.5g of anhydrous sodium sulphite (Na_2SO_3), in 125ml of MQ water and then add 2.5g of metol (p-methylaminophenol sulphate). Store in a plastic bottle.

3. Oxalic acid solution:

Prepare a saturated oxalic acid solution by shaking 10g of oxalic acid dehydrate ($(\text{COOH})_2, 2\text{H}_2\text{O}$ (AR) with 100ml of MQ water; decant the solution from the crystals for use.

4. Sulphuric acid solution (50%V/V):

Add slowly 100ml of conc. H_2SO_4 into 250ml of MQ water and allow to cool.

5. Reducing reagent:

Mix 100 ml of metol - sulphate solution with 60 ml of oxalic acid solution. Add 60 ml of 50% sulphuric acid solution slowly with mixing and make the mixture to 300 ml with MQ water.

6. Preparation of synthetic sea water:

Add 25 g NaCl + 8 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ in 1000ml MQ water.

Apparatus required

Spectrophotometer: With 1 cm path length cell.

Plastic containers: With marking at 25 ml volume.

Standard flasks: 100ml and 25ml

Standard pipettes: 5ml and 10ml.

Procedure

Preparation of standard solution

1. Dissolve 0.0188 g of sodium Silicofluoride (Na_2SiF_6) in 100 ml of synthetic seawater and store this stock solution in a polythene container. This solution contains 1000 $\mu\text{mol Si/L}$.
2. Prepare 5, 10, 15, 20 and 25 $\mu\text{mol Si/L}$ by transfer 0.5, 1, 1.5, 2.0 and 2.5 ml of the stock solution and make up the volume up to 100ml with same synthetic seawater. These solutions contain 5, 10, 15, 20 and 25 $\mu\text{mol Si/L}$ concentrations.

0.0188 g in 100 ml=1000 $\mu\text{mol Si /L}$

1 ml from 1000 μmol solution in 100 ml=1 $\mu\text{mol Si /L}$.

5 ml from 1000 μmol solution in 100 ml=5 $\mu\text{mol Si /L}$.

10 ml from 1000 μmol solution in 100ml=10 $\mu\text{mol Si /L}$.

15 ml from 1000 μmol solution in 100ml=15 $\mu\text{mol Si /L}$.

Calibration of standards and blank: Measure out 25 ml of MQ water for blank in triplicate. Similarly measure out 25 ml of working standard solutions in clean plastic container in triplicate. Add 10 ml of molybdate solution to each tube mix well and allow to stand for 10 min, add 15 ml of reducing reagent rapidly and mix immediately. After 2 hours incubation measure the absorbance of blank $A(b)$ and standard solutions $A(st)$ in a spectrophotometer using 1 cm cell at 810 nm against MQ water as reference.

Sample analysis: Measure out 25ml of the sample (triplicate) in a clean plastic container and add 10 ml of molybdate solution to each tube, mix well and allow to stand for 10 min, then add 15 ml of reducing reagent and mix immediately. Measure the absorbance $A(s)$ of the sample in 1 cm cell at 810 nm.

Calculation

Calculation for Factor value (F):

$$F = \frac{\text{Conc. of standard solution}}{A(st) - A(b)}$$

Where $A(st)$ = Mean absorbance of standards.

$A(b)$ = Mean absorbance of blanks.

Calculate the amount of reactive silicate present in the sample

$$\text{Silicate - Si } \mu\text{mol/L} = F \times A(s) - A(b)$$

Where A(s) = Mean absorbance of samples.

A (b) = Mean absorbance of blanks.

Results

12. DETERMINATION OF PARTICULATE ORGANIC CARBON (POC) AND PARTICULATE NITROGEN (PN)

Aim: To determine particulate organic carbon (POC) and nitrogen (PN) in suspended particulate matter and bottom sediment.

Outline of method

The particulate matter is filtered on to a pre-combusted glass-fiber (GF/F) filter (Whatman filter paper) and making these measurements by using Elemental Analyzer with facilities for analyzing the carbon and nitrogen.

Principle of Analysis

A dried, acidified sample of particulate matter is combusted at 960 °C. The organic carbon is converted to CO₂ and the nitrogen oxides are subsequently reduced to N₂ gas. Both gases are measured by thermal conductivity.

Apparatus

1. Elemental Analyzer
2. High-quality precision Electronic Balance (up to micro gram facility).

Reagents

Hydrochloric acid (concentrated HCl: reagent grade), *Certified reference materials* (CRM).

Sampling

The POC/PN samples are taken after oxygen, CO₂, salinity and nutrient samples have been removed, approximately 30–60 minutes after the CTD/rosette reaches the surface. Settling of large particles in the Niskin bottles will create a non-uniform distribution of the particles within this period of time. For best results, the Niskin bottle

should therefore be shaken before sampling or the entire volume filtered (including the volume below the spigot).

Samples are collected in 4 liter polypropylene bottles equipped with a 1/4" outlet at the base. The filtration is "in-line" with the filter mounted in a Delrin filter holder. The holder is connected to the outlet at the bottom of the 4 liter bottle on one end and a vacuum system (liquid container and pump) on the other. Two liters are normally filtered at all depths (although this volume may not be adequate for all systems) from surface to 1000 m onto precombusted (450°C, 5 hours) 25 mm Whatman GF/F filters (nominal pore size 0.45 µm). The filter is removed, wrapped in pre-combusted aluminum foil and stored frozen in a deep freezer (-20 °C) until processed.

Procedures

Sample Analysis

Prior to analysis, the filters are thawed, allowed to dry overnight at 65 °C in acid washed and pre-combusted (450 °C, 2 hours) scintillation vials and then placed overnight in a desiccator saturated with HCl fumes. The air in the desiccators is kept saturated by leaving concentrated HCl in an open container in the lower compartment of the desiccator. Thereafter, the filters are dried again at 65 °C and packed in recombusted (850 °C, 1 hour) nickel/tin sleeves. The samples are analyzed on Elemental Analyzer following the guidelines given by the manufacturer. A standard reference material should run in every ten samples to estimate the percentage of the errors deviated from calibration.

Standardization and blank determination: L-cystine and blanks (empty Ni sleeves) are measured prior to each batch run of samples (64 samples). A minimum of three empty filters are processed as an ordinary sample and analysed for each cruise as filter blanks. The L-cystine standard is weighed in nickel/tin capsules on Electro balance. Standard weights are usually between 0 and 2.0 mg. The nickel/tin capsule with the standard is put into a nickel sleeve and run on the Elemental Analyzer. The empty filter blanks

should be treated exactly like sample filters except that no sample water is passed through them.

Calculation and expression of results

The POC and PN weights of each of the samples are integrated and estimated automatically provided by the Elemental Analyzer manufacturer supplied with the instrument. The program automatically includes the latest nickel/tin sleeve blank into its calculations. The *in-situ* concentration is estimated:

$$\mu\text{g/kg} = \frac{S - B}{V_{\rho}}$$

Where:

S = the result for the filtered sample

B = the measured filter blank

V = volume filtered (liters)

ρ = density (a function of T, S and P, where T = model temperature at filtration, S = salinity of the sample, and P = atmospheric pressure)

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13. DETERMINATION OF DISSOLVED METALS AND IONS IN SEAWATER

The samples are collected by avoiding local heterogeneity and possible human influence. The samples are collected in 1000 ml pre-cleaned, high density polypropylene bottles that had been copiously pre-rinsed with MILLIQ and sample water before filling the samples. The samples are filtered through 0.45 μm cellulose nitrate membrane filter paper soon after collection. Each filtered sample is divided into two aliquots. One aliquot of 250 ml is kept un-acidified to measure ions and another aliquot is for trace metal analysis without any additions of acid. Water samples are kept in refrigerator (4 $^{\circ}\text{C}$) before analysis and equilibrated with ambient temperature prior to analysis. All the ions and metals samples are analyzed using Ion Chromatograph/ICP-MS with a precision of $\pm 5\%$. The accuracy of the measurement has to be checked by measuring freshly prepared standards of known concentrations made from analytical grade reagents. Report the results up to three places of decimal. As an alternative, samples of 500ml should be sent to ICMAM-PD for analysis of dissolved alkali and alkaline metals, viz., sodium, potassium, calcium, magnesium, chloride, sulphate, iron, manganese, aluminum, copper, lead, cadmium, zinc, chromium and nickel.

14. DETERMINATION OF PETROLEUM HYDROCARBONS IN SEAWATER

The procedure for the determination of dissolved / dispersed petroleum hydrocarbons in sea water is based on similarities between the fluorescence excitation and emission spectra of non-polar organic substances extracted from seawater and those present in most crude and residual fuel oils. These are characterized by a maximum excitation around 310 nm and broad peak around 360 nm in the emission spectrum. These features are primarily a reflection of the complex mixture of compounds containing two or more aromatic rings.

The procedure entails the collection of water from 1 m depth using an amber – glass bottle, extraction of the non-polar organic substances with pure hexane and quantification by fluorescence spectrophotometer. The procedure is not only highly sensitive, but at the same time simple, rapid and straight forward.

Apparatus

1. Amber –glass bottle 2 lit. capacity
2. Measuring cylinder 1 lit. capacity
3. Separatory funnel 2 lit. capacity
4. Separatory funnel 250 ml capacity

Reagents

1. Distilled hexane
2. Anhydrous sodium sulphate

Extraction procedure

Assuming a four litre of water sample, the following procedure is used for extraction. Half the sample is poured into 2 litre separatory funnel (No.1), 50 ml of hexane is then added to the separatory funnel and shaken vigorously, held up right and the cap loosened to allow hexane vapour to escape. The hexane phase is allowed to separate from the water and the water drained into funnel No.2. Then 50 ml of fresh hexane added to funnel No.2 and the extraction process repeated. The second half of the sample is then added to funnel No.1 (which still contains the first 50 ml of hexane) shaken, allowed to separate and the water phase drained into funnel No.2 where it is shaken again, allowed to separate and the water phase drained off and discarded. The hexane extracts in funnels 1 and 2 are combined and dried by adding Na_2SO_4 . The extract is then drained into a clean, glass stoppered bottle and stored for analysis. The quantification of the total petroleum hydrocarbon can be made in the fluorescence spectrophotometer by exciting the sample at 310 nm and measuring the emission spectrum at 360 nm.

Preparation of standards

The quantification of total petroleum hydrocarbon are determined against chrysene standard and expressed in terms of chrysene fluorescence units.

A stock solution is prepared by dissolving 1.0 mg chrysene in 100 ml hexane. From this stock solution a set of (0.1, 0.2, 0.5, 0.7, 1.0, 2.0 $\mu\text{g/ml}$) standards and prepared by serial dilution.

Calculation

The calculation of the sample extract, in chrysene units are read from the chrysene calibration curve and corrected for any blank fluorescence. The original sample concentration in chrysene equivalents is then calculated according to the formula.

$$\text{Concentration of PHC in the sample} = \frac{\text{Concentration in sample extract } \left(\frac{\mu\text{g}}{\text{ml}}\right) \times \text{Extract volume (5 ml)}}{\text{Volume of original sample in litres}}$$

The result is the chrysene equivalent concentration in $\mu\text{g/l}$.

15. DETERMINATION OF TOTAL ORGANIC CARBON IN SEDIMENTS

Preparation of samples

In order to remove salts present, marine mud should be carefully washed with distilled water on a sintered glass funnel (porosity 4). After drying at 105°C the sample should be ground to pass a 100 mesh size.

Preparation of reagents

Procedure

The sediment samples thawed at room temperature, be placed in glass jars with distilled water and acidified with 0.2 N HCl to eliminate the inorganic fraction. The pH be monitored throughout the process. Acidified samples are to be dried at 60°C, homogenized and prepared in capsules for elemental analyses (EA). Accuracy and precision be determined for the Elemental Analyser procedure described for POC and cystine is to be used as the calibration standard.

Calculations

The values will be expressed in % and can be calculated in the same process for POC calculation.

16. DETERMINATION OF HEAVYMETALS IN SEDIMENT AND BIOLOGICAL SAMPLES

Heavy metals in sediment and biological samples can be determined by acid digestion and subsequent quantification by Atomic Absorption Spectrophotometer. The acid digestion would help in removing the organic matter as well as in releasing the metals from particulates. The acid digestion involves two acids viz. Nitric acid and Perchloric acid (4:1). This method is originally developed by Walting (1981).

Apparatus

Beaker, Pipette, Hot plate.

Reagent

Concentrated HNO₃ and Concentrated HClO₄

Sampling

Sediment samples were collected with the aid of cleaned and dried Peterson grab. Sediment samples were transferred from the grab to cleaned polyethylene containers using cleaned plastics spatula. The samples were stored in frozen condition for further analysis. The preserved sediment subsamples were dried at 110°C to constant weight for estimation of metals.

Uniform size group of organisms should be collected and maintained in the filtered sea water for 24 hours to discard all the adventitious substances. Measurements on length, width and weight of the organisms should be made. The required tissue or organs from the animal should be dissected and dried to 60°C in an oven for 24 hours. This should be powdered and dried in a dessicator.

Procedure

Add 20 ml of concentrated HNO₃ to a known quantity of dried samples in the beaker, and leave the mixture for 24 hours. Then the sample has to be evaporated to dryness on a hot plate at 120°C, until the residue turns from yellow to white. After drying add 20 ml of a 4:1 mixture of nitric acid and perchloric acid. Evaporate the mixture to near dryness. If the supernatant solution is either clear or slightly yellow fume the sample of dryness. In case if the supernatant liquid is orange colour, add a further aliquot of nitric and perchloric acid and fume the sample to dryness. Care must be taken while adding perchloric acid as indiscriminate use can lead to explosions. Allow the dry residue to cool and add 10 ml of 10% nitric acid. Transfer the contents to a 20 ml polytop vial and allow this mixture to stand for 2 hours for the residue to settle. The supernatant liquid can also be filtered if necessary, by using cotton. The filtered samples in the vial can directly be aspirated for metal analysis into a AAS or ICP and quantified against a known standards.

17. BIOLOGICAL PARAMETERS

17.1. DETERMINATION OF CHLOROPHYLL

17.1.1. Spectrophotometric Method

Chlorophyll *a* is a common and abundant pigment in all photosynthetic organisms. It is used widely for estimating phytoplankton biomass.

17.1.2. Outline of the Method

A known volume of the seawater is filtered through a synthetic fiber (e.g. Millipore AA) or a glass fiber filter; pigments are extracted from filter in 90% acetone and kept overnight at 4°C and their concentration is estimated spectrophotometrically

17.1.3. Apparatus and Equipment Required

- Filter equipment designed to hold 47 mm diameter synthetic (e.g. Millipore) membrane or glass fiber filters.
- 15 mL Glass stoppered centrifuge tubes
- Spectrophotometer with 10 cm light path cuvette
- Centrifuge
- Stainless steel fine tip forceps

17.1.4. Sampling Procedure and Storage

Sample for chlorophyll estimation can be collected either from surface or sub-surface water in sampling bottle. The water sample should be filtered immediately or it can be brought to laboratory by keeping the bottle in ice box. If filtration is done, the filter should be taken out from the filter base after complete filtration and the filter should be folded and kept in a desiccator at -20°C up to 30 days if analysis cannot be done immediately.

Reagents

1. *90% Acetone*: Take 100 mL distilled water in 1 L volumetric flask and make up the volume to 1000 mL with analytical grade acetone. The reagent should be kept in tightly stoppered bottle and stored in the dark.
2. *Magnesium carbonate*: 1 g of MgCO₃ is added to 100 mL of distilled water and transferred to a wash bottle.

17.1.5. Procedure

1. Seawater sample of 0.5 to 1 L is filtered through a Millipore filter unit containing a membrane or glass fiber filter at $\frac{1}{2}$ atmospheric pressure vacuum. Two to three drops of MgCO_3 is added while the sample is being filtered.
2. After completion of filtration, the filter is removed and proceeded for extraction or stored in desiccators.
3. The filter is placed in a 15 mL centrifuge tube and 15 mL of 90% acetone is added and the tube should be shaken vigorously.
4. The centrifuge tube is tightly closed and allowed to stand overnight, preferably inside a refrigerator in dark for about 20 hrs.
5. It is desirable that the tubes are shaken once more after they have been 1 to 2 hrs after storing in the refrigerator.
6. The tubes are removed from the refrigerator and allowed to warm up in the dark nearly to room temperature.
7. The contents of the tubes are centrifuged at room temperature for 10 minutes at 3000 to 4000 rpm.
8. The extraction time depends on the model of centrifuge and the degree of the clarity obtained.
9. The supernatant is decanted into a 10 cm path length spectrophotometer cuvette without delay.
10. The sample should be kept in the room temperature to avoid misting on the cuvette. The readings are taken at wavelengths 750, 664, 647 and 630nm.
11. Correction for each extinction of small turbidity blank is done by subtracting the 750nm from 664, 647 and 630nm absorption.

17.1.6. Calculation and Expression of Results

The amount of pigment in the original seawater sample is calculated using the equation given below:

$$(Ca) \text{ Chlorophyll } a = 11.85 E_{664} - 1.54 E_{647} - 0.08 E_{630}$$

$$(Cb) \text{ Chlorophyll } b = 21.03 E_{647} - 5.43 E_{664} - 2.66 E_{630}$$

$$(Cc) \text{ Chlorophyll } c = 24.52 E_{630} - 1.67 E_{664} - 7.60 E_{647}$$

Where E stands for the absorbance at different wavelengths (corrected by the 750 nm reading) and Ca, Cb and Cc are the amounts of chlorophyll (in $\mu\text{g/mL}$ if a 1 cm light path cuvette), then

$$\text{mg chlorophyll/ m}^3 = \frac{C \times v}{V \times 10}$$

Where,

v is the volume of acetone in mL (15 mL)

V is the volume of the seawater in liters

Ca, Cb and Cc are the three chlorophylls which are substituted for C in the above equation, respectively.

Notes:

1. Millipore filters have the advantage that they dissolve in acetone completely, give no complications at the centrifugation stage and require no particular precautions during filtration.
2. The magnesium carbonate is added to ensure that the phytoplankton chlorophyll is prevented from becoming acid with the resulting decomposition to give phaeophytin pigments. Care should be taken that the Millipore filtration equipment, centrifuge tubes and spectrophotometer cells are kept free from acid and filters should not be contaminated with acid.
3. During the extraction period pigments are very photosensitive and neither extracts nor the un-extracted filters should be exposed to strong light or else chlorophyll values will be reduced to a small fraction of their initial level in less than 1 hour. Tubes must be stored in complete darkness.

4. Period of darkness should be about 15 to 20 hours. After this time the rate of further extraction is too slow. Pigmented extracts should preferably be kept chilled but they can be kept at room temperature for many hours without deterioration.
5. Centrifuge should be as efficient as possible when Millipore filters are used. In most small centrifuge 3000 to 4000 rpm for about 10 minutes is generally satisfactory but the efficiency should be tested with each instrument used.
6. 15 mL of 90% acetone is used here as the standard extraction volume so as to provide sufficient extract for the 10 cm spectrophotometric cell. However, if this cell requires less than 10 mL to fill it the method can be made more sensitive by adding only 10 mL of 90% acetone.
7. The method depends on highly accurate setting of the wavelengths on the spectrophotometer and these should be checked against standard hydrogen line emissions.
8. There are two chlorophylls C_1 and C_2 . In above equation, the chlorophyll C represents total chlorophyll c .

17.1.7. Reference

Strickland, J.D.H and T. R. Parsons. 1972. A practical handbook of seawater analysis. *Fish. Res. Board, Can. Bull.* No. 167(2nd ed.). 310p.

17.2. DETERMINATION OF PHAEOPHYTIN PIGMENTS

17.2.1. Spectrophotometric Method

Phaeophytin or chlorophyll degradation product may at times form a significant fraction of the total plant pigment in a seawater sample. The degradation products results from the digestion process of zooplankton which converts chlorophyll into phaeo-pigments (phaeophorbide and phaeophytin) as well as from decomposition process due to hydrolytic enzymes in the phytoplankton which may convert chlorophyll into chorophyllide. The absorption coefficient of the latter pigment is the same as the parent chlorophyll and it cannot, therefore be detected by spectrophotometric analysis. The absorption spectrum of the phaeo-pigments is considerably lower in the 665 nm region when compared with the parent chlorophyll. Thus it is possible to determine the amount of phaeo-pigments by measuring the extinction at 665 nm before and after destruction of all chlorophyll in the sample to phaeo-pigments.

17.2.2. Outline of the Method

The extinction of an acetone extract of plant pigment is measured before and after acidification with dilute acid. The change following acidification is used as a measure of the quantity of phaeo-pigments in the original sample.

17.2.3. Apparatus and Equipment Required

- Filter equipment designed to hold 47 mm diameter synthetic (e.g. Millipore) membrane or glass fiber filters.
- 15 mL Glass stoppered centrifuge tubes
- Spectrophotometer with 10 cm light path cuvette
- Centrifuge
- Stainless steel fine tip forceps

17.2.4. Sampling Procedure and Storage

Sample for chlorophyll estimation can be collected either from surface or sub-surface water using appropriate sampling bottle. The water sample should be filtered immediately or the sample can be brought to laboratory by keeping the bottle in ice box. If filtration is done, the filter should be taken out from the filter base after complete filtration and the filter should be folded and kept in a desiccator at -20°C up to 30 days if analysis cannot be done immediately.

Reagents

1. *90% Acetone*: Take 100 mL distilled water in 1 L of volumetric flask and make up the volume to 1000 mL with analytical grade acetone. The reagent should be kept in tightly stoppered bottle and stored in dark.
2. *Magnesium carbonate*: 1 g of MgCO_3 is added to 100 mL of distilled water and transferred to wash bottle.
3. *Hydrochloric acid*: Dilute 10 mL of concentrated hydrochloric acid to 100 mL of distilled water.

17.2.5. Procedure

1. Seawater sample of 0.5 to 1 L is filtered through Millipore filtering equipment containing a membrane or a glass fiber filter at $\frac{1}{2}$ atmospheric pressure vacuum.
2. Two to three drops of MgCO_3 is added while the sample is being filtered. After filtration the filter is removed dry and stored or can be extracted immediately as necessary.
3. The filter is placed in a 15 mL centrifuge tube and 15 mL of 90% acetone is added and the tube should be shaken vigorously. The centrifuge tube is tightly closed and allowed to stand overnight preferably inside a refrigerator in dark for about 20hrs.
4. It is desirable that the tubes are shaken once more after they have been 1 to 2 hrs in the refrigerator. The tubes are removed from the refrigerator and allowed to warm up in the dark nearly to room temperature.
5. The contents of each tube are centrifuged at room temperature for 10 minutes at 3000 to 4000 rpm.
6. The extract time of centrifuging depends on the model of centrifuge and the degree of the clarity obtained. The supernatant is decanted into a 10 cm path length spectrophotometer cuvette without delay.
7. The sample should be kept in the room temperature to avoid misting on the cuvette. For phaeophytin pigment the extinction of the extract at 665 and 750 nm are measured.

8. Two drops of dilute hydrochloric acid is added to the cuvette and mixed by putting the cuvette cover and the extinction is re-measured at 665 and 750 nm.
9. Each 750 nm reading is subtracted from the corresponding 665 nm extinction. The following equations are used to calculate the concentration of chlorophyll *a* and phaeophytin pigment in the sample.

17.2.6. Calculation and Expression of Results

$$\text{Chlorophyll } a \text{ (mg/m}^3\text{)} = \frac{26.7(665_0 - 665a) \times v}{V \times 1}$$

$$\text{Phaeophytin pigment } a \text{ (mg/m}^3\text{)} = \frac{26.7(1.7[665a] - 665_0) \times v}{V \times 1}$$

Where,

665₀ is the extinction at 665 nm before acidification,

665_a is the extinction at 665 nm after acidification,

v is the volume of acetone extract (mL),

V is the volume of the water filtered (liters) and 1 is the path length of the cuvette (cm).

Notes

1. If the extraction of pigment has been made using membrane filters, the addition of a small amount of hydrochloric acid will cause increase in turbidity which is undesirable if it occurs then it should be better to follow the procedure using glass fiber filters.
2. The samples are best mixed by holding a small piece of aluminum foil over the mouth of the cuvette and inverting it several times. The destruction of chlorophyll *a* to phaeophytin is not instantaneous and the sample should be allowed to stand for 4 to 5 minutes before being measured again. The destruction of chlorophyll *a* to phaeophytin is pH dependent and occurs best at pH 2.6 and 2.8 after 3 to 5 minutes.
3. Rinse the cuvette thoroughly with 90% acetone after each determination to ensure that no acid is carried over when the next 665₀ reading is taken.

17.2.7. Reference

Strickland, J.D.H and T. R. Parsons. 1972. A practical handbook of seawater analysis. Fish. Res. Board, Can. Bull. No. 167(2nd ed.). 310p.

17.3.DETERMINATION OF CHLOROPHYLL & PHAEOPHYTIN PIGMENTS

17.3.1. Fluorometric Determination

The fluorometric method is extensively used for the quantitative analysis of chlorophyll *a* and phaeophytin pigments. The procedure described here is appropriate for all levels of chlorophyll *a* concentration in the marine environments. Filtration volumes should be modified for the different environments.

17.3.2. Principle of Analysis

Chlorophyll *a*, fluoresce in the red wavelength after extraction in acetone when they are excited by blue wavelengths of light. The fluorometer excites the extracted sample with a broadband blue light and the resulting fluorescence in the red is detected by a photomultiplier. The significant fluorescence by phaeopigments is corrected for by acidifying the sample which converts all of the chlorophyll *a* to phaeopigments. By applying a measured conversion for the relative strength of chlorophyll and phaeopigment fluorescence, the two values can be used to calculate both the chlorophyll *a* and phaeopigment concentrations.

17.3.3. Apparatus

- Filtration system and Whatman GF/F
- Refrigerator for storage and extraction
- 15 mL glass centrifuge tube
- 4 Turner fluorometer, fitted with a red sensitive photomultiplier, a blue lamp, 5-60 blue filter and 2-64 red filter.

Reagents

1. Acetone
2. 90 % Acetone
3. 1.2M Hydrochloric acid (100mL HCl in 900 mL de-ionized water)

17.3.4. Sample Collection and Storage

Water sample is collected from desired depth using non metallic water sampler e.g, Niskins into clean polyethylene bottles with Tygon tubing. Samples are immediately filtered through 47 mm GF/F filters using polycarbonate in line filters and a vacuum of less than 100 mm Hg. Filters are folded in half twice and wrapped in aluminum foil and can be stored at -20°C (deep freezer) if analysis is not to be carried out immediately.

17.3.5.Procedure

Pigments are extracted by placing the filters in 5.0 mL 100 % acetone. For 47 mm GF/F filters, 0.8 ml of water is retained adjusting the final extraction solution to 86% acetone and the final extraction volume to 5.8 ml. The samples are covered with Parafilm to reduce evaporation, sonicated (OOC, subdued light) and allowed to extract for 4 hours in the dark at -20°C. Following extraction the samples are vortexed and filters are pressed to the bottom of the tube with a stainless steel spatula and spun down in a centrifuge for 5 minutes to remove cellular debris. For fluorometric analysis (not HPLC), decantation can replace centrifuging. The addition of 5.0 ml acetone for pigment extraction is necessary to completely submerge 47 mm GF/F filters in 15 ml centrifuge tubes. This volume should be altered depending on the size of the filter and volume of the extraction tube. The fluorometer is allowed to warm up and stabilize for 30 minutes before use. Before sample measurement, the fluorometer should be adjusted to zero with 90 % acetone for blank correction. Then 1.0 mL of pigment extract is mixed with 4.0 mL of 90 % acetone in a cuvette and read on the appropriate door to give reading between 30 and 100. The sample is then acidified with 2 drops of 1.2M HCl.

17.3.6. Standardization

Chlorophyll *a* standard (Anacystis nidulans, Sigma Chemicals) is dissolved in 90 % acetone for at least 2 hours and its concentration is calculated spectrophotometrically as follows:

$$\text{Chl } a = \frac{(A_{max} - A_{750nm})}{EXI} \times \frac{1000\text{mg}}{1 \text{ gm}}$$

Where,

A_{max} = Absorption maximum

A_{750nm} = Absorbance at 750 nm to correct for light scattering

E = Extinction coefficient for chl *a* in 90% acetone at 664 nm ($87.67 \text{ L g}^{-1} \text{ cm}^{-1}$)

l = Cuvette path length (cm)

From the standard, a minimum of five dilutions are prepared for each door. Fluorometer readings are taken before and after acidification with 2 drops of 1.2M HCl. Linear calibration factor (K_x) are calculated for each door (x) as the slope of the unacidified fluorometric reading vs. chlorophyll *a* concentration calculated spectrophotometrically. The acidification coefficient (F_m) is calculated by averaging the ratio of the unacidified and acidified readings (F_0/F_a) of pure chlorophyll *a*. Samples are read using *a* door setting that produces a dial reading between 30 and 100. The fluorometer should be adjusted to zero with 90 % acetone for blank correction.

17.3.7. Calculation and Expression of Results

The concentration of chlorophyll *a* and phaeophytin pigments in the sample are calculated using the following equations

$$\text{Chl } (\mu\text{g/l}) = (F_m/F_m - 1) * (F_0 - F_a) * K_x * (\text{vol}_{\text{ex}}/\text{vol}_{\text{filt}})$$

$$\text{Phaeo (chl equiv. weights)} = (F_m/F_m - 1) * [(F_m * F_a) - F_a] K_x - \text{vol}_{\text{ex}}$$

Where,

F_m = Acidification coefficient (F_0/F_a) for pure Chl *a* (usually 2.2)

F_0 = Readings before acidification

F_a = Reading after acidification

K_x = Door factor calibration calculation

Vol_{ex} = Extraction volume

Vol_{filt} = Sample volume

17.3.8. Reference

Knap, A., A. Michaels, A. Close, H. Ducklow and A. Dickson (eds.) 1996. Protocols for the Joint Global Ocean Flux Study (JGOFS) Core Measurements.

18. MARINE PHYTOPLANKTON SAMPLING METHODS AND ANALYSIS

Collection, Preservation, Identification and Enumeration

18.1. Introduction

Phytoplankton are microscopic, unicellular, autotrophic plants which are responsible for 90% photosynthesis in the sea and they bring about approximately half of the global (terrestrial and marine) net primary production. Phytoplankton are the primary producers in the sea which form the base of food chain and the ocean ecosystem is entirely dependent on them.

A total of about 4000 marine phytoplankton species have been described. Phytoplankton are the indicators of water quality. Their population and composition can be very well accounted for the prediction of the health of the water in which they are present. They are the direct indicators of human intervention in the marine environment. Any extreme changes in their population or composition can be taken as an alarm signal to check the source of pollution in the system. In such case, they alter the physico-chemical aspects such as pH, color, taste and odor of the water which can be observed in the form of bloom imparting color or sometimes discoloration of water.

Since several years, incidents of harmful algal blooms (HABs) have been encountered throughout the world. Dinoflagellates are the major group responsible for HABs causing anoxic condition and production of toxins. This has resulted in adverse impact on public health, economy and marine environment. Consumption of seafood contaminated by algal toxins results in various seafood poisoning syndromes such as paralytic shellfish poisoning (PSP), Neurotoxic shellfish poisoning (NSP), Amnesic shellfish poisoning (ASP), Diarrhetic shellfish poisoning (DSP), Ciguatera fish poisoning (CFP) and Azaspiracid shellfish poisoning (AZP). Most of these poisonings are caused by neurotoxins with highly specific effects on the nervous system of animals, including humans, by interfering with nerve impulse transmission. Saxitoxin is another neurotoxin produced by certain species of marine dinoflagellate such *Alexandrium* sp., *Gymnodinium* sp. and *Peridinium* sp. Domoic acid, an algal neurotoxin produced by diatoms of the genera

Pseudo-nitzschia can cause short-term memory loss, brain damage and, in severe cases, death.

18.2. Sample Collection

Phytoplankton population study in the marine environment is more difficult than in other systems. In the marine environment, sampling sites are dependent on water current, depth, water column stability, fresh water in flow, river runoff, off-shore sites prone for bloom etc. Accessibility is another important criterion to choose a sampling site. Water samples can be collected from four general areas of the water column such as the surface, integrated mixed layer, discrete depth and the water column profile, using different methods.

18.2.1. Qualitative and Quantitative Sampling

18.2.2. Surface Sampling

Phytoplankton from the surface water should be collected by using plastic bucket hauled from the sampling boat. Sample should be collected by allowing the bucket fully dipped into the water up to 0.3 m deep.

18.2.3. Sub-surface and Deep Water Sampling

Phytoplankton samples from the sub-surface should be collected at the depth of 0.5 to 1 m when the depth of the water column is about 2 to 3 m. While taking the samples from deeper waters, it should be collected at regular depth intervals.

18.2.4. Collection using Nets

Several plankton nets are available which can be used for phytoplankton sampling. Phytoplankton collection can be carried out either directly using the bolting silk cloth No. 25 with pore size 64 μ m. Water is taken in measured quantity (by using graduated container) and filtered through the bag net prepared with bolting silk or monofilament nylon material. Finally the sample is poured into a container by rinsing the bag in little amount of water and should be preserved according to analysis preferences. Bolting silk cloth or Nylon material mesh can be selected and used for construction of various types of phytoplankton nets. This method is more accurate for quantitative analysis of the phytoplankton sample. As the water filtered through the net can be accurately measured.

18.2.5. Standard Plankton Net

The standard plankton net is used for qualitative analysis of the phytoplankton. Simple plankton net is conical in shape. The diameter of the mouth is 15 cm and length of the net is 60 cm. The diameter and the length of the net should be increased according to the requirement. The ratio of net length to the net mouth diameter should be maintained between 3:1 and 5:1. It is made up of metal or plastic ring at its wide end. The narrow end of the net is aided with removable PVC container to collect the plankton sample filtered through the net. The ring at the mouth is bridled down through three nylon ropes tightened finally to the main towing sample in horizontal manner the same net can be towed in horizontal direction at the surface by removing the weight from the distal end. The net used for the purpose is made of monofilament nylon. The size of the mesh can be selected according to the target sizes of the phytoplankton groups. The standard size range is considered from 5 to 20 μm . The speed at which net is towed should be given consideration. The speed of the net towed should be restricted to < 5 knots. The net should be washed frequently after use and allowed to air dry. The condition of the net should be inspected for pin size holes and other wear and tear.

Advantages

- It is relatively less expensive, easy to handle and can be used for different types of surface waters.
- It can be well operated from small powered boat.
- It can be fitted with a flow meter to know the volume of the water filtered to get the semi-quantitative sample.

Disadvantages

- It requires towing through the long stretch of water with not very shallow depth.
- It is suited to qualitative sampling.
- Clogging due to extraneous materials or debris create problem in sampling and result.
- With fine mesh in use there should be reduction in collection efficiency and failure of the equipment due to tearing.

18.2.6. Calculation of the volume of Water Filtered through a net

To quantify the population or density it is essential that the volume of the water filtered through the net should be known. This can be determined approximately by using the formula:

$$V = r^2 \cdot d$$

Where,

V = Vol. of water filtered through net

r = Radius at the mouth of the net

d = Distance through which the net towed

18.2.7. Concentration of Sample

When large volume of water is collected through net sampling in which the number of phytoplankton cells appears less dense, the sample in volume can be reduced using one of the following methods.

- **By settling.** The sample can be kept in a measuring cylinder for settlement after preservation. Later settled portion can be collected by siphoning the top water out by using narrow tube.
- **By using plankton concentrator.** The sample can be filtered immediately after sampling using plankton concentrator. In this, the sample is passed through a stiff tube of PVC or perspex with filter of nylon monofilament net attached at the bottom.
- **By centrifugation.** Sample is centrifuged for 10 to 15 min at 1500 to 2000 rpm. The supernatant is removed by decanting.

18.3. Sample Preservation

To analyze the sample in live condition, it should be stored in a refrigerator in a partially filled container or in ice bags in the dark or at ambient temperature. In this case the samples should be examined immediately. If the samples are to be analyzed later in the lab, it should be preserved appropriately with suitable preservatives. Different types of phytoplankton preservatives are:

18.3.1. Preservation with Lugol's Iodine Solution

Acidic	Alkaline	Neutral	Modified
20 g Potassium iodide(KI)	20 g Potassium iodide	20 g Potassium iodide	10 g Potassium iodide
10 g Iodine(I ₂)	10 g Iodine(I ₂)	10 g Iodine(I ₂)	5 g Iodine(I ₂)
20 g conc. Acetic acid	50 g conc. Acetic acid	200 mL Distilled water	20 mL Distilled water
200 mL Distilled water	200 mL Distilled water		50 mL with 5 g anhydrous sodium acetate dissolved

- Add 0.2 to 0.8 mL of Lugol's solution for 100 mL of sample.
- Preserved samples should always be stored in dark to avoid reaction with sunlight.
- Different types of Lugol's solution can be used according to the requirement.
- Lugol's iodine is suitable for all phytoplankton but acidic Lugol's solution dissolves the coccoliths of Coccolithophores commonly present in marine water and estuaries.
- Modified Lugol's Iodine solution preserves the Coccolithophores but is not suitable for other flagellates.

18.3.2. Preservation with Formaldehyde

Formaldehyde ensures the long term preservation of phytoplankton. It is necessary to fix the phytoplankton sample immediately to prevent any change due to light, temperature, etc.

- Acidified Formaldehyde
- Buffered Formaldehyde
- Neutralized Formaldehyde

Acidic	Buffered	Neutral
20% formaldehyde solution (HCHO)	1 L 37% formaldehyde	20% formaldehyde solution (HCHO)
50 mL Glacial acetic acid (CH ₃ COOH)	20 g sodium borate, Na ₂ B ₄ O ₇ , Maintain pH=7.5	100 gm Hexamethylene tetramine

- For 100 mL of sample 2 mL of Formaldehyde should be added.
- For net phytoplankton formaldehyde is added to make one third of volume of the sample

18.4. Storing of Phytoplankton Sample

To store the sample for long time it should be appropriately preserved and kept in the plastic vials of good quality. Plastic vials should not absorb the color of the preservative. For long time storage, glass sampling bottles should be used in order to minimize the chemical reaction with preservative. Sample stored in glass bottle can be monitored frequently to check the status. The bottle should be tightly closed to avoid spillage and evaporation of the preservative. The storage bottle should be filled to 75-80% of its volume, so that the sample can be homogenized thoroughly before pouring it into sedimentation chamber.

18.5. Labelling of Sample

Labelling of the sample collected in the bottle should be done properly. It should have information such as place of collection, date and replication etc., clearly mentioned in the label so that the sample can be identified accurately. Label should be marked with pencil or water proof marker.

18.6. Sample Processing

18.6.1. Sedimentation

Sedimentation allows phytoplankton to settle down to the bottom of the sedimentation flask. Sedimentation of the sample can be done by using 1 liter conical measuring cylinder or in Utermohl's tubular chamber. Sedimentation should be done at the room temperature and out of direct sunlight. To avoid evaporation the chamber must be covered from the top. The settling time depends on the chamber and the preservative added. Different chambers are used depending on the density of the phytoplankton in the sample. The recommended settling times for Lugol's preserved samples are shown in the table

Chamber volume (mL)	Chamber height approx. (cm)	Settling time (hr)
2	1	3
10	2	8
25	5	16
50	10	24

18.6.2. Preparation of Chamber

Plexiglass tubes should be cut to desired length for the purpose and transparent glass piece can be cemented on the bottom with suitable adhesive. Loose circular glass plate

should be used to close the chamber from the top while processing the sample. These chambers should be from 2 to 50mL capacities while the bottom size remains same. Utermohl's compound chamber volume range from 10 to 50 mL and the top portion is a square shallow chamber of 2 mL capacity and a height of 4 mm and a tubular chamber with square brim similar to that of the bottom chamber with ends opening from both sides and water-tight when placed together.

18.6.3. Filling up of Utermöhl's Chambers

Filling of the sample chamber is done by placing the chamber on a petri plate. The sample is shaken well and the tubular chamber is filled till it overflows. The chamber is closed with cover plate without any air bubble trapped inside. The chamber is then allowed to settle down for observation. In case of the compound chamber, the upper chamber is filled with preserved sample and left undisturbed for minimum of 24 hours for settlement of the phytoplankton. Formaldehyde preserved sample needs setting time of up to 40 hours independent of the chamber size. After sedimentation, the top tubular portion is gently slid off from the bottom plate and replaced by a cover glass. Care should be taken that no air bubble enters at this stage. The bottom plate should be slowly transferred to the inverted microscope.

18.7. Counting

18.7.1. Sedgewick-Rafter Counting Slide

The Sedgewick-Rafter counting slide is a device used for plankton counting. This can be manipulated and provides reproducibility of results with calibrated microscope with eyepiece measuring device. This is a quick method for quantifying samples with high cell numbers. The slide is comprised of a transparent base, which has a centrally mounted chamber (50 mm x 20 mm x 1 mm deep) and can hold 1 mL of sample. The base of this chamber has a ruled 1 mm grid, so that the 1 mL sample is subdivided into single micro liters. This chamber is covered over by a cover glass, which protects the sample from drying out and disturbances by air currents. The sample is then counted using a compound microscope.

$$F = (1000/\text{Number of Squares Counted}) * 1000$$

To obtain a final result expressed as cells L⁻¹ the following equation is used to calculate the multiplication factor (*F*). *F* is dependent on the number of squares of the base of the cell counted during the analysis.

Examples of *F* for the Sedgewick-Rafter slide:

4 rows (200 squares) are counted.

$$\begin{aligned} F &= (1,000/200) * 1,000 \\ &= 5 * 1,000 \\ &= 5,000 \end{aligned}$$

50 rows (1000 squares) or the entire slide is counted.

$$\begin{aligned} F &= (1,000/1,000) * 1,000 \\ &= 1 * 1,000 \\ &= 1,000 \end{aligned}$$

- It allows quick analysis of samples with high density
- It has been proven for its reproducibility results between 10000 and 100000 cells L⁻¹
- It is economical in use
- It is difficult to use this with objectives having high magnification (40X)
- Not suitable for studying nannoplankton

18.8. Sample Identification

18.8.1. Microscopy

Since 150 years several techniques and methods for analysis of phytoplankton have been developed and adopted by the researchers throughout the world. Microscope based methods involve the identification of the phytoplankton based on the morphology. Taxonomists should have hands on experience and high degree of skill and patience for the identification of the species and adequate training should be included in the program. A high quality of microscope is essential for phytoplankton enumeration and identification. The ideal microscope should have phase contrast, oil immersion, and several magnifications (for example - 10X, 40X and 100X). Many species of phytoplankton appear transparent under light microscope. Hence, different techniques should be used to improve the contrast

for observation. Differential Interference Contrast (DIC) and Phase contrast are used. The two commonly used microscope for identification and counting are

The standard compound microscope

- Standard equipment is a set of 10X or 12.5X oculars and 10X, 20X, 40X and 100X objectives. Objectives are used to provide adequate working distance for the counting chamber. Magnification requirements vary with the plankton fraction being investigated, the type of microscope, counting chamber used and optics.

The inverted microscope

- The objectives for the inverted microscope are below a movable stage and the illumination comes from above, permitting view of organisms that have settled to the bottom of the chamber. The advantage of the inverted microscope is that by a simple rotation of the nosepiece a specimen can be examined directly in the settling chamber at any desired magnification.

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19. MARINE ZOOPLANKTON SAMPLING METHODS AND ANALYSIS

19.1. Introduction

Zooplankton (Greek: Zoon, animal; planktos, wandering) are myriads of diverse floating and drifting animals with limited power of locomotion. Majority of them are microscopic, unicellular or multicellular forms with size ranging from a few microns to a millimeter or more. In addition to size variations, there are differences in morphological features and taxonomic position.

In an aquatic ecosystem zooplankton form an important link in the food chain from primary to tertiary level leading to the production of fishery. It has been well established that potential of pelagic fishery either directly or indirectly depend on zooplankton. Herbivore zooplankter are efficient grazers of the phytoplankton and have been referred to as living machines transforming plant material into animal tissue. Hence they play an important role as the intermediaries for nutrients/ energy transfer between primary and tertiary trophic levels. Due to their large density, shorter life span, drifting nature, high taxa/species diversity and different tolerance to the stress, they are being used as the indicator organisms for the physical, chemical and biological processes in the aquatic ecosystem.

Based on the size, the plankton can be divided into following groups (Dussart, 1965).

	Group	Size limits	Major organisms
1	Ultrananoplankton	<2 μm	Free bacteria
2	Nanoplankton	2–20 μm	Fungi, small flagellates, small diatoms
3	Microplankton	20–200 μm	Most phytoplankton species, foraminiferans, ciliates, rotifers, copepod nauplii
4	Mesoplankton	200 μm –2 mm	Cladocerans, copepods, larvaceans
5	Macroplankton	2–20 mm	Pteropods, copepods, euphausiids, chaetognaths
6	Megaloplankton	>20 mm	Scyphozoans, thaliaceans

19.2. Methods of Collection

To investigate long-term variations in species composition and abundance of a plankton community in a particular region, it is best to choose a particular piece of equipment and standard sampling procedure. Such a method must be capable of sampling a wide range of plankton, including species that are both quantitatively and qualitatively representative of that region. Uniformity in sampling methods also makes comparison of the abundance of certain groups of plankton or of total plankton biomass in wide geographical areas possible.

The zooplankton collection involves primarily the filtration of water by net, collecting the water in bottles/ water samplers or by pumps. The sampling success will largely depend on the selection of a suitable gear; mesh size of netting material, time of collection, water depth of the study area and sampling strategy. The gear should be used keeping in view the objectives of the investigation. There are three main methods of zooplankton collection, which are as follows:

19.2.1. Sampling by Bottles

This type of sampling is preferred to sample small organisms- microzooplankton or less than that. Surface water can be obtained by gently scooping into a container of a suitable size from the leeward side of the ship. In inshore waters where plankton is very dense, a 100 ml water sample from surface water should be sufficient, but under the usual conditions of study it is advisable to sample 1-50 liters of water depending on the density of the plankton.

To sample the microplankton at desired depth, different water samplers like Niskin water sampler, Nansen reversing water sampler, Van Dorn sampler etc are used. These samplers work on a simple mechanism- when the sampler reaches to a desired depth; a messenger is sent which strikes the release to close the sampler at that depth with the water sample. If the wire from the ship is not vertical, the depth of the sampler is estimated by measuring the wire angle and the length of the wire paid out by means of a clinometer.

The main advantage of this method is that we can sample an accurate volume of water and the depth measured accurately. The disadvantage is that only small volume of water is sampled. So it is useful for sampling small organisms- unicellular animals and plants. After water has been sampled, the organisms can be separated by using a centrifuge or by pouring the water through a disc of very fine nylon or filter paper. One specialized method is to put a series of drops of water from the samples into a series of flasks

containing sterile sea water in which various nutrients have been dissolved. In a short while, the organisms present in the original drop of water will have increased their numbers many times; picking them and identifying them.

19.2.2. Sampling by Pumps

The pump is normally used on board the vessel or boat. The sampling can also be carried out from a dock. In this method, an open ended inlet hosepipe is lowered into the water and the outlet pipe is connected to a net of suitable mesh size. The net is particularly submerged in a tank of a known volume. This prevents damage to the organisms. The zooplankton is filtered through the net. A meter scale on the pump records the volume of water filtered.

This method is used for quantitative estimation and to study the small scale distribution of plankton. The main advantages of this method are ease of use, accuracy of sampling depth and ability to take accurate quantitative samples. The disadvantage is in limitation in the diameter of the inlet hosepipe enabling the larger organisms to escape the inlet current. The frictional resistance of the sampled water in the hose can cause turbulence; damaging the larger plankton especially the gelatinous forms viz. medusae, ctenophores and siphonophores etc. The sampling depth is limited to a few meters and it is difficult to obtain samples from deeper layers.

19.2.3. Sampling by Nets

The most common method of zooplankton collection is by a net. The amount of water filtered is more and the gear is suitable both for qualitative and quantitative studies. The plankton nets used are of various sizes and types. The different nets can broadly be put into two categories, the open type used mainly for horizontal and oblique hauls and the closed nets with messengers for collecting vertical samples from desired depths.

A net is towed vertically, horizontally or obliquely. Shapes of nets commonly used are (a) conical, (b) conical-cylindrical (conical net with a collar), and (c) conical with a mouth-reducing cone (Fig.1). Rectangular conically shaped nets have also been designed. The net is attached to the wire directly or, in most net designs, with a bridle. At the cod-end of plankton net, a sampling bucket is attached. The cone is made up of nylon mesh or made by monel metal (Ni+Cu).

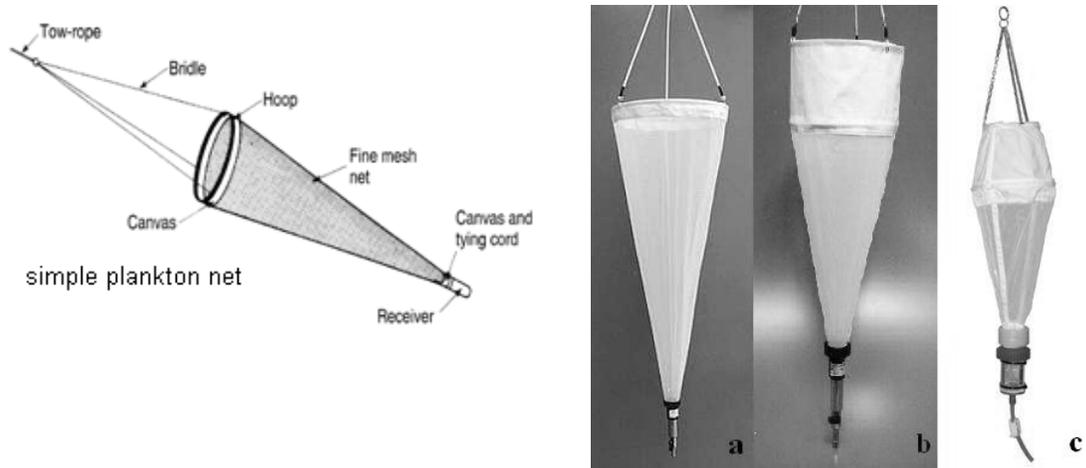


Fig. 1. Shows the different shapes of net simple plankton net, (a) conical, (b) conical-cylindrical and (c) conical with a mouth-reducing cone

In net sampling a comparatively large amount of water is filtered and, as a result, a representative sample of organisms in a given volume is obtained. But certain problems are associated with this method, such as loss of organisms through the meshes, net avoidance, and variation in filtration efficiency. Filtration efficiency differs for different types of nets, and the sampled organisms vary in relation to the mesh size and mouth area of the sampler as well as towing speed. By net sampling we can only measure the average density (often underestimated because of net avoidance or extrusion) of organisms integrated over a certain volume but cannot measure the density or distribution of organisms on small spatial scales.

There is a great variety of mesh available from the finest to the coarse pore sizes. The mesh size of 0.2 mm of monofilament nylon is usually used for collecting zooplankton for taxonomic and productivity studies. In addition to the mesh size, the type, length and mouth area of the net, towing speed, time of collection and type of haul will determine the quality and quantity of zooplankton collected.

The standard UNESCO mesh size for sampling zooplankton is 200 μm mesh (Harris *et al.*, 2000) but, it was found that a 100 μm mesh is useful in estuaries as small zooplankton respond to environmental variability more rapidly than large zooplankton. A major reason for is the belief that samples remain comparable even though the absolute values are biased. Many larval fish biologists use 500 μm mesh, knowing fully well that fish eggs and small, unidentifiable larvae will be extruded through the mesh. Ultimately net size should be determined in accordance with the objectives of the study.

Closing type nets are used to collect samples from various layers separately. Samplers have been devised to close either at the mouth or body or at the cod end bucket.

The choice of net and type of haul to be taken should be determined by the objectives of the study. Whatever type of net is used for sampling, it should be thoroughly washed after each tow so that any planktonic material adhering to the mesh of the filtering cone or other part of the plankton net should be pushed into the collecting bucket to prevent contamination of samples with collections from the previous hauls. The washing of the nets will also prevent clogging especially when there is a bloom or the finer mesh is used for obtaining the samples. The nets should also be checked for torn or holes through which the plankton can escape resulting in the loss of sample. After each haul the zooplankton sample should be transferred into a clean beaker of half to one litre capacity. The debris or extraneous material should be removed. Replicate hauls should be made whenever possible.

For quantitative plankton sampling it is very important to know the actual amount of water passed through the net. A flow meter is used for this purpose. The flow meter has a multi bladed propeller, which is rotated by the flow of water.

19.2.4. Various Types of Plankton Sampler

Various types of net are available to collect zooplankton samples. These are

- Heron- Tranter net
- WP net
- Bongo net
- Indian Ocean Standard Net (IOSN)
- Hensen net
- Clarke-Bumpus Horizontal closing net
- Nansen Vertical closing net
- Beyer's Epibenthic closing net
- Hardy's continuous plankton recorder etc.

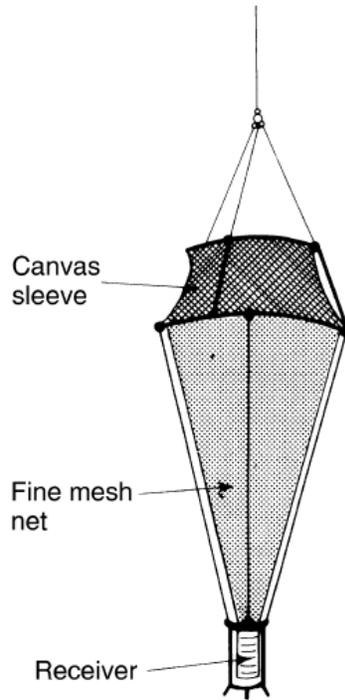


Fig. 2: The Hensen Net

Principal characteristics of some contemporary nets (Omori & Ikeda, 1984):

Net	Mouth area (m ²)	Form	Mesh opening (mm)	Open area ratio
ORI 100	2.00	Conical-cylindrical	1.00	3.8
Indian Ocean Standard	1.00	Conical-cylindrical	0.33	4.3
Large tropical Juday	1.00	Reducing cone	0.45	3.1
CalCOFI Standard	0.79	Conical-cylindrical	0.55	3.2
Bongo	0.38	Conical	0.50	6.8
UNESCO WP-2	0.25	Conical-cylindrical	0.20	6.0
NORPAC Standard	0.16	Conical	0.33	3.7
Marutoku A	0.16	Conical	0.33	1.7
Kitahara	0.05	Reducing cone*	0.10	4.2

*Reducing cone means conical net with a mouth-reducing cone.

- **Indian Ocean Standard Net (IOSN)**

This net was used in the International Indian Ocean Expedition (IIOE), one of the main purposes of which was to study the qualitative and quantitative distribution of planktonic organisms in the Indian Ocean. The design of this net has taken into consideration the requirements and preferences of several nations and of various working conditions. The net consists of a round section of galvanized iron, 2.5cm thick with an internal diameter of 113 cm. Three bridles made of 4 mm diameter hydrographic wire, each 152 cm in length are attached to the ring. The length is 5 m and the mesh size is 0.33 mm. The speed of hauling should be 1 m/sec.

19.2.5. Flow meter Calculation

The flow meter consists of an impeller and a counter. The impeller is directly connected to the counter which records each revolution of the impeller. The flow meter has to be attached to the mouth region of the zooplankton net.

In market, different types of flow meters are available. For calculation the manufacturer provides different formula specific to the flow meter. The flow meter of General Oceanic has a 6 digit counter. The company gives specific formula for each flow meter type for calculation.

The following calculation is given for the Hydro-Bios digital flow meter. This flow meter has a three blade impeller which is coupled directly to a five digit counter. The pitch of the impeller is 0.3 meter per each revolution. Therefore the number of revolutions multiplied by 0.3 gives the towing distance in meters.

Example 1: How to calculate towing distance in metre?

Initial reading of the flow meter I = 00121

Final reading after towing F = 00280

No. of revolutions (F-I) = 00280 – 00121 = 159

Towing distance = 0.3 X (F-I) = 0.3 X 159 = 47.7 metre

Then Volume of water passing through the net is to be calculated. For this, formula of volume of the cylinder ($\pi r^2 h$) is used.

To find out the volume of the water passed through the net by applying the formula $\pi r^2 h$

Where r = radius of the net (mouth region)

πr^2 = Mouth area of the net

h = Towing distance (Length of the cylinder)

Volume of water = Mouth area of the net X Towing distance(m)

Example 2:

Let the diameter of net (mouth region) = 0.5 metre

Radius = $0.5/2 = 0.25$ metre

Towing distance = 47.7 metre

$$\begin{aligned}\text{Volume of water passed through the net} &= \pi r^2 \times 47.7 \text{ m} \\ &= 22/7 \times (0.25)^2 \times 47.7 \\ &= 9.37 \text{ m}^3\end{aligned}$$

To express the biomass and population per m^3 , the biomass and population should be divided by the volume of the water passed through the net.

Note: For calculation, all the numbers should be in same units (meter or cm or km). For conversion of volume in m^3 to Litre: $1 \text{ m}^3 = 1000$ Litre

19.3. Method of Zooplankton Preservation

Zooplankton intended for taxonomic study need to be narcotized, fixed and preserved immediately after being caught. Otherwise autolysis, bacterial action, cannibalism or chemical deterioration will set in. Narcotics which are soluble in either water or lipids are mainly used with zooplankton to prevent their contraction and distortion at fixation, thereby ensuring ready identification of the organisms preserved. The different narcotizing agents are magnesium chloride, carbonated water, chloroform, methyl alcohol etc.

The different preservatives used for preserving zooplankton samples are formaldehyde (4-5%), ethanol (70%), isopropanol (40%) etc. Formaldehyde is the cheapest

among the known preservatives. The commercial formalin usually comes as 37 - 40%. Add formalin to zooplankton sample in the ratio of 1:7 or 1:8 (formalin: sample volume) to make final concentration of 4-5%. As the commercial formaldehyde has considerable acidity, it is always advisable to use the formaldehyde solution after neutralizing it. To neutralize the acidity of formaldehyde solutions, excess CaCO_3 should be added when the pH of these solutions rises to 7.

It would be better to store the preserved zooplankton samples in well ventilated room at temperature less than 25°C . The samples should be kept in the wide mouth jars. A good quality preprinted labels, on which the collector's name, fixative and preservative used and other field information are written should be put into the jars for ready reference at the time of sample analysis.

19.4. Method of Zooplankton Analysis

The basic analysis consists of measurements of biomass (standing stock), enumeration of common taxa and species.

19.4.1. Measurement of Biomass

The term biomass denotes the live weight or the amount of living matter present in the zooplankton sample. The value obtained is used to evaluate the secondary productivity and fishery potentials of the study area. The biomass is estimated by the following methods.

- *Volumetric (displacement volume and settling volume) method*
- *Gravimetric (wet weight, dry weight and ash free dry weight) method*
- *Chemical method*

Prior to determination of biomass, larger zooplankters such as medusae, ctenophores, salps, siphonophores and fish larvae should be separated from the zooplankton sample and their biomass taken separately. The total biomass would be the biomass of bigger forms plus the biomass of the rest of the zooplankton.

Volumetric method

The volume measurements are easy to make in the field or laboratory. The total zooplankton volume is determined by the displacement volume method. In this method the zooplankton sample is filtered through a piece of clean, dried netting material. The mesh size of netting material should be the same or smaller than the mesh size of the net used for

collecting the samples. The interstitial water between the organisms is removed with the blotting paper. The filtered zooplankton is then transferred with a spatula to a measuring cylinder with a known volume of 4 % buffered formalin. The displacement volume is obtained by recording the volume of fixative in the measuring jar displaced by the zooplankton. The settled volume is obtained by making the sample to a known volume in the measuring jar. The plankton is allowed to settle for at least 24 hours before recording the settled volume.

Gravimetric method

For the gravimetric method, the weight measurements should be done preferably in laboratory. It is carried out by filtering the zooplankton. The interstitial water is usually removed by a blotting paper. While blotting, due care should be taken not to exert too much pressure as to damage the delicate organisms or specimens. The zooplankton weight is taken on predetermined or weighed filter paper or aluminum foil. The wet weight is expressed in grams. The dry weight method is dependable as the values indicate the organic content of the plankton. Analysis such as the dry weight is determined by drying an aliquot of the zooplankton sample in an electric oven at a constant temperature of 60°C. The whole or total sample shouldn't be dried because subsequent analyses such as enumeration of common taxa and identification of the species wouldn't be possible after drying the sample. The dried aliquot is kept in a desiccator until weighing. The values are expressed in milligram. Ash free dry weight method is also occasionally used for biomass estimation.

Chemical method

In this method, the live zooplankton samples are dry frozen. Before analysis, the samples are rinsed with distilled water. Measurement of constituent elements such as carbon, nitrogen, phosphorus and biochemical elements viz. protein, lipid and carbohydrates are made. Sometimes the biochemical values of a particular taxon and species are undertaken to evaluate food energy transfer at higher trophic levels. The calorific content of the plankton can be used as an index of zooplankton biomass.

19.4.2. Enumeration of Zooplankton Population

Information on the faunal composition and the relative abundance of different zooplankton taxa and their species is obtained by counting the plankton present in the samples. The enumeration of specimens in the total sample is laborious, time consuming and mostly impractical. The number of common zooplankton groups and their species

observed in the samples may vary from tens to thousands. For enumeration it is recommended that the sub-sample or an aliquot is taken for the common taxa. However, for the rare groups, the total counts of the specimens in the samples should be made. For enumeration of zooplankton the sub-sample or aliquot of 10 to 25% is usually examined. However, the percentage of aliquot can be increased or decreased depending on the abundance of zooplankton in the sample.

Abundance determination should be done following the coefficient of variation stabilization technique, as this ensures that all enumerated taxa are determined with equal precision with the minimum required analytical time (Alden et al., 1982). With this technique, a given sample is split into sub-samples until the 3 most numerous species are present in numbers of at least 30 animals per sub-sample. All organisms in the sub-sample are then counted, including all rare species. Alden et al. (1982) supply a table in which the count for each species in this sub-sample can be looked up, and the sub-sample needed to obtain a count of at least 30 individuals per species can be read off the chart (i.e., the $\frac{1}{2}$ sub-sample, the $\frac{1}{4}$ sub-sample, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, etc.). Thus, each of the rare species can be counted to the minimum required precision (i.e. 30 animals per sub-sample) by counting only one more sub-sample. Counting 30 animals per sub-sample gives a 95% confidence limit of $\pm 30\%$ of the mean.

Identification should be made to the species level, following the latest taxonomic authority. In case of doubt, type specimens should be sent via mail to experts for verification. In all cases a reference collection should be established and maintained in small glass vials.

19.4.2.1. Sub-sample for Zooplankton Analysis

Count entire samples having low zooplankton number (<200 zooplankter) without sub sampling. However, most zooplankton samples will contain more organisms than can be enumerated practically; therefore, use a sub sampling procedure. Before sub sampling, remove and enumerate all large uncommon organisms such as fish larvae, coelenterates, decapods, etc. Sub-sample by the pipette or splitting method.

In the pipette method, adjust sample to a convenient volume in a graduated cylinder or Inhofe cone. Concentrating the plankton by using a rubber bulb and clear acrylic plastic tube with fine mesh netting fitted on the end is convenient and accurate. For picoplankton and the smaller microzooplankton, use sedimentation techniques. Transfer sample to a

beaker or other wide mouth vessel for subsampling with a Hensen-Stempel or similar wide-bore pipette. Gently stir sample completely and randomly with the pipette and quickly withdraw 1 to 5 mL. Transfer to a suitable counting chamber.

Alternatively, sub-sample by splitting with any of a number of devices. Instruments are available for splitting the sample into the fractions. These are generally made of plastic with internal partitions. Folsom plankton splitter is widely used and its level should be checked before use.

The zooplankton sample to be sub-sampled is poured into the drum and the drum is rotated slowly back and forth. Internal partitions divide the samples into equal fractions. The fraction should be poured again into the drum for further splitting. The process is repeated until a suitable sub-sample is obtained for counting. The splitter is thoroughly rinsed to recover the organisms, which should be sticking onto the wall of the drum. The sample is usually splitted into 4 sub-samples. One of the sub-samples is used for estimation of dry weight, the second for counting the specimens of common taxa, the third for relative abundance of species and the fourth fraction is kept as reference collection. Plastic or glass pipettes are also used to take the sub-sample for counting. The Stempel pipette is used to obtain a certain volume (0.1 to 10 ml). The zooplankton sample in a glass container is diluted to a known volume and is stirred gently. The Stampel pipette is then used to remove the sub-sample or aliquot for counting.

Another method permits abundance estimates of more equivalent levels of precision among taxa than obtained with either the Hensen-Stempel pipette or the Folsom splitter. Normal counting procedures tally organisms on the basis of their abundance in a sample. Therefore, in a sample with a dominant organism making up 50% of total numbers, the tally of dominant taxon will be large and may introduce a small error. However, error about the subdominants will increase as the tally of each taxon decreases. By accepting on a level of precision, the technique has been developed to obtain the same error about dominants and subdominants, permitting quantitative comparisons between taxa over successive times or between stations.

19.4.2.2. Enumeration of Zooplankton under Microscope

Using a compound microscope and a magnification of 100X, enumerate small zooplankton (protozoa, rotifers, and nauplii) in a 1- to 5- mL clear acrylic plastic counting cell fitted with a glass cover slip. For larger, mature microcrustacea use a counting chamber

holding 5 to 10 mL. A Sedgwick-Rafter cell is not suitable because of size. An open counting chamber 80 by 50 mm and 2 mm deep is desirable; however, an open chamber is difficult to move without jarring and disrupting the count. A mild detergent solution placed on the chamber before counting reduces organism movements or special counting trays with parallel or circular grooves or partitions can be used. Count microcrustacea with a binocular dissecting microscope at 20X to 40X magnification. If identification is questionable, remove organisms with a microbiological transfer loop and examine at a higher magnification under a compound microscope.

19.4.2.3. Expression of Zooplankton Population Result

After enumeration, report the smaller zooplankton count as number per litre (Nos./l) and larger zooplankton count as number per cubic meter (Nos./m³).

Example: 1 To find zooplankton population in whole sample

Count in 5ml = 200

Count in sub-sample = 200 * 20 (If the volume of sub-sample is 100ml)
= 800 Nos.

Count in whole sample = 800 * 4 (No. of splitted sub-samples)
= 3200 Nos.

<p style="margin: 0;">Count in whole sample</p> <p style="margin: 0;">Report the count as Nos./m³ = -----</p> <p style="margin: 0; text-align: center;">Vol. of water filtered through the net</p>

Example: 2 To find zooplankton count in Nos./m³, calculate by following steps

zooplankton count in Nos./m³

= 3200 / 9.37 m³(Vol. of water)

= 342 Nos./m³

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20. MARINE BENTHOS SAMPLING METHODS & ANALYSIS

20.1. Introduction

The term “Benthos” refers to the benthic community that lives on or in the bottom of any body of water. They are divided into two groups, namely infauna and epifauna. Infaunal organisms live in the sediment and their numbers and kinds are reliable and sensible indicators of benthic habitat. Epifauna live on the sediment surface and are usually associated with surface structures such as shells, vegetation and animal colonies. The composition of benthic communities differs substantially according to sediment type conventionally; these organisms are sub-divided on the basis of size (Table 1, from McIntyre, 1978). Size categories, though somewhat arbitrary, identify the major functional groups of organisms, each of which requires different approaches to sampling and analysis.

Table 1. Size categorization of benthos (from McIntyre, 1978)

Category	Size	Biological features	Sampling techniques	Taxonomic position
Microbenthos	Pass finest sieves	High rates of respiration and reproduction	Plating and culturing. Cores of < 2 cm diam.	Most Protozoa
Meiobenthos	Pass 0.5-1 mm sieves	Medium respiration rates Two or more generations per year	Cores of 2 – 10 cm diam.	Large Protozoa Small Metazoa
Macrobenthos	Retained on 0.5-1 mm sieves	Low respiration rates. Two or less generations per year. Mostly infauna	Grab sampling at least about 0.1 m ²	Medium-sized Metazoa
Megabenthos	Handpicked from samples	As above, mostly epifauna	Towed gear, trawls, dredge	Large Metazoa

Macrobenthos, the ones which are retained on 0.5 mm mesh size (polychaetes, bivalves, gastropods, isopods & amphipods).

20.2. Sampling Methods

20.2.1. Macrobenthos -Sub-tidal sampling

Benthos sampling is most commonly carried out using a grab or corer. The nature of the sea bed will determine the type of sampling apparatus. For soft bottom substrates, a grab of standard design is an appropriate sampler. A van Veen grab (biting area of 0.1 m² or 0.025 m²) can be used to take sediment samples from the sea floor. The grab is lowered to the sea bed on a steel cable or nylon rope with its "jaws" open. As soon as the buckets touch the bottom, the valve keeping them open is released. As the grab is pulled back the jaws close, scooping up sediment from the seafloor. The grab is opened over a sieving platform on the ship and the contents are sieved through 500 micron (0.5 mm) sieve. For coarse sediments, 1 mm sieve is used prior to a 0.5 mm sieve. Flow chart of sampling and analysis of marine benthic fauna are shown in Fig.1.

Van Veen grab can be employed as a standard benthos sampler, since it is (i) an efficient sampler for the range of soft sediments encountered in the near shore area, (ii) reliable and simple to operate and (iii) widely applied, which allows data comparison with other marine areas. Grabs should be equipped with hinged inspection ports. The biting depth of grabs can vary with sediment conditions. Weights can be added to adjust according to the sediment conditions.

The survey vessel should be appropriately and adequately equipped for bottom sampling, with sufficient deck space. The size of vessel required should be chosen as appropriate to the conditions in the sampling area and the type of sampling gear to be employed. In all cases where heavy sampling gear is deployed the vessel must be fitted with a suitable power winch, wire of the appropriate dimensions rigged to a meter wheel and an 'A' frame or gantry. The vessel should also be equipped with echo-sounder and satellite global positioning device.

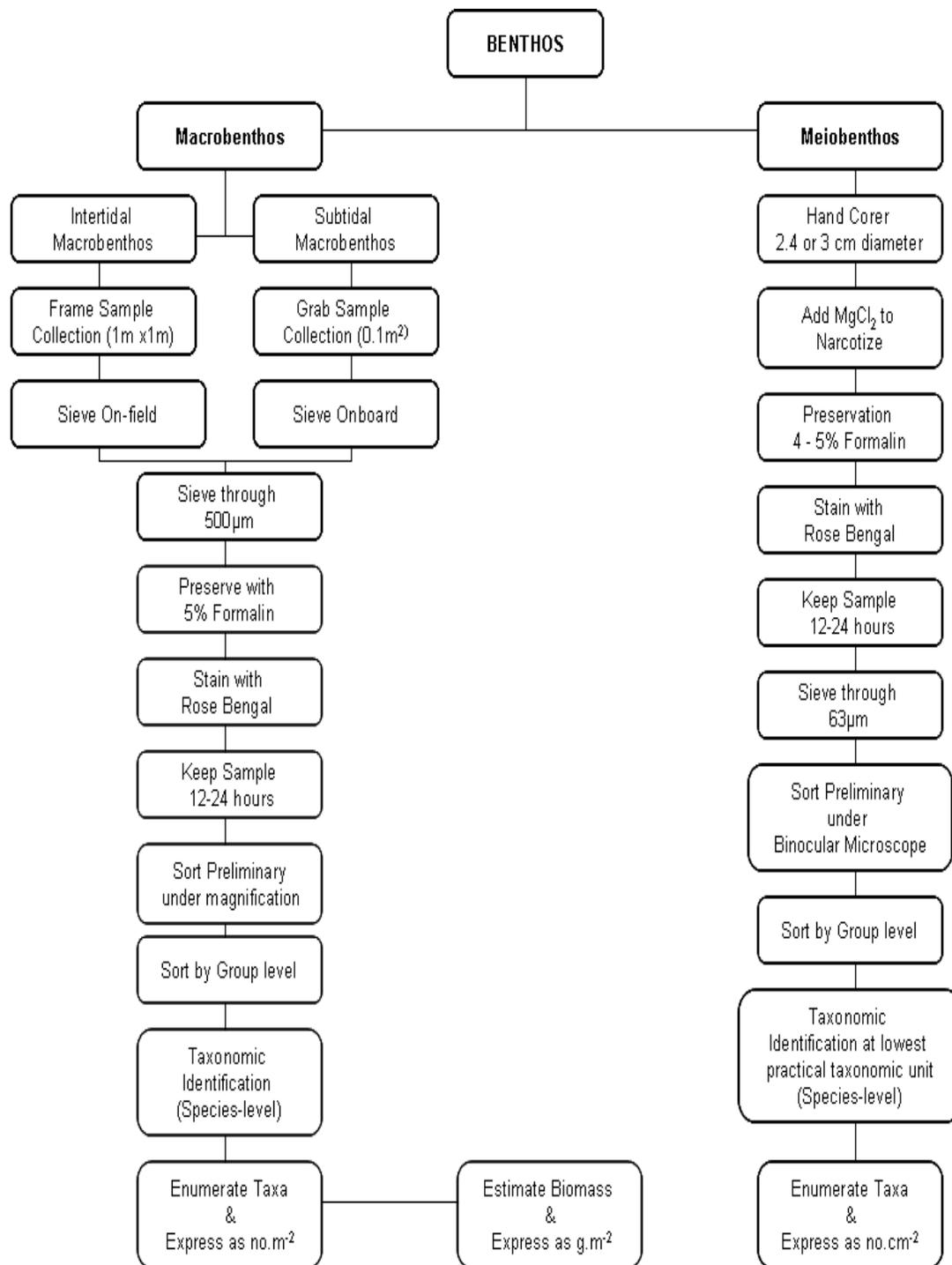


Fig. 1. Flow-chart of sampling and analysis of marine benthic fauna

20.2.2. Macrobenthos - Inter-tidal Sampling

Intertidal macrobenthos samples are collected by frame-quadrate (1x1m; divided to squares of - 25cm²) (Fig.2) methods at two transects parallel to the coast and also maintaining these transects laid left and right side of the sewage outlet/land run off in to the bay/ open sea.

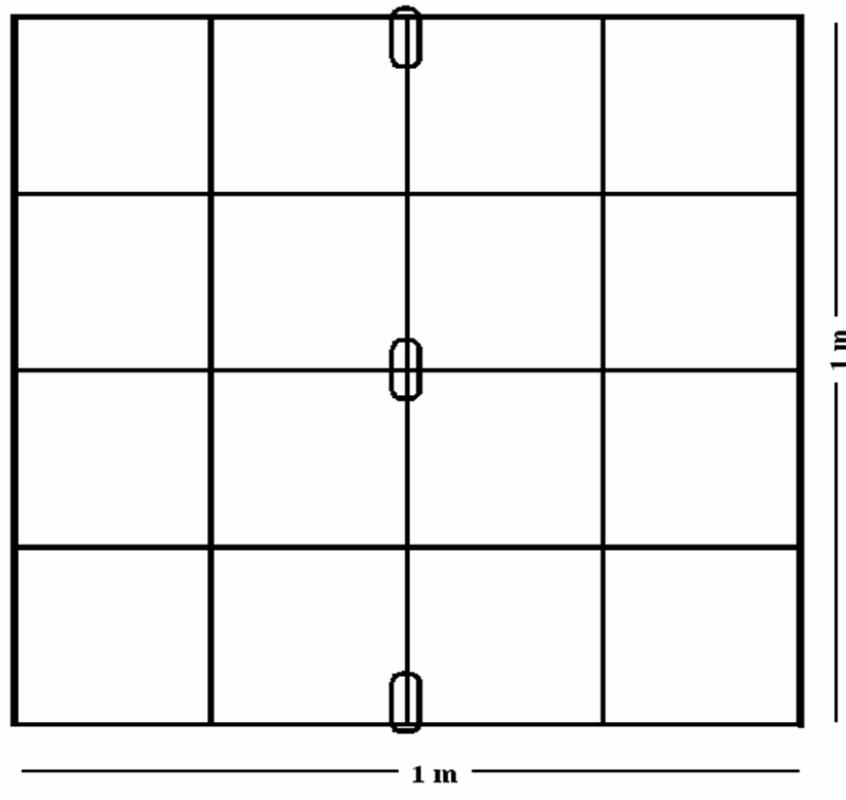


Fig. 2. Quadrate frame (1m x 1m) for inter-tidal macrobenthos sampling

In the case of flat beach (i.e. where the distance between high-water line and low-water line is appreciably high), three frame-samples are collected to cover the horizontal distribution of benthic organisms. Each transects samples are collected from high-water line; mid-water line and low-water line (Fig.3, see also Fig.4).

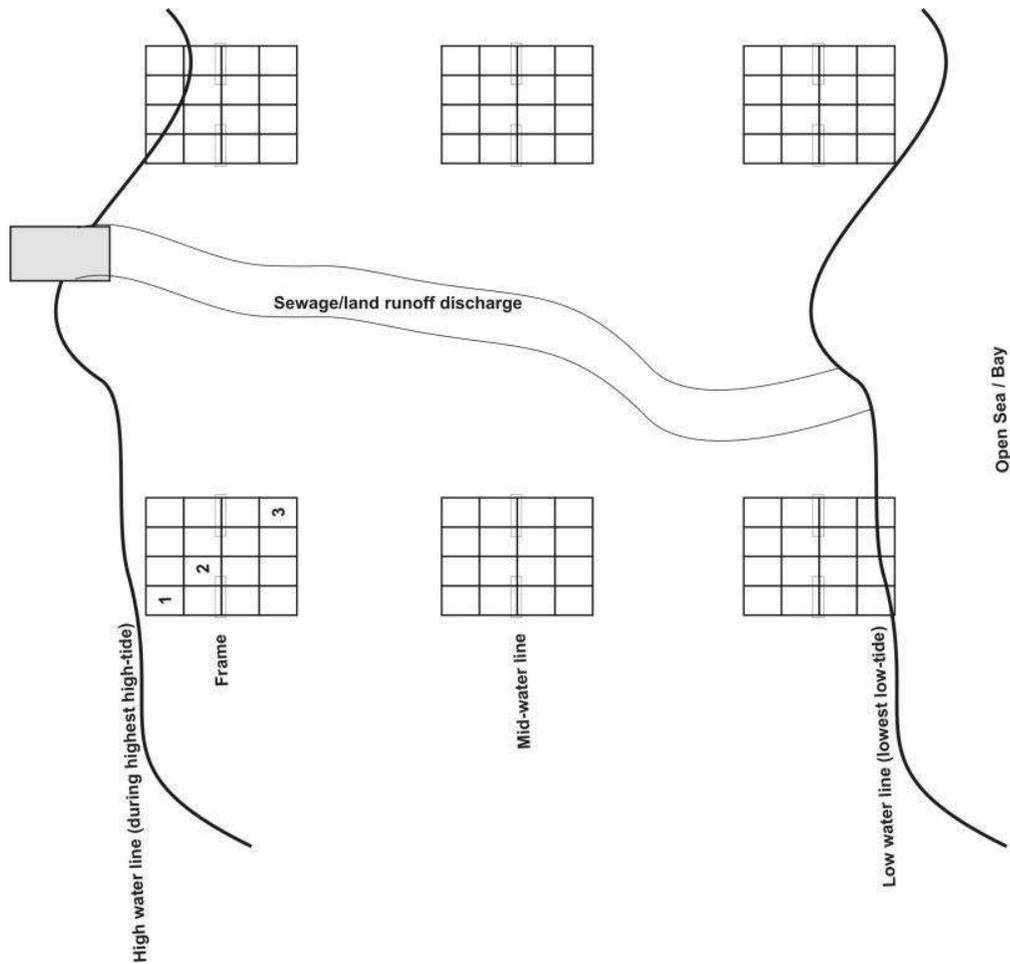


Fig. 3. Inter-tidal sampling of macrobenthos by quadrat frame method

Positioning Equipment

A Differential Geographical Positioning System (DGPS) with monitor should be used in all sea areas if possible. If the differential is not available, accuracy should be assessed and a minimum of Global Positioning System (GPS) should be used.

Personnel

Van Veen Grab can be operated by two survey staff in addition to a winch operator. At least one of the survey team should be experienced with handling grabs and have experience of sampling and sieving marine invertebrates.

Ship-Board Routines

Sampling on shallow stations (70 m or less) is recommended to be conducted during daytime, since some benthic species have semi-pelagic activity during the night.

Grab Deployment

At each site the grab should be set down gently at a speed that avoids triggering the mechanism. The winch wire should remain vertical (wire angle must be kept as small as possible) to ensure an even bite of the grab. In the case of deep or fast-moving water this may require additional weights on the grab and maintaining position by motoring into the current or anchoring. Between approximately 5 m and 10 m above the sea floor, the lowering speed should be decreased (< 0.5 m/s) in order to further reduce the bow-wave and water turbulence in front of the grab. Contact with the sea floor is observed by the slack on the wire, after which the grab is gently raised for approximately the first 5 m. Then the recovery can proceed with maximum safe speed.



Fig.4. Area covered



Fig.5. Views of benthic sample collection

Appropriate equipment for receiving and processing the samples should be ready on deck. On retrieval the grab should be placed on stable landing table. The sample should be examined for adequacy via the top inspection ports immediately upon retrieval on deck. If the sediment depth in the grab is less than 7 cm in mud or 5 cm in sand, the sample is rejected. If sediment characteristics make it impossible to collect approved samples, the best available samples should be retained, and the circumstances noted in the field record.

The faunal samples should be gently decanted into a receiving container (barrel). The grab is to be rinsed thoroughly before redeployment. Each laboratory shall regularly check the exact sampling area of its grab in order to make possible a correct calculation of the number of individuals per square metre. (The area of the grab has a tendency to increase,

especially when sampling in stiff clayey sediments). Sediment characteristics and background information should be recorded before sieving.

Sieving

Approved samples should be sieved in the field using seawater to remove the fine sedimentary material. Each sample must be sieved, stored and documented separately (Fig. 5)

The standard sieve shall be of metal gauze (stainless steel, brass or bronze) and have a mesh size of 1.0 x 1.0 mm. In order to collect quantitatively developmental stages of the macrofauna and abundant smaller species (longer but thinner than 1 mm) it is, however, recommended using an additional sieve with mesh size of 0.5 x 0.5 mm. The additional sieve also ensures against lost of specimens when sieving because of using too high water pressure. The mesh size of the sieves has to be checked from time to time for damage and wear. The use of large sieves is encouraged because:

- the risk of clogging is kept low;
- the risk of spilling is reduced when transferring samples from the receiving container to the sieves.

Water should be added gently to the receiving container to produce a water sediment suspension. The use of sprinklers and hand-operated douches to suspend the sample is recommended. Very stiff clay can be gently fragmented by hand. The sample is transferred in small quantities to the sieve as a sediment-water suspension.

The sieving of the sample has to be done carefully in order to avoid damage of fragile animals. Sieving is done by washing the material in the sieve with gentle jets of seawater and shaking by hand. Deck hoses must be provided with shower nozzles. Visible fragile animals, e.g. some polychaetes, echinoderms, etc. or large, heavy molluscs shall be hand-picked during the sieving, placed in separate plastic bags/jars and fixed before being placed in the container along with the rest of the sample. Stones and big shells should be picked out and kept in separate containers or discarded if devoid of encrusting fauna to avoid the grinding effect.

In order to reduce damage of delicate organisms sieving should be done by placing the sieve in a water bath deep enough to cover the mesh screen and “paddled” until the sediments are washed out. However, this process is time consuming and therefore is not

recommended in case of limited resources. Furthermore, long duration of sieving time should be avoided because small animals may actively pass through the sieve.

All residues retained on the sieve should be carefully flushed off the sieve with water from below into appropriate sample containers (e.g. plastic jars, plastic buckets with watertight lids).

Between the sample portions the sieves must be checked and cleared of trapped fauna and any sediment to avoid clogging and thus to ensure an equal mesh size during the whole sieving procedure.

According to the UKNMMP Green Book (2003) samples should be sieved to 0.5 mm and 1 mm fractions either in the field or in the laboratory and analysed separately. Whether separated in the field or laboratory, the sieving method employed should remain consistent from year to year.

Separation of 0.5 mm and 1 mm fractions in the laboratory is recommended because:

- The time for sieving a sample onboard is kept shorter, which is important in time-limited cruises and in bad meteorological conditions (rain, cold, rough sea).
- Since the fractions must be kept separately, onboard separations will double the number of specimen containers, which is inconvenient for storage and transportation.
- The risk of mixing up fractions from different samples increases if a sample is split in two specimen containers (for two fractions) onboard.

Fixation

Samples (hand-picked animals and the sieving residue) should be fixed as soon as possible after sieving using buffered 37-41 % formaldehyde (formalin). For small sample volumes, where no particularly large animals, 4% formaldehyde:seawater solution should be appropriate. Where the sample contains debris, tube-dwelling polychaetes, large animals or a lot of residual sediment, especially in compact clay sediments, formalin concentration should be increased to 10 % even 20 %.

There should be at least the same amount of solution in the sample container as solid material. Large shells should be opened to allow the fixative to penetrate to animal tissues.

Fixative and sample material should be gently mixed by stirring or inverting the sample containers.

For buffering, 100 g of hexamethylenetetramine (Hexamine = Urotropin) shall be used per 1 dm³ of 40% formaldehyde or sodiumtetraborate (= Borax) at a ratio of 1.5g/dm³ formaldehyde. Buffering is necessary to prevent the leaching of calcium from shell material within the sample.

All necessary measures should be taken to avoid health damage by formalin.

Staining

Staining facilitates the sorting process and increases the sorting efficiency. However, over-staining may hinder identification of species. Staining is optional according to staff preference.

Rose Bengal (1 g/dm³ of 40% formaldehyde), which turns animal protein red is added to the fixation fluid. Alternatively the stain can be applied in the laboratory, where the sample should be washed free from formalin and then immersed in stain (1 g Rose Bengal/dm³ of tap water + 5 g of phenol for adjustments to pH 4-5) for 20 minutes.

Labelling

The sample containers should be indelibly pre-marked with the unique sample information (station designation, sample number, replicate number and date) externally. In addition samples should be properly labelled internally. The information filled in labels should be sufficient to identify the sample with certainty. The mandatory fields are date, station designation, depth, sample number, replicate number (additionally the cruise and vessel designations, type of grab, time, sediment type, etc. should be indicated). Labels made of heavy weight and chemically resistant paper should be filled in with a soft carbon pencil, which will not fade in Formalin. Filled in labels are placed inside the sample containers.

Sample Registering

Samples should be properly registered in sample recording sheets (the standard pro-forma for on-site records is given below:

The following information should be recorded in the field:

- project or contract identifier (code);
- geographical coordinates for each sampling station;
- type of positioning system and its accuracy;
- whether or not a buoy was used;
- whether or not the ship was anchored;
- date and time of each sampling station/sample;
- the water depth from which the sample was taken (if more than one sample is taken at a station, the depth range of samples should be recorded);
- the name, type and sampling area of the sampler;
- sieve mesh aperture sizes;
- number of replicate samples;
- depth of sediment in grab as a measure of sample volume;
- comments such as rejected/unapproved samples together with the causes;
- a visual sediment description, including:
 - a description of sediment type (e.g. sand, silt, clay, etc. and their relative proportions), including important notes, e.g., main groups of large, easily visible species present, occurrence of concretions, stones, dead shells, etc.;
 - surface colour and colour change down the sediment profile, if visible;
 - smell, e.g. presence and severity of H₂S;
 - anthropogenic debris, rubbish, plastics.
- near-bottom temperature and salinity;
- person responsible for sampling.

20.3. LABORATORY ANALYSIS

Sorting and Taxonomic Identification

For qualitative enumeration, each sample is examined under a binocular microscope. The organisms are separated into different taxonomic groups for further identification. All taxa are identified to their specific, generic or other higher levels to the extent possible with the help of standard taxonomic references (e.g. Polychaeta: Fauvel, 1953; Day, 1967; Decapoda: Alcock, 1985; FAO Species Catalogue, 1991; Sethuramalingam and Ajmal Khan, 1992; Jayabaskaran, et al., 1999; Caprellids: Guerra-García et al (pers. comn.) Mollusca: Satyamurty, 1952; 1956; Cernohorsky, 1967, 1972a & 1972b, Abbott and Dance, 1982; Oliver, 1984; Subba Rao *et al.*, 1991 & 1992; Apte, 1998; Subba Rao and Dey, 2000; Pisces: Smith and Heemstra, 1986).

The three major taxonomic groups – Polychaeta, Mollusca and Crustacea should be identified to the species level. Since these are the richest groups and generally contribute mostly to the abundance and biomass of macrozoobenthos. Anthozoa, Echinodermata, Cephalochordata, Phoronidea and Pantopoda shall also be identified to the species level, since sufficient taxonomic expertise and keys are available. Nemertini, Turbellaria, Oligochaeta, Chironomidae and insects in general should be identified to higher taxonomic level (Phylum or Class) for routine monitoring purposes.

Taxonomic guides and keys used for the identification of organisms should be reported with the data. A taxonomic reference collection should also be available for training and verification purposes.

Abundance Determination

Broken animals shall only be counted as individuals by their heads (e.g. polychaetes) or hinges of bivalves with adhering pieces of tissue. Taxa that are not sampled quantitatively or that are not truly indicative of sediment conditions shall not be quantified but their presence should be noted. These taxa include Foraminifera, Nematoda, planktonic organisms, benthic fish, and colonial epifauna (Porifera, Bryozoa, etc.).

20.4. Meiobenthos

The main difference between sampling of macrobenthos and meiobenthos is the much higher numerical density of the Meiobenthos. Smaller samples are usually adequate to study the meiobenthic taxa. In general, a diameter of 2.5 to 4 cm corer (tube or pipe) has been found to cover the entire sample. From each grab haul, duplicate samples of meiobenthos are collected with the help of transparent glass or PVC hand corer. The hand corer is gently inserted or tapped into the sediment of grab haul. The sediment sample inside the corer is removed slowly with the help of a plunger. The sediment core is divided into two halves 0 - 5 cm and 5 - 10 cm to find out the vertical distribution of meiobenthic fauna. The samples are collected in a container with narcotizing agents (e.g. Magnesium chloride solution). After one hour, slowly add 4 % formaldehyde containing Rose Bengal stain in the sample. It will take 24 hours for staining the organisms. Sorting is performed at group level under binocular microscope. Taxonomic identification of meiobenthic organisms is carried out using standard taxonomic literature (Giere, 1993; Atkins, 2002).

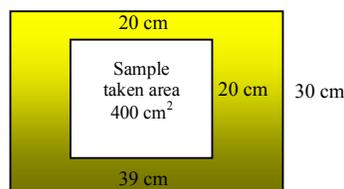
20.5. Biomass

Prior to sorting (identification), wet weight of each sample is determined using a mono-pan electronic balance. The biomass (wet weight) is estimated and expressed as gm m^{-2} .

20.6. Calculation

20.6.1. Macrobenthos

1. Grab size (Sagar Purvi) : 39cm (L) , 30cm (B)

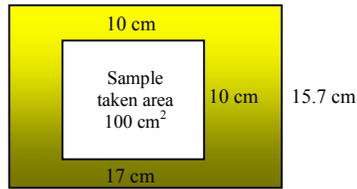


$$1 \text{ m}^2 = 10000 \text{ cm}^2$$

$$\begin{aligned} \text{Area} &= L \times B \\ \text{Sample taken area} &= 20 \text{ cm} \times 20 \text{ cm} = 400 \text{ cm}^2 \end{aligned}$$

$$\text{Macrobenthos} = 10000/400 = 25$$

2. Hotspot Grab size : 17cm (L) , 15.7cm (B)

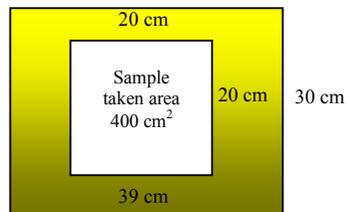


$$\text{Area} = L \times B$$
$$\text{Sample taken area} = 10 \text{ cm} \times 10 \text{ cm} = 100 \text{ cm}^2$$

$$\text{Macrobenthos} = 10000/100 = 100$$

20.6.2. Meiobenthos

1. Grab size (Sagar Purvi) : 39cm (L) , 30cm (B)

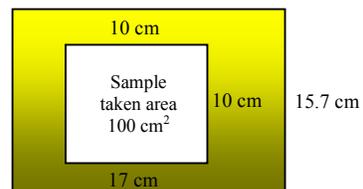


$$1 \text{ m}^2 = 10000 \text{ cm}^2$$

$$\text{Area} = L \times B$$
$$\text{Sample taken area} = 20 \text{ cm} \times 20 \text{ cm} = 400 \text{ cm}^2$$

$$\text{Meiobenthos} = 100/400 = 0.25$$

2. Hotspot Grab size : 17cm (L) , 15.7cm (B)



$$\text{Area} = L \times B$$
$$\text{Sample taken area} = 10 \text{ cm} \times 10 \text{ cm} = 100 \text{ cm}^2$$

$$\text{Meiobenthos} = 100/100 = 1$$

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21. MONITORING OF MICROBIOLOGICAL PARAMETERS IN MARINE ENVIRONMENTS (WATER & SEDIMENTS)

21.1. Introduction

The marine environment is the largest contiguous habitat on earth. Many distinct marine ecosystems and their microbial assemblages have been identified and studied ranging from ice-swept polar seas to deep-sea thermal vents. Although water covers the majority of earth surface, there is still an increasing demand of pure water. The coastal marine environment around India's 7500 km coastline supports a variety of marine ecosystems including the fragile mangroves and coral reefs. The generic and species diversity existing in some of the marine ecosystems of the Gulf of Mannar (Bay of Bengal) are unique and extremely valuable.

Demographic pressure in the urban cities and towns as well as an increase in the rural population and rapid industrialization have resulted in the generation of enormous amounts of waste materials (both organic and inorganic). These waste materials ultimately reach the marine environment either directly or indirectly through rivers, creeks, bays, etc., posing great threat to the ecosystems and bio-resources. Therefore, water quality assessment has become mandatory.

Microorganisms present in the polluted areas are used as health indicators to identify the polluted sites and also for evaluating the impact of pollution. Following are the name of health indicator bacteria

1. *Escherichia coli*
2. *Citrobacter freundii*
3. *Klebsiella pneumonia*
4. *Salmonella* serotype *typhimurium*
5. *Pseudomonas aeruginosa*
6. *Staphylococcus aureus*
7. *Vibrio cholerae*
8. *Streptococcus faecalis*

Enumeration of the health indicating bacterial population will be useful in the activities related to prevention and control of pollution.

Sediment is collected using a Grab (van Veen grab for shallow depths and Peterson for deeper areas). Most grabs penetrate to depth of 10 cm or more (less on hard-packed sand) and collect the sample, which is semicircular in cross section. The

upper 5-10 cm of the sediment which is considered to be microbiologically most active is of interest for analyses of microbial pathogens.

NOTE: Sediment samples are collected using van Veen grab (0.1m² area) from shallow depths. In this grab the long arms attached to grab bucket exert a considerable leverage for closing the jaws.

1a. Water

Materials required

- Niskin Sampler or bucket
- Sterile bottles (plastic/glass)
- Labels
- Marker
- Ice box
- Plastic bags
- Rubber bands

Collection

Water samples are collected using either a clean bucket (surface water) or Niskin sampler (subsurface and deep water). Water from the Niskin sampler or bucket is transferred into a sterile glass or plastic bottle, immediately following collection.

The bottle is labeled with following details:

- Station No
- Date
- Time
- Depth (m)

In case the sampling area is turbid filter the water sample through 200µm bolting silk / Whatman No. 1 filter paper)

1b. Sediment

Materials required

- Van Veen grab
- Plastic bags/Petri plates
- Spatula
- Absorbent Cotton
- Alcohol

Labels
Rubber bands
Marker
Ice Box

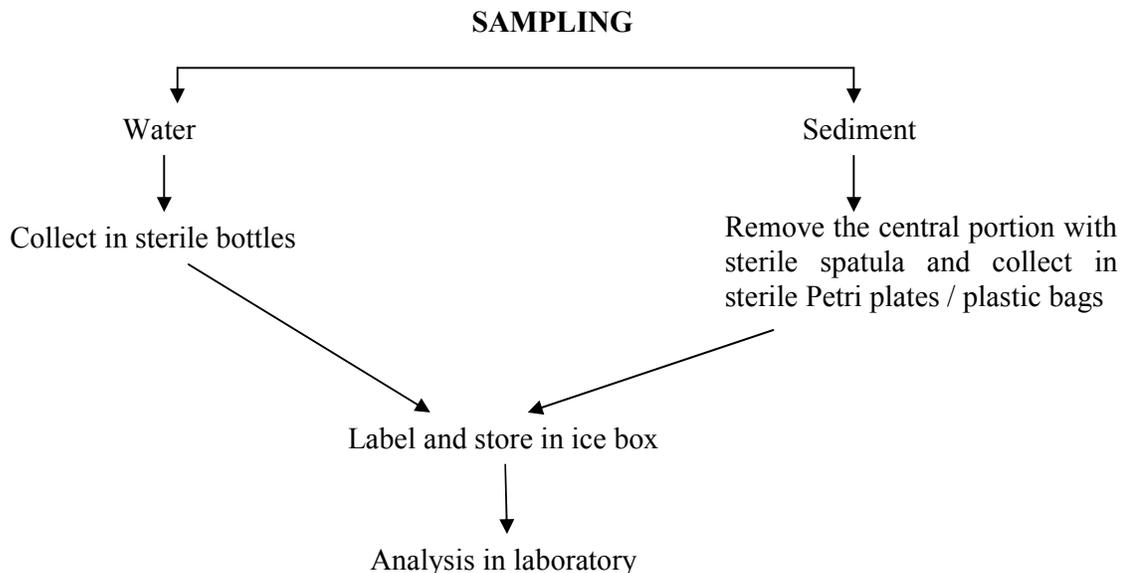
Collection

Sediment sample is collected using a van Veen grab (from shallow depths up to 100 m). Central portion of the sediment up to a depth of 10 cm is removed with a sterile spatula and transferred into a plastic bag or sterile Petri plate; label the samples as mentioned above.

Transportation

The samples are brought to the laboratory under ice-cold condition as quickly as possible for further processing.

Flow Chart



21.2. Enumeration of Marine Bacteria

Counting bacteria in natural environment has been a long standing endeavour for aquatic microbial ecologist. Estimation of culturable bacteria has evolved in several stages: (1) Enumeration of culturable bacteria based on ability of single bacterium to form colonies on marine agar plates.

For enumeration and isolation of all health indicator bacteria, culture method is adopted under COMAPS programme.

21.3. Total Viable Counts (TVC) (retrievable count (RC)/ colony forming unit (CFU)

21.3.1. Plate method (Culture method)

The bacteria are allowed to grow on seawater nutrient agar medium for a fixed period of time and at room temperature. Each single cell grows into a colony and the numbers of colonies are counted.

Water

Material required

Petri plates (Himedia)

Nutrient agar (Himedia Cat no-M 001)

Glass spreaders

Erlenmeyer flask (250 ml)

Test tubes (20 ml)

Incubator

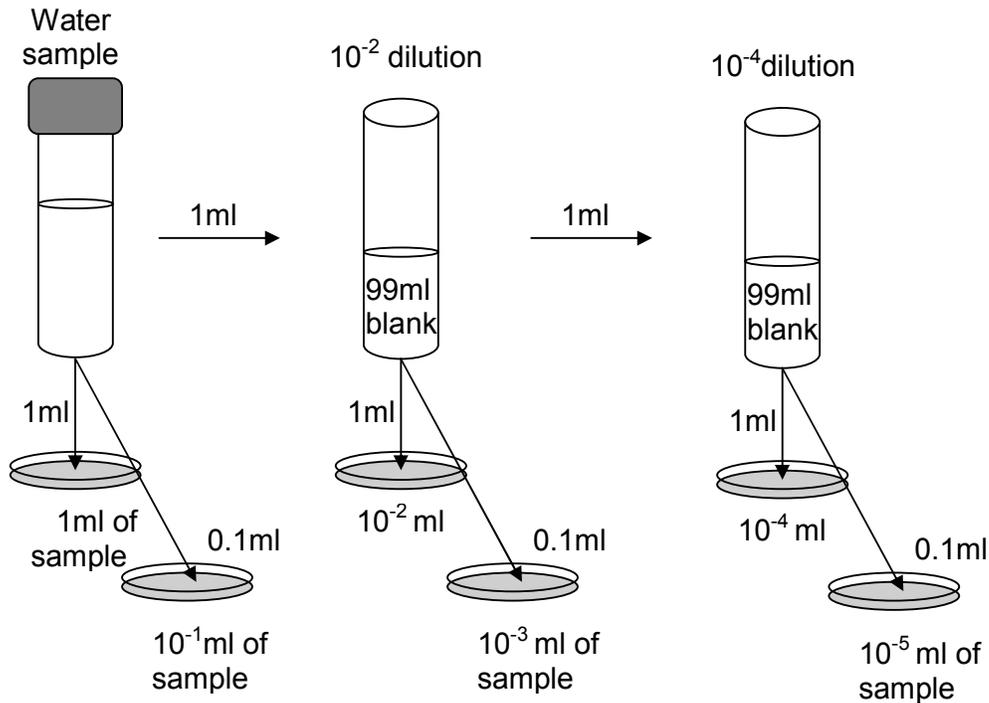
Procedure

Preparation of Nutrient agar:

Suspend 28 grams in 1000ml of half strength sea water. Adjust the pH to 7.5 ± 0.2 of the medium. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121° C). Transfer 15-20 ml/plate under sterile condition.

Note: Test the salinity and decide the strength of the sea water depending on the location of sampling. Salinity of the sample is measured with a salinometer (you may use a refractometer also).

21.3.1a. Preparation of serial Dilution-water



WATER SAMPLE

Pipette 0.1 ml onto Nutrient agar plate
(in duplicate)

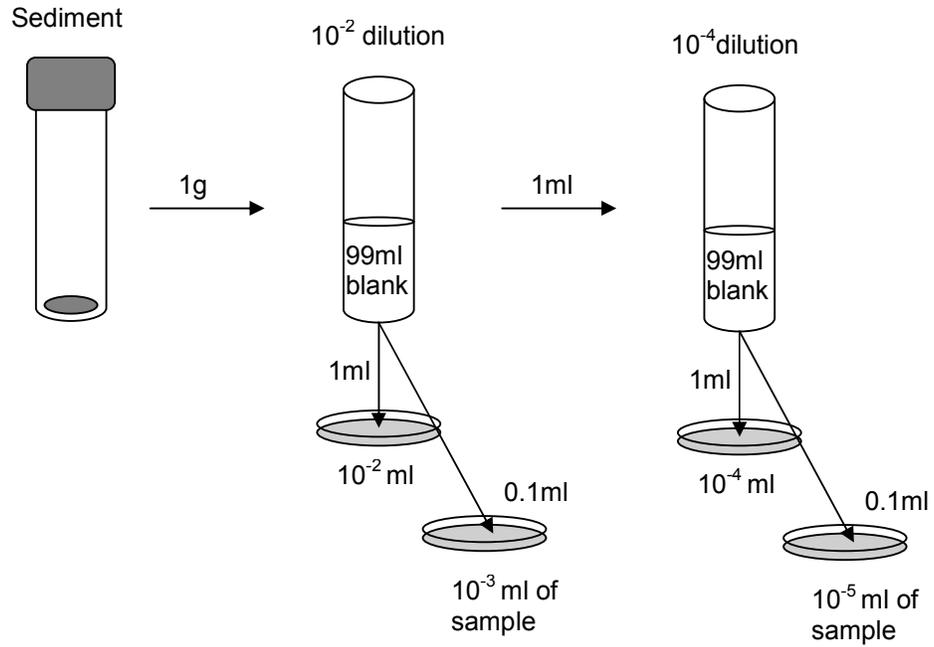
Using sterile glass spreaders spread the
sample uniformly until the plate is dry. The
plates are sealed using Para film

Incubate at RT for 24 to 48 hrs.

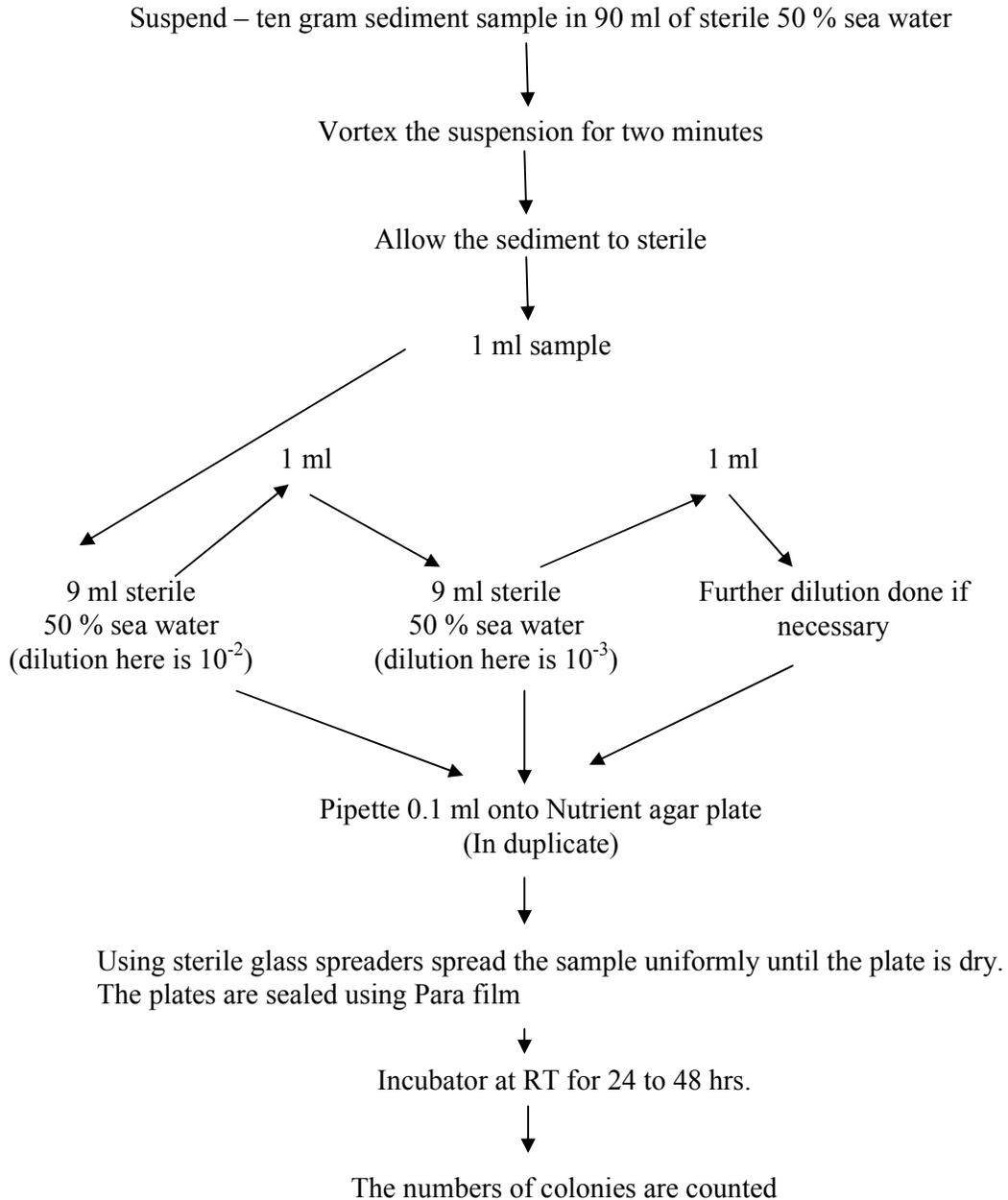
The numbers of colonies are counted

$$\text{Total Viable count / ml} = \frac{\text{No. of colonies}}{0.1 \text{ ml}}$$

21.3.1b. Preparation of serial Dilution-sediment



Procedure



$$\text{Total Viable count / g dry wt} = \frac{\text{No. of colonies}}{\text{Dilution factor}}$$

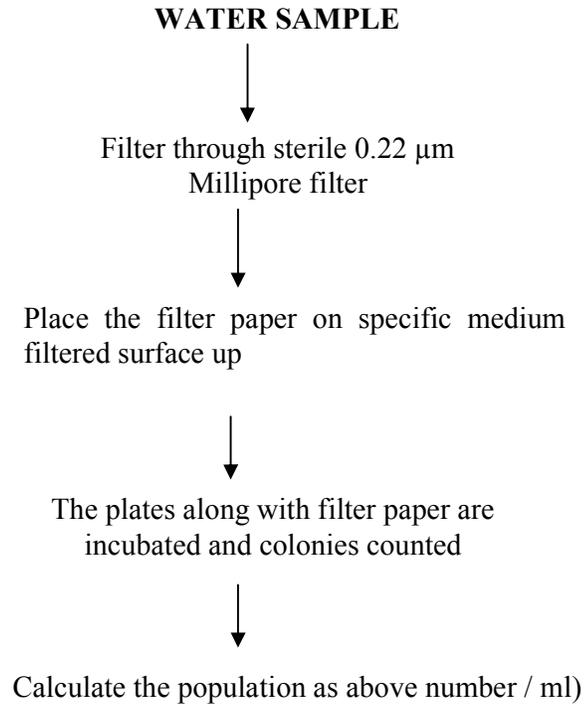
Note : The water in which sediment is suspended is filtered using a pre – weighted filter paper and it is dried and weighed.

21.3.2. Membrane filter method

Materials Required

Filtration Unit

Millipore filter paper (0.22 μm)



21.4. Enumeration of Health Indicator Bacteria

Health indicator bacteria are enumerated on selective media. Himedia have different types of selective media as shown below

1. HiCrome E.coli Agar(M 1295)
2. HiCrome Salmonella Agar(M1296)
3. M-Enterococcus Agar

Advantages of HiCrome media

1. Abundance of colonies with desired characteristics representing is more compared to other selective media.
2. Easy identification based on colour of the colony.
3. Single medium can be used to differentiate various groups of bacteria

Procedure

1. For plating the water samples, follow the procedure outlined in 19.3.1a and for sediment, follow the procedure given in 19.3.1b.

21.5. Isolation and Purification of Bacterial Strains

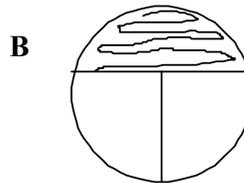
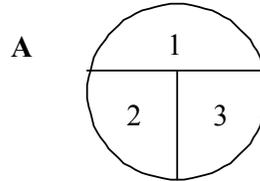
Isolation of bacteria in pure culture is the first and the important step. For that, one should clearly observe the plates and try to distinguish colonies based on their morphological parameters like colour (on special media), shape, size, opacity, pigmentation, etc. A random selection mechanism is also followed when the colonies appear to be similar and too numerous to count (TNTC). Often, plates with 30 to 300 colonies are ideal for counting and for choosing colonies for purification. Once selection is made, then the purity of the isolate is tested by using quadrant streaking method outlined in figures A to D. Purification is to obtain the desired bacteria from the plate using a loop and streaking on to solid medium to get contaminant free pure culture. A pure culture should yield colonies that appear similar to one another and microscopic observation of the culture should reveal cells that are reasonably similar to each other in appearance, particularly in regard to cell diameter and Gram reaction

Material required

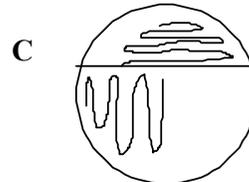
- Nutrient agar plates
- Inoculation needle (loop)
- Light Microscope.

Procedure

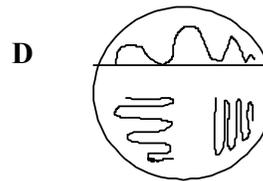
1. The desired colonies are marked.
2. Using a sterile loop the individual colonies are picked out and transferred on to nutrient agar plate.
3. The isolated culture is streaked on the agar plate as shown. Streak a loopful of culture slightly back and forth on the surface of the agar over section 1 as shown in diagram B.



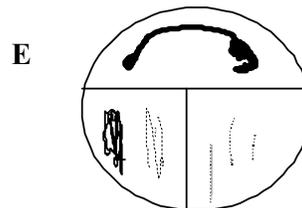
4. Flame the needle and allow it to cool. Draw the loop over section and then streak back and forth as mentioned earlier



5. Repeat the same.



6. Incubate the dish in an inverted position to prevent drops of condensed water on lid from falling onto the agar surface. Section 1 will develop the thickest amount of growth, while the following sections will usually have well isolated colonies.



21.6. Preparation of Culture for Sending

After the identification of the respective culture streak it on a nutrient agar slants.

Preparation of agar slants

1. Transfer about 1ml of nutrient agar into the storage vial (Laxbro cat no: SV- 2R) for stab or screw cap tubes 5ml for slant, autoclave and keep it in tilted position at an angle of 45⁰ for slants.
2. Leave it for two days to check any contamination.
3. Pick a single pure colony from nutrient agar plate and transfer it into these vials/slants.
4. Streak it on the slant or stab inoculate.
5. Incubate it for one day.

21.7. Colour chart for identification of hicrome media recommended

Bacteria	M1295	M1296
<i>E.coli</i>	Blue	Pink red
<i>Streptococcus faecalis</i>	M-Enterococcus agar	Red colour

21.8. References

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